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brother of cdo (*umleitung*) is cell-autonomously required for Hedgehog-mediated ventral CNS patterning in the zebrafish

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SUMMARY

The transmembrane protein Brother of Cdo (Boc) has been implicated in Shh-mediated commissural axon guidance, and can both positively and negatively regulate Hedgehog (Hh) target gene transcription, however, little is known about in vivo requirements for Boc during vertebrate embryogenesis. The zebrafish *umleitung* (*uml*^{ty54}) mutant was identified by defects in retinotectal axon projections. Here, we show that the *uml* locus encodes Boc and that Boc function is cell-autonomously required for Hh-mediated neural patterning. Our phenotypic analysis suggests that Boc is required as a positive regulator of Hh signaling in the spinal cord, hypothalamus, pituitary, somites and upper jaw, but that Boc might negatively regulate Hh signals in the lower jaw. This study reveals a role for Boc in ventral CNS cells that receive high levels of Hh and uncovers previously unknown roles for Boc in vertebrate embryogenesis.

KEY WORDS: Boc, Cdo, Shh, Morphogen, Pituitary, Jaw, Zebrafish

INTRODUCTION

Hedgehog (Hh) signaling is required for numerous developmental processes in vertebrates, including neurogenesis, axon guidance, myogenesis, organogenesis and limb formation (Fuccillo et al., 2006; Hughes et al., 1998; Panman and Zeller, 2003; Reidy and Rosenblum, 2009; Ruiz i Altaba et al., 2002; Sanchez-Camacho and Bovolenta, 2009). Sonic Hedgehog (Shh) regulates several distinct cellular behaviors in the embryo, including cell differentiation, proliferation and survival (Jacob and Briscoe, 2003; Tannahill et al., 2005). Shh functions in the central nervous system (CNS) as a morphogen, being secreted by the notochord and floor plate of the spinal cord to elicit concentration-dependent transcriptional responses in neural precursors that then determine neural fates along the dorsoventral axis (Ericson et al., 1997). Shh from the floor plate also serves as a chemoattractant for ventrally migrating axonal growth cones in the neural tube (Charron et al., 2003).

Hh signaling gradients in developing embryos are influenced by numerous positive and negative regulators, some of which directly interact with the Shh protein. The Hh receptor Patched and Hedgehog-interacting protein (Hhip) can bind Shh and help shape the Shh gradient (Briscoe et al., 2001; Dessaud et al., 2007; Ochi et al., 2006). Both of the genes that encode these proteins are Class II Hh target genes (transcriptionally activated by Shh signaling) and both are expressed in cells that require Shh for proper differentiation (Chuang and McMahon, 1999; Goodrich et al., 1996).

Shh binding proteins that are transcriptionally repressed by Hh signaling (Class I Hh target genes) include Cell adhesion molecule-related/downregulated by oncogenes (Cdo) and Brother of Cdo (Boc) (Tenzen et al., 2006). These proteins belong to a group of immunoglobulin (Ig) and fibronectin type III (FNIII) domain-containing integral membrane proteins (Kang et al., 1997; Kang et al., 2002). Boc acts as a receptor for Shh and mediates commissural axon guidance in the spinal cord (Okada et al., 2006). Boc is also required to repel retinal ganglion cell (RGC) growth cones from regions expressing Shh in the mouse forebrain (Fabre et al., 2010) and for the proper formation of ventrally projecting axon tracts in the zebrafish brain (Connor et al., 2005).

Roles for Boc and Cdo in Hh-mediated CNS patterning have begun to be elucidated, but the details of how these proteins affect the cellular Hh response in vivo is poorly understood. Cdo normally promotes Hh signaling, and Cdo mutant mice display phenotypes consistent with a loss of Hh signaling (Tenzen et al., 2006; Zhang et al., 2006). Although in vitro studies indicate that Cdo and Boc can both bind Shh to positively modulate Hh signaling, Boc mutant mice are reported to have normal ventral CNS patterning (McLellan et al., 2008; Okada et al., 2006). Intriguingly, both *Boc* and *Cdo* are primarily expressed in dorsal CNS tissues that do not respond to Hh signals, suggesting a negative role in Hh signal regulation in these regions (Mulieri et al., 2002; Mulieri et al., 2000; Tenzen et al., 2006). Consistently, overexpression of Boc and Cdo in CNS explants results in non-cell-autonomous inhibition of the Shh response (Tenzen et al., 2006). These Hh binding proteins might also function to amplify Shh signals at the dorsalmost limits of the Shh gradient, and/or might help to refine Shh gradient formation (Dessaud et al., 2008).

The *Drosophila* homologs of *Cdo* and *Boc*, *interference Hedgehog* (*iHog*) and *brother of iHog* (*boi*), respectively, were recently shown to be required for Hh signaling in the developing wing disc. *boi* and *iHog* mutants have defects in Hh-mediated embryonic patterning and have reduced Hh target gene expression that is enhanced in double mutants (Yao et al., 2006). Both of these proteins work at the level of Ptc and can bind directly to Hh via an extracellular FN domain. iHog overexpression enhances the localization of the Ptc receptor at the cell surface of Hh-receiving cells (Zheng et al., 2010). It appears that a high level of Hh

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signaling might involve interactions between Hh-bound protein complexes with a number of cell-surface receptors (Wilson and Chuang, 2006).

A large-scale forward genetic screen in zebrafish identified mutations in many components of the Hh signaling pathway (Haffter et al., 1996). These mutations were isolated because of defects in body axis formation, slow muscle differentiation and/or retinal axon guidance (Baier et al., 1996; Brand et al., 1996; Karlstrom et al., 1996; Trowe et al., 1996; van Eeden et al., 1996). Known components in the Hh pathway that were identified in this screen include Disp1 (Nakano et al., 2004), Shh (Shha – Zebrafish Information Network) (Schauerte et al., 1998), the transmembrane proteins Smo (Varga et al., 2001), Hhip, Ptch2 and Ptc2 (Ptch1 – Zebrafish Information Network) (Koudijs et al., 2008; Koudijs et al., 2005; Lee et al., 2008), the cytoplasmic regulator Sufu (Koudijs et al., 2005), and the Hh responsive transcription factors Gli1 (Karlstrom et al., 2003) and Gli2 (Gli2a – Zebrafish Information Network) (Karlstrom et al., 1999). Novel components identified include the extracellular protein Scube2 (Kawakami et al., 2005; Woods and Talbot, 2005) and the intracellular protein Dzip1 (Sekimizu et al., 2004; Wolff et al., 2004).

Here, we present the molecular characterization of *umleitung* (*uml*), the last of the ipsilateral class of zebrafish mutants from this screen (Karlstrom et al., 1996) to reveal itself. We show that *uml* encodes Boc and that Boc is cell-autonomously required for Hh signaling in the ventral CNS. These studies uncover new roles for Boc in vertebrate development.

MATERIALS AND METHODS

Genetic mapping and fish lines

uml^{ty54} was generated in the Tü strain and crossed to the TL, AB and Brian's wild-type strains for genetic mapping (Talbot and Schier, 1999) using simple sequence repeat markers (Knapik et al., 1996). Other fish lines used were *yot*^{ty119}*(gli2)* (Karlstrom et al., 1999), *dtr*^{ts269}*(gli1)* (Karlstrom et al., 2003) and *Tg(nkx2.2a:megfp)*^{vu17} (Kirby et al., 2006).

