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Boc and Gas1 each form distinct Shh receptor complexes with Ptch1 and are required for Shh-mediated cell proliferation

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SUMMARY

Hedgehog (Hh) proteins regulate important developmental processes including cell proliferation and differentiation. While Patched acts as the main Hh receptor, in *Drosophila*, Hh signaling absolutely requires the additional Hh-binding proteins Ihog and Boi. Here we show that, unexpectedly, cerebellar granule neuron progenitors (CGNPs) lacking Boc and Cdon, the vertebrate orthologs of Ihog and Boi, still proliferate in response to Hh. This is because in their absence, Gas1, a Hh-binding protein not present in *Drosophila*, mediates Hh signaling. Consistently, only CGNPs lacking all three molecules Boc, Cdon, and Gas1 have a complete loss of Hh-dependent proliferation. In a complementary manner, we find that a mutated Hh ligand which binds Patched1 but neither Boc, Cdon, nor Gas1 cannot activate Hh signaling. Together, this demonstrates an absolute requirement for Boc, Cdon, and Gas1 in Hh signaling and reveals a distinct requirement for ligand-binding components that distinguishes the vertebrate and invertebrate Hh receptor systems.

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INTRODUCTION

Hh proteins are key molecules for diverse tissue patterning processes in both invertebrates and vertebrates. For example, in *Drosophila*, Hh is crucial for the development of a segmented body plan and the patterning of imaginal tissues, whereas in vertebrates Sonic hedgehog (Shh) functions to pattern limb buds and promote cell fate specification, proliferation, and axon guidance in the central nervous system (Charron and Tessier-Lavigne, 2005; Dessaud et al., 2008; Ingham and Placzek, 2006; Jiang and Hui, 2008). Shh is synthesized as a 45 kDa pro-protein and post-translational modifications generate a biologically active 19 kDa N-terminal fragment. Shh initiates signaling by binding the 12-pass transmembrane protein Patched1 (Ptch1). Upon Shh binding, the inhibition exerted by Ptch1 on the 7-pass transmembrane protein Smoothed (Smo) is relieved, eliciting a signaling cascade which ultimately leads to Gli-mediated transcription.

In addition to Ptch1, several membrane-associated proteins are thought to function as accessory receptors that promote Shh signaling. The related molecules Cdon (cell-adhesion-molecule-related/downregulated by oncogenes) and Boc (biregional Cdon-binding protein) positively regulate Shh signaling, promoting Shh-dependent cell fate specification and axon guidance (Okada et al., 2006; Tenzen et al., 2006; Yao et al., 2006; Zhang et al., 2006). They are single-pass transmembrane proteins of the Immunoglobulin (Ig) superfamily and contain an extracellular region consisting of four (Boc) or five (Cdon) Ig repeats and three Fibronectin type 3 repeats (FNIII). In *Drosophila*, Ihog (Interference hedgehog) and Boi (Brother of Ihog), the orthologs of Boc and Cdon, are redundant with one another and are absolutely required for Hh-dependent patterning (Camp et al., 2010; Zheng et al., 2010). However, whether Boc and Cdon are absolutely essential for Shh signaling in vertebrates is unknown.

Furthermore, additional membrane-associated proteins have been shown to positively modulate Shh signaling in vertebrates. Growth Arrest Specific 1 (Gas1), a GPI-linked protein bearing no structural resemblance to Boc and Cdon and with no orthologues in *Drosophila*, binds Shh and regulates ventral specification of neural progenitors during neural tube development by promoting Shh signaling (Allen et al., 2007; Martinelli and Fan, 2007a, b). Interestingly, analysis of *Gas1* mutant mice shows that it is a positive regulator of cerebellar size and CGNP proliferation (Liu et al., 2001), two processes that are normally driven by Shh during early postnatal development. However, whether Gas1 acts in a Shh-dependent manner in CGNP proliferation has yet to be determined.