Sequencing and *uml(boc)* genotyping

cDNA from wild-type or *uml* mutant individuals was PCR amplified using four sets of overlapping primers (Connor et al., 2005): cdsBOC5′UTR.fw, ATGCAAGAAGTTGGGGAGCCG; EXON5.rv, ATTCCTCAAGTCCAGGCCGTC; EXON4.fw, TGCTGCTTACAACCCCGTCAC; EXON10.rv, CGTCACATAAGCGGAGGTCTCGGT; EXON9.fw, GAAGAGGGGGACCAATAGGAC; EXON15.rv, AGCGGGACCATGGTATACTGG; EXON14.fw, CCTGGAGATCTGCCTTACCTC; EXON18.rv, TCCATCTTCTGGATCATGTGT. DNA fragments were gel purified (Qiagen Gel Extraction Kit) and sequenced commercially (GeneWiz).

For genotyping, fin clip DNA was PCR amplified using primers designed to discriminate between the mutant and wild-type sequence (umlEXON5.MUT.FW, GAAGCGGCTCGTATCATCTAA; UMLEXON5.RV, TCAGCGAGACACACGTAAGTG) resulting in a 207 bp PCR product only in mutant carriers. The wild type control primer was: umlEXON5.WT.FW, GAAGCGGCTCGTATCATCTAT. Differences in the wild type and mutant primers are underlined.

In situ hybridization, immunohistochemistry and imaging

Whole-mount in situ hybridization (ISH), immunohistochemistry, cryostat sectioning and imaging were performed as described previously (Karlstrom et al., 1999; Guner and Karlstrom, 2007; Barresi et al., 2005) using published probes. Bone and cartilage were labeled as described by Walker and Kimmel (Walker and Kimmel, 2007). DiI/DiO axon labeling was performed as described by Karlstrom et al. (Karlstrom et al., 1996). ISH probes for *cdo* and *foxa2* were made by PCR using gene specific primers containing the T7 or SP6 RNA polymerase binding sites (indicated in capitals): CDO.fw.Sp6, ATTTAGGTGACACTATAGAAtacgatctggagtggagagc; CDO.rv.T7, TAATACGACTCACTATAGGGAaagagatgtgaggcccagtg; Foxa2.fw1.Sp6, ATTTAGGTGACACTATAGAAacagcgttaagagccagcag; Foxa2.rv1.T7, TAATACGACTCACTATAGGGAtgcccgtgttgacataggac.

Embryo injections, cell transplantation and cyclopamine treatments

shh and *boc* mRNA were synthesized (Message Machine, Ambion) using the *shh*/T7TS (Ekker et al., 1995) and *boc*/PCS2^{+} (Connor et al., 2005) plasmids as templates. The translation-blocking *boc* morpholino (MO1-boc: 5′-AATCCAATTCAACGTCCCAGACATC-3′) has been described previously (Connor et al., 2005). Embryos were injected at the two-cell stage as described previously (Westerfield, 2000).

Cell transplantation was performed as described previously (Carmany-Rampey and Moens, 2006) with donor embryos injected with or without the *boc* morpholino. Approximately 20 donor cells were transplanted into the presumptive medial spinal cord region at the gastrula stage (Woo and Fraser, 1995). Paired donor/host embryos were grown in 12-well plates and fixed at 24 hours postfertilization (hpf). Cyclopamine (Toronto Chemicals) treatments were carried out in 12-well plates with 40 embryos per well as described previously (Guner and Karlstrom, 2007).

RESULTS

Axon guidance and forebrain patterning defects in *uml* mutants

The *umleitung* (*uml*) (meaning 'detour' in German) mutant was identified in a large-scale screen of N-ethyl-N-nitrosourea (ENU)-generated mutations based on defects in retinal axon projections to the tectum (Karlstrom et al., 1996). In 5 days postfertilization (dpf) *uml* mutants, some retinal ganglion cell (RGC) axons failed to cross the ventral midline of the forebrain and instead projected ipsilaterally (Fig. 1A,B). Despite these midline crossing errors, RGC axons reached their correct topographic targets in the optic tectum (Fig. 1B). At 2 dpf, *uml* mutant RGC axons approached, but did not cross, the midline (Fig. 1C,D). The post optic commissure (POC) failed to form in the diencephalon of *uml* mutants, whereas the anterior commissure (AC) in the telencephalon formed normally (Fig. 1C-F). These axon phenotypes are similar to, but less severe than, those seen in other ipsilateral class mutants such as *yot(gli2)* and *dtr(gli1)* (Barresi et al., 2005; Culverwell and Karlstrom, 2002; Karlstrom et al., 1996).

To better understand these forebrain axon guidance defects, we assayed axon guidance cues in the region. Specialized glial fibrillary acidic protein (Gfap)-expressing glial cells span the midline prior to axon crossing (Barresi et al., 2005). In *uml* mutants, the glial bridge in the post optic area (POA) was disrupted, whereas the glial bridge in the AC region appeared to form normally (Fig. 1E,F insets). Slit guidance molecules are thought to act primarily as growth cone repellents, with bands of *slit* expression helping to position correctly the optic nerves and POC in the forebrain (Fricke et al., 2001). *slit2* and *slit3* expression domains were expanded into the POA in *uml* mutants (Fig. 1G,H; data not shown). Interestingly, *uml* mutants also had reduced *slit1a* and *sema3d* expression in the ventral forebrain (Fig. 1I,J; data not shown). These defects in axon guidance molecule expression were very similar to those seen in *yot(gli2)* mutants (Fig. 1H,J) (Barresi et al., 2005) suggesting that axons failed to cross the midline in *uml* mutants because of glial patterning defects and/or the expansion of *slit* repellent molecules into the POA (Barresi et al., 2005).

Next, we determined whether earlier forebrain patterning events are disrupted in *uml* mutants. The homeodomain transcription factor *dlx2* (*dlx2a* – Zebrafish Information Network) serves as a good marker for patterning within the telencephalon and diencephalon (Akimenko et al., 1994). *dlx2a*

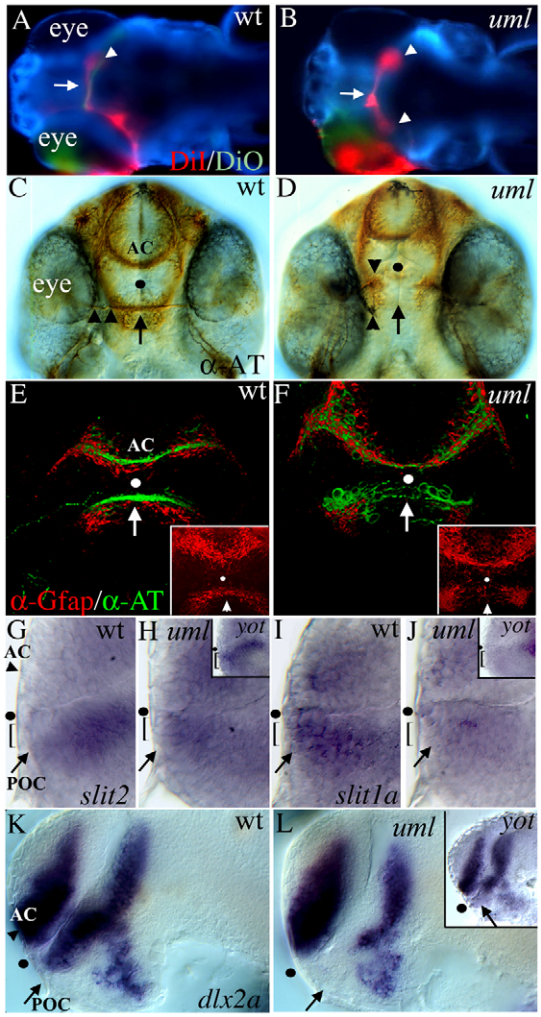

Fig. 1. Axon guidance and forebrain patterning defects in *umleitung (uml)* mutants. (**A**,**B**) DiI (red) and DiO (green) labeling of retinal ganglion cell (RGC) axons from the left eye at 5 days postfertilization (dpf). Dorsal views, anterior to the left. In wild-type zebrafish (A), all RGC axons cross the midline (arrow) to innervate the contralateral tectal lobe (arrowhead). In *uml* mutants (B) some RGC axons fail to cross the midline (arrow) and instead project to the ipsilateral tectal lobe, leading to bilateral projections (arrowheads). (**C**,**D**) Anti-acetylated tubulin (AT) labeling of forebrain axons at 48 hpf. Ventral views, anterior uppermost. In wild-type embryos (C), axons cross the midline to form the anterior commissure (AC) in the telencephalon and the post optic commissure (POC, arrow) in the diencephalon. RGC axons (arrowheads) grow along the POC (arrow) to the midline to form the optic nerve and optic chiasm. In *uml* mutants (D), RGC axons fail to reach the midline at this age (arrowheads) and the POC fails to form (arrow), whereas the AC forms normally in the telencephalon. (**E**,**F**) Anti-Gfap (red) and anti-AT (green) labeling of glia and axons in the forebrain at 36 hpf. Ventral views, anterior at the top. Insets show Gfap labeling alone. In wild-type embryos (E), glial cells span the midline adjacent to the AC and POC (arrow). In *uml* mutants (F), the posterior glial bridge is disrupted, leaving a gap at the midline (arrow). (**G-L**) In situ hybridization (ISH) showing gene expression in the anterior forebrain at 30 hpf. (G) Lateral views, anterior to the left, eyes removed. *slit2* (and *slit3*, not shown) is expressed posterior and ventral to the POC (arrow), but not in cells (bracket) that lie between the POC and optic recess (dot). (H) In *uml* and *yot* (inset) mutants, *slit2* expression is expanded into the region between the POC and the optic recess (black dot). (I) In contrast to *slit2* and *slit3*, *slit1a* is expressed between the POC and optic recess (bracket) in wild-type embryos. (J) *slit1a* expression in this region is reduced in *uml* mutants (bracket) and absent in *yot* mutants (inset). (K) *dlx2a* is regionally expressed in the AC region (arrowhead) of the telencephalon and POC region (arrow) of the diencephalon. (L) In *uml* mutants, *dlx2a* expression is absent in the POC region. These expression defects are nearly identical to those seen in the *yot* (inset). Dots mark the optic recess in C-L.

expression was absent in the POA of *uml* mutants, whereas *dlx2a* expression appeared normal in the telencephalon and the more posterior diencephalon (Fig. 1K,L), indicating regional patterning defects in the forebrain. This misexpression was nearly identical to that seen in the Hh signaling pathway mutants *yot(gli2)* (Fig. 1L, inset) and *dtr(gli1)* (data not shown). Together, these data suggest that the *uml* mutation might affect Hh-mediated forebrain patterning.

Reduced Hh signaling in *uml* mutants

Given these phenotypic similarities with known Hh pathway mutants, we examined the expression of several Class I and Class II Hh target genes in *uml* mutant embryos. *ptch2* (previously Ptc1, now Ptch2 – Zebrafish Information Network) expression is a well-established read-out of Hh signaling (Concordet et al., 1996). In *uml* mutants, *ptch2* expression was reduced but not absent throughout the CNS (Fig. 2A,B) and somites (not shown), suggesting an overall reduction in Hh signaling levels. Consistently, expression of the floor plate marker *foxa2* was reduced in the brain and absent in lateral floor plate cells but remained in the medial floor plate (Fig. 2C,D), as is the case in the Shh mutant *syu(shh)* (Schauerte et al., 1998).

The Class II genes *nkx2.9* and *nkx2.2a* are expressed in the ventral brain and lateral floor plate (Guner and Karlstrom, 2007). *nkx2.9* expression was completely absent in *uml* mutants (Fig. 2F), whereas *nkx2.2a* expression was regionally absent in the brain and completely absent in the spinal cord (Fig. 2H). This is similar to the pattern seen in *yot(gli2)* and *dtr(gli1)* mutants (Guner and Karlstrom, 2007). In wild type, the Class II genes *olig2* and *nkx6.1* were regionally expressed in the brain and were expressed slightly more dorsally than *nkx2.2a* in the ventral spinal cord (Fig. 2I,K). In *uml* mutants, expression of *olig2* and *nkx6.1* was reduced in the brain and spinal cord (Fig. 2J,L). In the spinal cord, the expression domains of both genes were shifted ventrally by a few cell diameters (Fig. 2J′,L′). By contrast, expression of the Class I Hh target genes *pax3a* and *pax7a* (Seo et al., 1998) appeared to be unaffected in *uml* mutants (Fig. 2M,N), similar to the situation in *yot(gli2)* and *dtr(gli1)* (Guner and Karlstrom, 2007). Together, these results indicate that mutations at the *uml* locus lead to reduced Hh signaling levels, similar to other zebrafish Hh pathway mutants.

The *uml^{ty54}* mutation results in loss of Boc function

To determine the molecular cause of these Hh signaling defects, we next identified the gene encoded at the *uml* locus. Using simple sequence repeat markers (Knapik et al., 1996) and a mapping panel encompassing over 3200 meiotic events, we mapped *uml* to a 2.5 cM genetic interval on zebrafish chromosome 24. Given the lack of genomic sequence