Thus, Boc, Cdon and Gas1 have each been proposed to function as positive modulators of Shh signaling (Allen et al., 2007; Martinelli and Fan, 2007a; Okada et al., 2006; Seppala et al., 2007; Tenzen et al., 2006). We hypothesized that they are obligate Shh receptors with Ptch1, and not simply modulators of the signaling cascade. If this model were correct, simultaneous disruption of all three molecules would completely abrogate Shh signaling. We tested this by investigating the proliferation of CGNPs in response to Shh in the developing cerebellum. We found that CGNPs express Boc and Gas1, but not Cdon. Interestingly, the cerebellum is smaller in *Boc*^{-/-} mice, and *Boc*^{-/-} CGNPs have lower proliferation than wild-type CGNPs in response to Shh. Similarly, *Gas1*^{-/-} CGNPs are also less responsive to Shh, while *Gas1*^{-/-};*Boc*^{-/-} CGNPs are completely unable to proliferate in response to Shh. We further demonstrated that Boc and Gas1 interact with Ptch1 and form distinct receptor complexes. Finally, we generated a Shh mutant protein that binds Ptch1 but not Boc, Cdon nor Gas1 and found that this molecule could not elicit Shh-dependent signaling and CGNP proliferation. Together, our data indicates that Boc, Cdon and Gas1 are necessary components of the Shh receptor complex and are essential for Shh signal transduction in vertebrates. While Ihog and Boi are necessary to mediate Hh signaling in

Drosophila (Camp et al., 2010; Zheng et al., 2010), we show that Gas1 is an additional component of the Hh receptor system in vertebrates that can compensate for the absence of Boc and Cdon.

RESULTS

Boc, but not Cdon, is expressed in proliferating CGNPs of the cerebellum

To investigate the receptor requirements for the proliferative effect of Shh in CGNPs, we first analyzed Boc and Cdon expression in the developing cerebellum. CGNPs arise from the rhombic lip (RL) between embryonic day (E) 13.5–14.5 and migrate anteriorly over the cerebellar anlage, forming the highly proliferative external germinal layer (EGL) (Roussel and Hatten, 2011). Starting at E17.5 and continuing during early postnatal development, Purkinje cells (PCs) lining the EGL stimulate CGNP proliferation by secreting Shh (Dahmane and Ruiz i Altaba, 1999; Kenney and Rowitch, 2000; Wallace, 1999; Wechsler-Reya and Scott, 1999). Following a proliferative burst, CGNPs stop dividing, differentiate into granule neurons, migrate inwards past the PC layer and populate the internal granular layer (IGL).

We first examined Boc and Cdon expression in the cerebellar anlage of E14.5 mouse embryos. Immunostainings of sagittal sections showed that while Boc was expressed in the presumptive EGL, RL and the ventricular zone of the roof of the 4th ventricle, Cdon expression was restricted to the RL (Fig. 1A). At E18.5, a stage at which CGNPs proliferate in response to Shh, we detected Boc expression in the EGL and, albeit at lower level, in the PC layer of the developing cerebellum. In contrast, Cdon expression was limited to the tip of the RL.

Analysis of post-natal day (P) 6 *Boc*^{+/-} and *Cdon*^{+/-} gene-targeted mice encoding a β -galactosidase (β -Gal)-neomycin reporter gene fusion (β -geo) (Okada et al., 2006) revealed strong β -Gal activity in *Boc*^{+/-} cerebellum, but was limited to the choroid plexus of *Cdon*^{+/-} cerebellum. Immunostainings confirmed this expression pattern and revealed that Boc localized to cells expressing Lim1, a marker for CGNPs and PCs (Fig. 1B,C). Interestingly, while highest levels of Boc were detected in the outer proliferative region of the EGL (Lim1⁺, Pax6⁺ and TAG1⁻ cells), lower levels were observed in differentiated migratory granule cells (TAG1⁺ cells) and in PCs (Calbindin⁺ cells). These results show that Boc, but not Cdon, is highly expressed in proliferating CGNPs of the cerebellum.