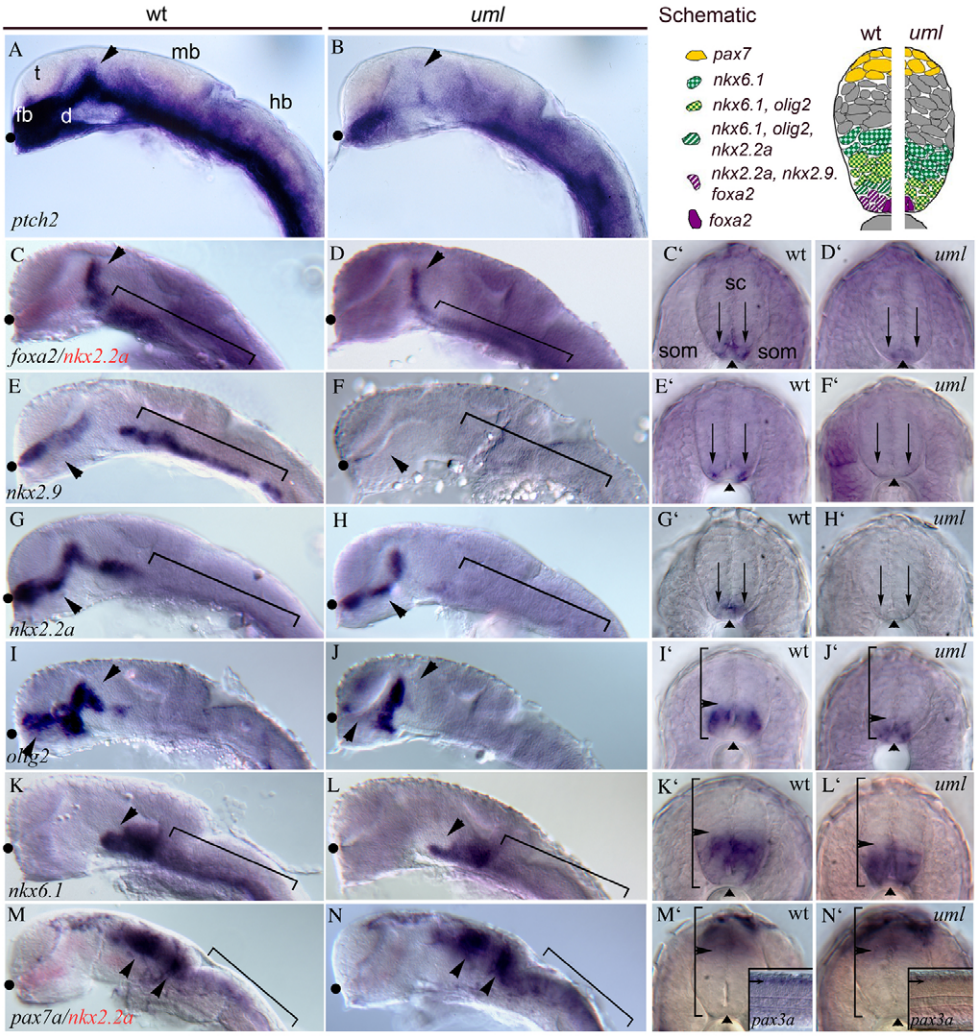

Fig. 2. Reduced Hh signaling in *uml* mutants. (**A**) The Hh target gene *ptc1* (now known as *ptch2*) is expressed throughout the ventral CNS in wild-type embryos with a dorsal peak of expression in the zona limitans interthalamica (ZLI) (arrowhead). (**B**) In *uml* mutants, *ptch2* expression is greatly reduced throughout the brain and spinal cord, and is nearly absent from the ZLI (arrowhead). (**C**) *foxa2* is expressed in the ZLI (arrowhead) and ventral midbrain/hindbrain (bracket). (**C'**) In the spinal cord, *foxa2* is expressed in the medial floor plate (FP, arrowhead) and lateral FP cells that correspond to the V3 region of the mammalian spinal cord (arrows). (**D**,**D'**) In *uml* mutants, *foxa2* expression is reduced in the brain and is absent from the lateral floor plate (D′ arrows). (**E**,**E'**) *nkx2.9* is expressed in the diencephalon (arrowhead in E), ventral midbrain/hindbrain (bracket) and in lateral FP cells (arrows in E′). (**F**,**F'**) In *uml* mutants, *nkx2.9* expression is completely absent throughout the CNS. (**G**,**G'**) *nkx2.2a* is expressed in the diencephalon (arrowhead in G), ventral midbrain/hindbrain (bracket) and lateral floor plate of the spinal cord (arrows in G′). (**H**,**H'**) In *uml* mutants *nkx2.2a* expression is reduced in the diencephalon (arrowhead in H), ventral midbrain/hindbrain (bracket), and spinal cord (arrows in H′). (**I**,**I'**) *olig2* is regionally expressed in the diencephalon (arrowheads) and in a band of cells in the spinal cord just dorsal to the floor plate (arrowhead in I′). (**J**,**J'**) In *uml* mutants, *olig2* expression is regionally absent in the diencephalon (arrowheads in J) and expression is reduced and shifted ventrally in the spinal cord (arrowhead in J′). (**K**,**K'**) *nkx6.1* is expressed in the ventral midbrain (arrowhead in K), hindbrain (bracket in K) and in the ventral half of the spinal cord (bracket with arrowhead in K′). (**L**,**L'**) In *uml* mutants, *nkx6.1* expression is reduced in the brain (arrowhead and bracket in L) and shifted ventrally in the spinal cord (bracket with arrowhead in L′). (**M**,**M'**) *pax7* is expressed in the dorsal midbrain (arrowheads in M), hindbrain (bracket in M), and spinal cord (bracket with arrowhead in M′). Inset shows *pax3* expression. (**N**,**N'**) In *uml* mutants, *pax7* (and *pax3*, inset) expression appears unchanged. A-N are lateral views of the heads of 24 hpf zebrafish, anterior to the left, eyes removed. Black dots mark the optic recess. C′-N′ are spinal cord cross-sections of embryos shown in C-N. In C′-N′, arrows mark the lateral floor plate and arrowheads mark the floor plate. In I′-N′, brackets with arrowheads mark the dorsoventral boundary of gene expression within the spinal cord. In C, D, M and N *nkx2.2a* labeling (red) was used to identify *uml* mutants. Upper right panel is a schematic representation of Hh target gene expression in the spinal cord of wild-type (left) and *uml* mutant (right) embryos. d, diencephalon; fb, forebrain; hb, hindbrain; mb, midbrain; sc, spinal cord; som, somites; t, telencephalon.

information available in this region, we identified a syntenic region on *Tetraodon nigroviridis* chromosome 6 and identified genes likely to be located near the *uml* locus. By combined synteny and recombinant analyses we further refined the *uml* genetic interval to a 0.3 cM region containing approximately nine genes, including *brother of cdo* (*boc*) (Fig. 3A).

Co-segregation analysis showed that *boc* was tightly linked to the *uml* locus (0 recombinants/3242 meioses). Sequencing of the *boc* coding region in *uml* mutants revealed a single point mutation in exon 5 (T to A) that is predicted to lead to a severely truncated protein (Tyr238 to a STOP codon) (Fig. 3B) containing only the signal sequence and two immunoglobulin (Ig) domains (Fig. 3D).

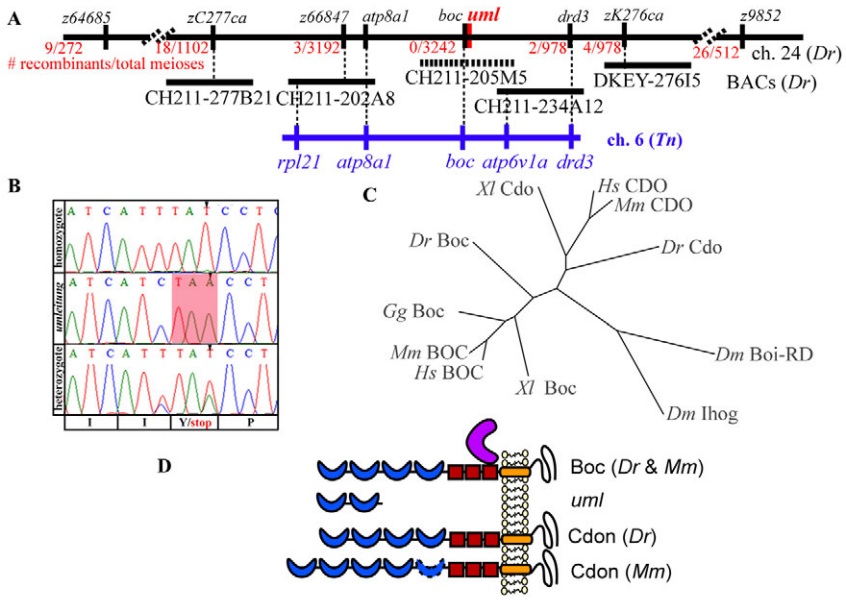

Fig. 3. *uml*^{ty54} encodes a nonsense mutation in *brother of cdo* (*boc*). (**A**) Genetic map of chromosome 24 near the *uml* locus. The number of recombination events between *uml* and different gene or microsatellite markers are shown in red. Linked bacterial artificial chromosome (BAC) clones are shown in black (dashed bar indicates an unsequenced BAC). A syntenic region of puffer fish (*Tetraodon nigroviridis*, *Tn*) chromosome 6 is shown in blue. (**B**) Sequencing the *boc* gene in *uml* mutant embryos revealed a nonsense mutation in exon 5 (T714→A) that would lead to a premature STOP codon (shaded in pink) in place of Tyr238. Sequence from heterozygous individuals contains both bases at this position, as expected. (**C**) Unrooted tree showing the sequence relatedness of Boc and Cdo from the fruit fly (*Dm*), mouse (*Mm*), frog (*Xl*), chicken (*Gg*), human (*Hs*) and zebrafish (*Dr*). (**D**) Schematic representation of the Boc protein from zebrafish and mouse. The *uml* allele is predicted to encode a truncated Boc protein that lacks two of four immunoglobulin (Ig) domains (blue), the Shh (purple) binding fibronectin type III domains (red), the transmembrane region (orange), and the intercellular C-terminal tail (black line). A splice variant of mouse Cdo is predicted to contain an additional Ig domain (dashed).

Sequence alignments indicate that zebrafish Boc is closely related to mammalian Boc, but is also similar to the closely related Cdo protein. Both of these proteins are conserved across multiple species from *Drosophila* to humans (Fig. 3C).

To further confirm that the *uml(boc)* mutation leads to a loss of Boc function, we injected translation blocking *boc* morpholinos (Connor et al., 2005) into wild-type and *uml* mutant zebrafish. Injecting 5 ng of *boc* morpholino into wild-type embryos produced early defects indistinguishable from those observed in the *uml* mutants, including curved body axes (Fig. 4A-C) and regional loss of the Class II Hh target gene *nkx2.2a* (Fig. 4D-F). We also reproduced the RGC axon path-finding defects seen in 48 hpf *uml* mutants, although not as robustly as the earlier phenotypes (Fig. 4G-I). Injection of *boc* morpholinos (MOs) into *uml* mutants did not enhance any of these phenotypes (data not shown). Together, these results strongly suggest that *uml*^{ty54} represents a *boc* loss-of-function mutation that results from a severe truncation in the Boc extracellular domain.

To further investigate the role of Boc in Hh signaling, we overexpressed *boc* and *shh* in *uml* and wild-type embryos. *boc* mRNA injections (250 pg) partially rescued the loss of *nkx2.9* and *nkx2.2a* (Fig. 5E,K) in 100% of *uml* mutants. Interestingly, whereas *boc* mRNA injection restored expression of *nkx2.9* and *nkx2.2a* in the normal ventral domain of *uml* mutants, little ectopic Hh target gene expression was seen either in *uml* mutants or wild-type embryos (Fig. 5B,H). In fact, ectopic expression of *nkx2.9* was not detectable by in situ hybridization (Fig. 5B,E), and ectopic expression of *nkx2.2a* was only detected in the *Tg(nkx2.2a:megfp)* transgenic line (Ng et al., 2005), which allows for amplification of GFP fluorescence in *nkx2.2a*-expressing cells (Fig. 5H). By contrast, injecting 100 pg of *shh* mRNA into wild-type individuals led to a massive expansion of *nkx2.9* and *nkx2.2a* expression (Fig. 5C,I). Injecting this same amount of *shh* mRNA into *uml* mutants led to a much less robust expansion of *nkx2.9* and *nkx2.2a* expression but did rescue *nkx2.9* and *nkx2.2a* expression in ventral regions (Fig. 5F,L). Unlike the case in *yot*(*gli2*) and *dtr*(*gli1*) mutants, cells throughout the CNS of *uml* mutants responded to increased levels of *shh*, but only weakly. Together, these results suggest that (1) both Boc and Shh overexpression can compensate for the loss of Boc function in the ventral CNS and (2) cells can respond to Shh in the absence of Boc function but this response is much weaker than in the presence of Boc.

Expression of *boc* during zebrafish embryogenesis

To begin to assess how Boc influences Hh-mediated embryonic patterning, we examined *boc* expression in relation to Hh-mediated embryonic patterning events (see Fig. S1 in the supplementary material). Expression of *boc* was first seen in the ventral neural plate and spinal cord at 8-10 hpf, just prior to the first expression of the Hh target gene *nkx2.2a* (see Fig. S1A-C in the supplementary material). By 12 hpf, *boc* was predominantly expressed in the dorsal spinal cord, although weak labeling was still present ventrally (see Fig. S1D in the supplementary material). *boc* expression in the dorsal spinal cord generally overlapped with expression of the closely related *cdo* gene (see Fig. S1E,I in the supplementary material). Expression of *boc* was maintained dorsally in the brain and spinal cord and was expressed regionally in the eye throughout embryonic and larval stages (see Fig. S1F-Q in the supplementary material). *boc* was weakly expressed in cells that give rise to the pituitary gland (see

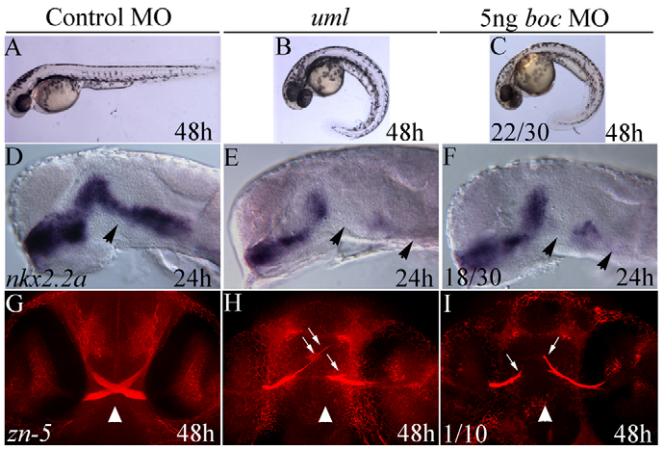

Fig. 4. *boc* morpholino (MO) injections cause *uml*-like defects. (**A**) A control MO-injected 48 hpf embryo. (**B**) *uml* mutants have curved body axes at this stage. (**C**) Injection of a translation blocking *boc* MO results in a curved body axis similar to that seen in *uml* mutants. (**D**) Normal *nkx2.2a* expression in the ventral forebrain and midbrain (arrowhead) of a control MO-injected embryo. (**E**) Regional loss of *nkx2.2a* expression seen in *uml* mutants (arrowheads). (**F**) Injecting *boc* MO results in the loss of *nkx2.2a* expression in the same regions as in *uml* mutants (arrowheads). (**G**) Zn-5 antibody labeling at 48 hpf showing RGC axons that have crossed the midline by 48 hpf to form the optic chiasm (arrowhead). (**H**) In *uml* mutants, RGC axons fail to cross the midline (arrowhead) and grow aberrantly in the ventral forebrain (arrows). (**I**) *boc* MO injections can result in specific retinal axon defects (arrowheads) that are remarkably similar to those seen in *uml*. A-C are lateral views of live zebrafish. D-F are lateral views of the head, eyes removed, anterior to the left. G-I are ventral views of the head, anterior uppermost.