Boc is important, but not absolutely required, for Shh-mediated CGNP proliferation

To investigate the role of Boc in cerebellum development, we examined the gross morphology of *Boc*^{-/-} cerebella. While *Boc*^{-/-} mice are viable and cannot be distinguished from their littermates, their cerebellum is smaller than *Boc*^{+/-} or WT animals (Fig. 2A and data not shown). *Boc*^{-/-} cerebella were 14.3±0.05% (p<0.001) lighter than that of *Boc*^{+/-} cerebella (Fig. 2B). When the mass of the cerebellum was normalized to the body weight (p<0.001), the relative cerebellar mass was still reduced, indicating that this difference is not due to an overall decrease in total body weight (Fig. 2B). The cerebellum and IGL surface areas measured from sagittal sections of *Boc*^{-/-} adult mice were also reduced when compared to *Boc*^{+/-} animals (Fig. 2C,D; p<0.001 and 0.05, respectively). Although the IGL surface area is diminished in adult mice, migration of granule neurons and cerebellum foliation did not appear to be affected in *Boc*^{-/-} mice.

The decrease in cerebellum size in the absence of Boc could be due, at least in part, to reduced cell proliferation and/or enhanced cell death. TUNEL staining showed no significant difference in the number of apoptotic cells between *Boc*^{-/-} and *Boc*^{+/-} cerebella (Fig. 2E,F). In contrast, measurement of BrdU incorporation in the EGL of *Boc*^{-/-} and

Boc^{+/-} mice showed that 40±1% of *Boc*^{+/-} CGNPs were actively dividing, compared to only 30±3% of *Boc*^{-/-} CGNPs (p<0.05) (Fig. 2G,H). Phospho-histone H3 (pH3) staining also showed a significant reduction in the number of mitotic pH3-labeled cells per mm² of EGL in *Boc*^{-/-} mice compared to *Boc*^{+/-} mice (p<0.05) (Fig. 2H). Together, these *in vivo* data indicate that *Boc* plays a role in CGNP proliferation.

Since *Boc* modulates Shh signaling (Okada et al., 2006; Tenzen et al., 2006; Zhang et al., 2006), we next tested whether *Boc* mediates Shh-induced CGNP proliferation. We cultured CGNPs purified from *Boc*^{-/-}, *Boc*^{+/-} and *Boc*^{+/+} mice in the presence of varying concentrations of recombinant Shh (ShhN) (Fig. 2I). While Shh treatment induced the proliferation of WT CGNPs over 6 fold compared to unstimulated CGNPs, Shh stimulation increased *Boc*^{-/-} CGNP proliferation only about 3 fold. Significant differences in the proliferation of *Boc*^{+/+}, *Boc*^{+/-} and *Boc*^{-/-} CGNPs was observed at all concentrations of ShhN used (Fig. 2I), indicating that *Boc* promotes proliferation of CGNPs in a gene copy-number dependent manner. Together with our *in vivo* data, these results indicate that *Boc*^{-/-} mice have a smaller cerebellum due to a decrease in Shh-dependent CGNP proliferation and that *Boc* acts cell-autonomously in CGNPs to regulate their proliferation.

Gas1 is important, but not absolutely required, for Shh-mediated CGNP proliferation

Whilst inactivation of *Boc* in CGNPs, which do not express *Cdon*, lead to a partial decrease in their proliferation, it did not abolish their response to Shh. Moreover, CGNP proliferation is not further decreased when *Cdon* is inactivated in *Boc*^{-/-} mice (Fig. S1). These results are not consistent with a model where *Boc* and *Cdon* act like their *Drosophila* orthologues *Ihog* and *Boi* and are absolutely required for Hh signaling in vertebrates (Camp et al., 2010; Zheng et al., 2010). This raises the possibility that, unlike *Drosophila*, additional or different Shh binding molecules (other than *Ptch1*, *Boc* and *Cdon*) are required for vertebrate cells to respond to Shh.

Given that *Gas1* binds Shh and modulates Shh signaling (Allen et al., 2007; Martinelli and Fan, 2007a, b; Seppala et al., 2007), we hypothesized that *Gas1* may be this additional receptor. We first characterized the expression pattern of *Gas1* in the developing cerebellum. Immunofluorescence stainings showed that *Gas1* is restricted to the presumptive EGL of the cerebellar primordium at E14.5 and continues to be expressed in the EGL at E18.5 (Fig. 3A). At P6, like *Boc*, *Gas1* localizes to *Lim1*⁺ cells in the EGL (Fig. 3B). *Gas1* staining is most intense in the outer proliferative layer of the EGL (*Lim1*⁺, *Pax6*⁺, *TAG1*⁻ cells) and was not detected in *TAG1*⁺ migratory granule neurons and in *Calbindin*⁺ PCs (Fig. 3C).