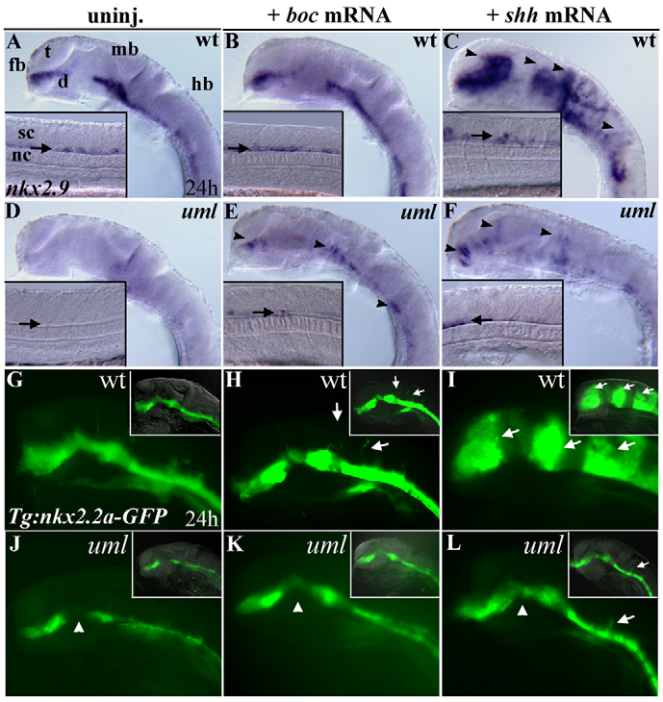

Fig. 5. *boc* mRNA injections rescue Hh signaling defects in *uml* mutants and weakly activate Hh signaling ectopically. (**A**) Wild-type *nkx2.9* expression in the brain and lateral floor plate (inset, arrow). (**B**) Ectopic *nkx2.9* expression was not detected by in situ hybridization after injecting 250 pg of *boc* mRNA. (**C**) By contrast, injecting 100 pg of *shh* mRNA led to ectopic *nkx2.9* expression throughout the CNS (arrowheads). (**D**) *nkx2.9* is absent in *uml* mutants. (**E**) Injecting 250 pg of *boc* mRNA into *uml* mutants partially rescued *nkx2.9* expression (arrowheads) in the brain and spinal cord. (**F**) Injecting 100 pg of *shh* mRNA into *uml* mutants partially rescued *nkx2.9* expression defects and led to ectopic *nkx2.9* expression (arrowheads), but at much lower levels than in wild-type embryos. (**G**) Wild-type *nkx2.2a* expression in the brain and floor plate visualized in the *Tg(nkx2.2a:megfp)* reporter line (Ng et al., 2005). (**H**) Injecting 250pg of *boc* mRNA led to weak ectopic *nkx2.2a* expression only in regions close to the Shh responsive domain of the CNS (arrows). (**I**) Injecting 100 pg of *shh* mRNA led to strong ectopic *nkx2.2a* expression throughout the CNS (arrows). (**J**) Regional absence of *nkx2.2a* expression in *uml* mutants visualized using the *Tg(nkx2.2a:megfp)* reporter line. (**K**) Injecting 250 pg of *boc* mRNA into *uml* mutants rescued *nkx2.2a* expression defects in the brain (arrowhead). (**L**) Injecting 100 pg of *shh* mRNA into *uml* mutants also rescued *nkx2.2a* expression defects (arrowhead) and led to ectopic *nkx2.2a* expression in the brain (arrows), but at much lower levels than in wild-type embryos (compare with I). All panels show lateral views the head at 24 hpf, anterior to the left, eyes removed. Insets show lateral views of the trunk (A-F, arrows indicate the floor plate) or combined DIC and fluorescent images (G-L). d, diencephalon; fb, forebrain; hb, hindbrain; mb, midbrain; nc, notochord; sc, spinal cord; som, somites; t, telencephalon.

Fig. S1G in the supplementary material) and was more strongly expressed in the somites (see Fig. S1D,E,J in the supplementary material) and brachial arches (see Fig. S1P,Q in the supplementary material). The restriction of *boc* expression to the dorsal CNS was reported previously (Connor et al., 2005) and is consistent with *boc* being a class I Hh target gene. This was confirmed by the loss of *boc* expression following injection of *shh* mRNA (see Fig. S1M in the supplementary material) and by the ventral expansion of *boc* expression seen in *smoothened* mutants (see Fig. S1N in the supplementary material). Interestingly, no ventral expansion of *boc* or *cdo* expression was seen in the spinal cord of *uml(boc)* mutants (see Fig. S1H,O in the supplementary material), consistent with the normal expression of the class I Hh target genes *pax3a* and *pax7a* in the dorsal CNS of *uml(boc)* mutants (Fig. 2). *boc* gene expression itself was slightly reduced in the dorsal CNS of *uml(boc)* mutants (see Fig. S1H in the supplementary material).

Boc function is necessary for pituitary development, early myogenesis and jaw formation

Given the expression of *boc* in tissues outside the CNS and the requirement for Hh/Gli signaling during zebrafish pituitary, jaw and slow muscle fiber differentiation (Du et al., 1997; Eberhart et al., 2006; Sbrogna et al., 2003), we examined these tissues more carefully for defects in *uml(boc)* mutants. Similar to *dtr(gli1)* mutants, expression of the Hh target gene *nkx2.2a* was reduced in the anterior adenohypophysis in *uml(boc)* mutants, whereas posterior *pax7a* expression was not affected (see Fig. S2A,B, in the supplementary material; data not shown). Our previous work showed that Hh dosage regulates endocrine cell differentiation, with Prolactin- (Prl), Adrenocorticotropic hormone- (Acth; Pomca – Zebrafish Information Network) and Growth hormone- (Gh; Gh1 – Zebrafish Information Network) secreting cells being the most sensitive to reduced Hh signaling levels (Guner et al., 2008). Consistent with a mild loss of Hh signaling in *uml(boc)* mutants, *gh*-expressing cells were absent, *acth*- and *prl*-expressing cells

were greatly reduced, whereas more posterior endocrine cells were largely unaffected (see Fig. S2C-G in the supplementary material). *pomc*-expressing cells in the ventral hypothalamus were disorganized, as in other Hh pathway mutants (see Fig. S2F in the supplementary material), consistent with Hh-related forebrain patterning defects.

Reduced Hh signaling leads to fused upper jaw cartilages and reduced lower jaw structures (Brand et al., 1996; Schwend and Ahlgren, 2009; Teraoka et al., 2006). In *uml(boc)* mutants, neurocranial elements, including the trabeculae, were fused, consistent with a loss of Hh signaling in the upper jaw (see Fig. S2 in the supplementary material). Surprisingly, the lower jaw was expanded in *uml(boc)* rather than reduced or absent. As Hh signaling is needed for proliferation and chondrogenesis during jaw growth (Schwend and Ahlgren, 2009; Teraoka et al., 2006), this phenotype might point to a repressor role for Boc in Hh-mediated craniofacial development.

Although somite borders appeared morphologically normal in *uml(boc)* mutants (Fig. 4), the fact that *boc* was expressed in developing adaxial cells and somites (see Fig. S1 in the supplementary material) led us to examine muscle differentiation in *uml(boc)* mutants more carefully. Expression of *myoD* (*myod1* – Zebrafish Information Network) in early slow muscle fiber precursors (adaxial cells) was reduced but not absent in *uml* (see Fig. S2R,S in the supplementary material), similar to *yot(gli2)* heterozygotes (data not shown). Expression of *ptch2* was also generally reduced in the somites (data not shown), suggesting a reduction in Hh signal levels in this tissue. The number of slow muscle fibers, which require Hh signaling for differentiation (Barresi et al., 2000), was slightly reduced in *uml* mutants (see Fig. S2T,U in the supplementary material). These data suggest that Boc plays a minor role in slow muscle fiber differentiation, and might act as an activator of the Hh response during adaxial cell specification.

Hh signal levels are reduced in the absence of Boc function

The ventral shift in *olig2* and *nkx6.1* expression in *uml* mutants (Fig. 2) suggested that Hh signal levels are generally reduced in the ventral CNS. To further examine how loss of Boc function affects overall levels of Hh signaling, we treated progeny from *uml(boc)* heterozygous parents with varying concentrations of the dose-dependent Hh signaling inhibitor cyclopamine (CyA) (Chen et al., 2002). In the absence of CyA, ~17% of embryos from a *uml* clutch had reduced *olig2* expression, indicating that this phenotype is not completely penetrant (see Fig. S3 in the supplementary material). Low doses of CyA (20 μM) had almost no effect on embryos from wild-type clutches, but increased the number of affected embryos in *uml(boc)* clutches from 17% to 33%. Furthermore, 13% of the embryos from these clutches had a more severe loss of *olig2* expression. Slightly higher doses of CyA (40 μM) increased the severity of defects, with 45% of the embryos in *uml* clutches having a severe loss of *olig2* expression. These CyA doses caused severe *olig2* expression defects in less than 5% of embryos from wild-type clutches. Both wild-type and *uml* mutant embryos had more severe *olig2* expression defects when Hh signaling was blocked further (see Fig. S3C,D in the supplementary material). These data suggest that *uml* homozygous and heterozygous embryos are more sensitive to the loss of Hh signaling than wild-type embryos and points to a general attenuation of Hh signaling in the absence of Boc function.

Boc function is required cell-autonomously in Hh-responding cells

To determine the site of action for Boc we generated genetic mosaic embryos by cell transplantation. To simplify the assay for the Hh transcriptional response, we performed these experiments using *Tg(nkx2.2a:megfp)* transgenic embryos (Ng et al., 2005). When targeted to the lateral floor plate region of *uml* mutant (or *boc* MO-injected) host embryos, isolated wild-type cells were able to respond to Hh signals, as indicated by expression of GFP from the *nkx2.2a:megfp* transgene (Fig. 6C). In the opposite experiment, *boc* mutant or morphant cells carrying the *nkx2.2a:megfp* transgene were never found to express GFP, even when located in the lateral floor plate region where *nkx2.2a* is normally expressed (Fig. 6D). These results show that Boc function is required cell-autonomously for cells to respond to maximal Hh levels in the ventral CNS (Fig. 6E,F). Importantly, normal Boc function in adjacent cells did not compensate for the lack of Boc function in transplanted cells.

DISCUSSION

Boc as a positive regulator of Hh signaling

Our analysis of the zebrafish *uml(boc)* mutant shows for the first time that Boc is required for the transcriptional response to Hh in the ventral vertebrate CNS. Boc appears to be required for the transcriptional activation of Class II Hh target genes that require maximal Hh signal levels, as transcription of the most ventrally expressed Hh-regulated genes fails to be activated in the absence of Boc function (Fig. 2). Increasing Hh signals artificially in *uml(boc)* mutants rescued gene expression in the normal expression domains (Fig. 5), suggesting that Boc acts to amplify Hh signals that are otherwise subthreshold for the activation of these genes (Fig. 6E). The ventral shift in the *olig2* and *nkx6.1* expression domains suggests that the overall Hh signal gradient is also shifted ventrally in *uml(boc)* mutants (Fig. 6F). Consistent with this idea, Boc dosage seems to affect cellular sensitivity to Hh, as indicated by the increased sensitivity to the Hh-inhibiting drug CyA seen in heterozygous *uml(boc)* mutants (see Fig. S3 in the supplementary material).

Consistent with an activator function for Boc in Hh signaling, we show that overexpression of *boc* or *shh* can rescue Hh target gene expression in the lateral floor plate in *uml(boc)* mutants (Fig. 5). The fact that this rescue is not uniform could indicate that the dose of Boc, which is variable in mRNA injection experiments, is crucial for a proper Hh response. Shh injections into *uml(boc)* mutants resulted in minimal ectopic Hh target gene activation, suggesting that Boc can affect the Hh response throughout the CNS. Given the fact that Boc can directly bind to Shh (Tenzen et al., 2006) and our data that Boc is required cell-autonomously for maximal Hh signaling (Fig. 6), we suggest that Boc amplifies early Hh signaling by increasing local Hh concentrations and/or the duration of Hh binding to responding cells (Fig. 6E). Intriguingly, *boc* expression quickly becomes downregulated in the ventral CNS, suggesting that this amplification serves as a transient kick-start needed for maximal Hh signaling in the ventral CNS.

Studies using neural explants indicated that Boc can function cell-autonomously to activate Hh signaling (Tenzen et al., 2006); however, loss of Boc function in mice did not appear to grossly affect Hh-mediated CNS patterning (Okada et al., 2006), hindering a closer examination of this issue. Recent work in *Drosophila* uncovered a subtle requirement for the Boc homolog (*boi*) in the induction of Hh target genes, and this phenotype was enhanced when the closely

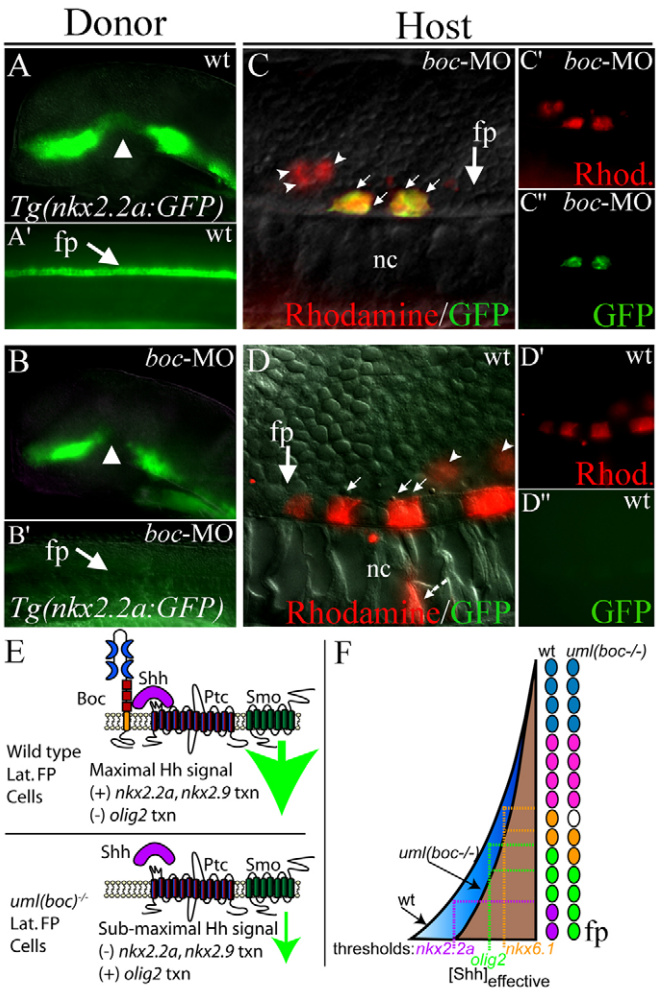

Fig. 6. *boc* is required cell-autonomously for Hh target gene expression in the ventral spinal cord. (**A**,**A′**) GFP expression in a *Tg(nkx2.2a:megfp)* transgenic donor embryo reports wild-type *nkx2.2a* expression in the forebrain (A) and spinal cord (A′, lateral view of trunk). (**B**,**B′**) Loss of Boc function (*boc* MO injection) leads to the regional loss of *nkx2.2a* expression in the forebrain (arrowhead in B) and complete loss of *nkx2.2a* expression in the floor plate (arrow in B′) in 100% of injected embryos (see Fig. 4). (**C-C″**) Example of transplanted wild-type cells (Rhodamine; red) in a host embryo that lacks Boc function (*boc* MO-injected). Wild-type donor cells that are located in the floor plate region (arrows) express *nkx2.2a* despite the lack of *boc* function in surrounding cells. More dorsally located cells do not express the *nkx2.2a:megfp* transgene (arrowheads). *nkx2.2a* expression was seen in 23 cells in four embryos in this experimental paradigm. For clarity, C′ and C″ show Rhodamine and GFP fluorescence alone. (**D**) A wild-type host embryo containing cells that lack *boc* function (from *boc* MO-injected donors as seen in B). In this scenario, no cells were found that expressed the *nkx2.2a:megfp* transgene, even when transplanted cells were located in the floor plate region. A total of 59 cells in the floor plate region of six embryos were assayed in this experimental paradigm. For clarity, D′ and D″ show Rhodamine and GFP staining alone. (**E**) Schematic model of Hh signal levels in wild-type or Boc deficient cells. Boc binding to Shh could facilitate the binding of Shh to Ptc, increasing local concentrations of Shh or the length of binding time, thus generating a maximal Hh signal (large green arrow). Without Boc, the Hh signal is reduced (small green arrow). (**F**) Schematic model of spinal cord Hh signaling levels in the presence (blue) or absence (brown) of Boc function. Colored circles depict individual cells in the zebrafish spinal cord. Loss of Boc function might lower the effective concentration of Shh in the ventral spinal cord, eliminating expression of genes that require the highest levels of Hh signaling for transcriptional activation (e.g. *nkx2.2a*). Expression domains for genes with slightly lower Shh thresholds are shifted ventrally. fp, floor plate; nc, notochord.