To determine whether *Boc* and *Gas1* are co-expressed in CGNPs, we performed immunostainings on consecutive sections of cerebellum from *Math1-Cre; mTmG*^{E18.5} mice, where the CGNPs express GFP following Cre-mediated recombination. We used this strategy instead of double immunostainings as both anti-*Boc* and anti-*Gas1* antibodies are produced in the same species. We found that both *Boc* and *Gas1* co-localize with GFP⁺ cells, indicating that *Gas1* and *Boc* are co-expressed in the same CGNPs (Fig. 3D).

Although the gross morphology of *Gas1*^{-/-} cerebella appears normal, they are smaller in size compared to control cerebellum and have decreased proliferation in the outer EGL (Liu et al., 2001). While this phenotype is reminiscent of that of *Boc*^{-/-} cerebella, no direct link has been made between the phenotype and the ability of *Gas1*^{-/-} CGNPs to respond to Shh. To directly test this, we performed proliferation assays on purified CGNPs from *Gas1*^{-/-} mice and control littermates. Our results show that *Gas1* is essential for normal CGNP proliferation in response to Shh (Fig. 4C). Interestingly, the mutation of *Gas1*, similarly to the mutation of *Boc*, is not sufficient to abrogate the response of CGNPs to Shh.

Shh-dependent proliferation is completely lost in *Gas1*^{-/-};*Boc*^{-/-} CGNPs

To determine whether Boc and Gas1 might have partially redundant functions in Shh-dependent CGNP proliferation, we examined the cerebellum of E18.5 *Gas1*^{-/-};*Boc*^{-/-} embryos, since these animals die at birth. Hematoxylin-eosin staining of *Gas1*^{-/-};*Boc*^{-/-} cerebella revealed a significant loss of the EGL compared to controls (Fig. 4A). Although *Gas1*^{+/-};*Boc*^{-/-} and *Gas1*^{-/-};*Boc*^{-/-} cerebella showed no significant difference in the cross-sectional area of the whole cerebellum, the overall area of *Gas1*^{-/-};*Boc*^{-/-} EGL was reduced by about 30% compared to controls ($p < 0.001$) (Fig. 4B). Quantitation of the EGL along the postero-anterior axis showed that the difference in EGL thickness is greatest towards the anterior pole of the cerebellum (Fig. 4B and S2D). Marker analysis showed that *Lim1* and *Pax6* were properly expressed in the EGL of *Gas1*^{-/-};*Boc*^{-/-} embryos compared to controls (Fig. S2A,B), thus, CGNPs are specified and localize normally. Furthermore, *Cdon* expression was not changed in the absence of Gas1 and Boc (Fig. S2C). However, the proliferation of *Gas1*^{-/-};*Boc*^{-/-} CGNPs was severely decreased compared to *Gas1*^{+/-};*Boc*^{-/-} CGNPs ($p < 0.001$) (Fig. 4B). Moreover, the number of pH3⁺ cells per μm^2 of EGL surface area was lower in *Gas1*^{-/-};*Boc*^{-/-} than *Gas1*^{+/-};*Boc*^{-/-} animals ($p < 0.05$) (Fig. 4B), demonstrating that the decrease in pH3⁺ cells in the EGL is not simply due to a total decrease in EGL area. These results indicate that Gas1 and Boc account for a large part of CGNP proliferation at this stage *in vivo*.