related iHog protein was also removed (Zheng et al., 2010), suggesting overlapping functions for these two molecules. Given our results and the *Drosophila* data, the lack of obvious ventral CNS phenotypes in Boc mutant mice might be explained by a functional overlap with the closely related molecule Cdo. Although Cdo is expressed in the floor plate in both mouse (Tenzen et al., 2006) and fish (see Fig. S1E in the supplementary material), the timing of expression appears to be slightly later in fish, which might prevent Cdo from compensating for the loss of Boc function. If true, we would expect that roles for these proteins in activating Hh signaling would be revealed in mouse Boc-Cdo double mutants.

Boc as a negative regulator of Hh signaling

Transcription of *boc* is clearly repressed by Hh in zebrafish and mouse (see Fig. S1 in the supplementary material), consistent with negative regulators of the Hh response such as *cdo* and *gas1* (Tenzen et al., 2006). Gas1 and Cdo can bind Shh and might act to shape the Hh response gradient by limiting diffusion or activity of Hh proteins. Indeed, explant studies show that Boc can act in a non-cell-autonomous manner to negatively regulate Hh signaling in cells adjacent to Boc-expressing cells (Tenzen et al., 2006). We did not see changes in the expression of dorsal Hh-regulated genes in the spinal cord of *uml(boc)* mutants. One possibility is that Cdo, Gas1 and/or other dorsally expressed Hh-binding proteins could mask a role for Boc as a negative regulator of Hh signals.

The fact that the lower jaw is present in *uml(boc)* mutants clearly indicates that Boc is not required as an activator of Hh-mediated cell specification and proliferation in this tissue (Brand et al., 1996; Schwend and Ahlgren, 2009; Teraoka et al., 2006). In fact, the expansion of jaw tissue in *uml* mutants suggests that Boc could be acting as a negative regulator of Hh signaling in this tissue. Given the direct role for Hh at later stages of jaw development (Schwend and Ahlgren, 2009) and the high level of *boc* expression in the jaw at this time (see Fig. S1 in the supplementary material), we propose that high levels of Boc in the jaw could bind Shh and limit the ability of Hh to activate chondrogenesis and proliferation in this tissue.

Boc in forebrain patterning and axon guidance

Forebrain patterning defects in *uml(boc)* mutants are strikingly similar to those seen in the Hh pathway mutants *yot(gli2)* and *dtr(gli1)* (Figs 1, 2) (Karlstrom et al., 1996; Karlstrom et al., 2003), consistent with a role for Boc in Hh-mediated forebrain patterning. This is supported further by the rescue of forebrain gene expression following overexpression of *boc* or *shh* in *uml(boc)* mutants (Fig. 5). Although a role for Boc in mammalian forebrain development has not been reported, loss of the closely related Cdo protein leads to reduced Hh target gene expression in the forebrain and defects in midline facial and brain structures associated with holoprosencephaly (HPE) that are enhanced by reductions in Hh

signaling (Zhang et al., 2006). Ventral *boc* expression in the early zebrafish brain (see Fig. S1A in the supplementary material) is consistent with an early cell-autonomous role for Boc in Hh-mediated forebrain patterning, as we show for the spinal cord (Fig. 6). This suggests that *boc* might be a candidate gene for human HPE (Ming and Muenke, 1998).

Despite the fact that Boc is known to play a direct role in axon guidance of mouse commissural neurons (Okada et al., 2006) and retinal ganglion cells (Fabre et al., 2010), retinal axon guidance defects in *uml(boc)* mutants (Fig. 1) are probably indirect. In fact, we saw no evidence of *boc* expression in zebrafish RGCs up to 5 dpf, suggesting that Boc does not play a direct role in RGC axon guidance. Our work in the Hh pathway mutants *yot(gli2)* and *dtr(gli1)* showed that expanded expression of Slit-repellent cues accounts for the ipsilateral RGC axon projections seen in these mutants (Barresi et al., 2005). Similar to these mutants, *slit* expression is expanded across the POA in *uml(boc)*. Midline-spanning glia are reduced in *uml(boc)*, as they are in *yot(gli2)* and *dtr(gli1)*, suggesting that POC and RGC axon guidance defects are likely to be caused by earlier defects in the forebrain growth substrate.

Boc and pituitary development

The reduced expression of *nkx2.2a* in the developing pituitary placode, combined with reductions in the pituitary endocrine cell types that require the highest levels of Hh signaling (see Fig. S2 in the supplementary material) (Guner et al., 2008; Devine et al., 2009), suggest a novel role for Boc in Hh-mediated pituitary patterning. Low-level *boc* expression was detected in the placode region prior to 24 hpf, however *boc* expression was not seen in the placode by 36 hpf. Thus, Boc might be transiently required for the maximal response to Hh in the early pituitary placode, similar to the situation in the ventral spinal cord.

Boc and muscle differentiation

Although no muscle phenotypes have been reported for *Boc* mutant mice, *Cdo* mouse mutants have delayed development of all somitic musculature (Cole et al., 2004). In cultured cells, Boc was shown to bind Cdo at its extracellular domain to form a myogenesis-promoting complex (Kang et al., 2002). The number of slow muscle fibers is slightly reduced in zebrafish *uml(boc)* mutants (see Fig. S3 in the supplementary material), confirming a role for Boc in muscle differentiation. The reduction in *myoD* expression in adaxial cells (see Fig. S3 in the supplementary material) and of *ptch2* expression throughout developing somites (data not shown) in *uml(boc)* mutants is similar to that seen in *yot(gli2)* heterozygotes (Karlstrom et al., 2003), and is consistent with a reduction in Hh signal levels in these cells. Starting at 20 hpf, *boc* is expressed in superficial muscle fibers that are distant from the midline source of Shh (see Fig. S1J in the supplementary material) suggesting that Boc could continue to play a role in myogenesis that is independent of Hh signaling, as was recently shown for Cdo (Lu and Krauss, 2010).

From axon guidance to Hh signaling

The first large-scale zebrafish genetic screen uncovered 13 mutations in the Shh pathway (to date), including six mutations that affect retinotectal axon guidance. Here, we show that the last of the original ipsilateral mutants (Karlstrom et al., 1996) affects a poorly understood component of the Hh signaling pathway, Boc. Ironically, despite a known role for Boc in Hh-mediated axon guidance in the mouse spinal cord (Okada et al., 2006), our studies indicate that the retinal axon guidance errors seen in *uml* mutants are likely to be an indirect outcome of earlier Hh-mediated forebrain patterning defects, with Boc acting as an early activator of Hh signaling. A later role for Boc as a repressor of Hh signals is consistent with *boc* expression in the dorsal CNS and with the increased lower jaw structure seen in *uml* mutants. The *uml(boc)* mutant thus provides a new tool for dissecting the overlapping roles of multiple Hh repressor proteins in development. Given the key role Hh signaling plays in regulating neural and cancer stem cell proliferation, a detailed knowledge of Hh inhibitory proteins might be particularly important for the treatment of human cancers as well as for stem cell therapies.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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