In addition to Shh, Insulin Growth Factor (IGF) and Notch signaling also promote CGNP proliferation (Corcoran et al., 2008; Solecki et al., 2001). Residual CGNP proliferation is observed in other mutant cerebella that lack Shh signaling (Corrales et al., 2004), thus, the proliferation observed in the EGL of *Gas1*^{-/-};*Boc*^{-/-} cerebellum is probably independent of Shh signaling. To test whether *Gas1*^{-/-};*Boc*^{-/-} cells have completely lost Shh responsiveness, we cultured CGNPs purified from E18.5 *Gas1*^{+/-};*Boc*^{-/-} and *Gas1*^{-/-};*Boc*^{-/-} cerebella with various ShhN concentrations. We found that while *Gas1*^{+/-};*Boc*^{-/-} CGNPs proliferate *in vitro* in response to Shh, *Gas1*^{-/-};*Boc*^{-/-} CGNPs show no enhanced proliferation in response to Shh (Fig. 4D). Importantly, the proliferative response of *Gas1*^{-/-};*Boc*^{-/-} CGNPs to IGF-I, another factor able to stimulate CGNP proliferation, remained similar to that of control cells (Fig. 4E). Furthermore, treatment with purmorphamine, a Smo agonist, induced the proliferation of *Gas1*^{-/-};*Boc*^{-/-} CGNPs ($p < 0.01$) (Fig. 4F), indicating that Boc and Gas1 function upstream of Smo. Together, our data indicates that the presence of either Gas1 or Boc is absolutely required for Shh to promote CGNP proliferation. Given that Shh signaling in the cerebellum begins only at E17.5 and that Shh signaling plays an even more important role in CGNP proliferation after birth than at E18.5 (Corrales et al., 2004; Flora et al., 2009; Lewis et al., 2004), we anticipate that the EGL of *Gas1*^{-/-};*Boc*^{-/-} mice would be much more severely reduced postnatally. However, because *Gas1*^{-/-};*Boc*^{-/-} mice die at birth, conditional alleles will be required to directly test this.

To test whether the lack of a proliferative response of *Gas1*^{-/-};*Boc*^{-/-} CGNPs to Shh *in vitro* is consistent with loss of Shh signaling *in vivo*, we examined the expression of *Gli1*, a Shh transcriptional target (Corrales et al., 2004), by RNA *in situ* hybridization. While control cerebella had intense *Gli1* signal in the EGL, *Gli1* expression was not detected in *Gas1*^{-/-};*Boc*^{-/-} cerebella (Fig. 4A), confirming the inactivation of Shh signaling in *Gas1*^{-/-};*Boc*^{-/-} cerebella.

Boc and Gas1 interact with Ptch1 and form distinct receptor complexes

We next investigated the molecular mechanism by which Boc and Gas1 act and, more specifically, whether they associate with Ptch1 to constitute the Shh receptor complex. We found that Boc and Gas1 can each co-immunoprecipitate with Ptch1, indicating that Boc and

Gas1 can physically interact with Ptch1 (Fig. 5A). Importantly, these interactions are specific to Ptch1, as both Dispatched-1 (Disp1) and Smo, two multi-span transmembrane proteins also involved in Shh signaling, failed to interact with either Boc or Gas1 (Fig. S3). Furthermore, the addition of Shh did not modify the ability of Ptch1 to interact with Boc, suggesting that their interaction is constitutive (Fig. 5B).

Mapping studies showed that the second large extracellular loop of Ptch1 (L2), which is necessary for binding to Shh (Marigo et al., 1996), was not required for the interaction with Boc. Ptch1 Δ L2-HA, a Ptch1 construct where L2 is deleted, interacted with Boc to an extent similar to full length Ptch1-HA (Fig. 5C). This is consistent with the binding of Shh to Ptch1 not being necessary for Ptch1 to interact with Boc. We next mapped the domain(s) of Boc mediating its interaction with Ptch1. Boc Δ Cyto-GFP, a mutant lacking the cytoplasmic domain of Boc, interacted with Ptch1 as strongly as full-length Boc-GFP (Fig. 5D), indicating that the cytoplasmic domain is not required for its association with Ptch1.

To further characterize the region of Boc that interacts with Ptch1, we performed binding assays with various derivatives of Boc-Fc fusion proteins encompassing the Boc extracellular domain and cells expressing Ptch1-GFP. Deletion analysis of the Boc extracellular domain revealed that removal of the FNIIIc domain (mutant Boc FNIII(ab)), shown to be required and sufficient for Shh binding (Okada et al., 2006), only marginally affected Ptch1 binding, while truncation of both the FNIIIa and FNIIIb domains (mutant FNIII(c)) abolished it almost entirely (Fig. 5G). Boc-Fc constructs containing either the FNIIIa or FNIIIb domains alone bound to Ptch1 at levels that were about 60% of that of Boc ecto-Fc. Together our data indicate that the Boc FNIIIa and FNIIIb domains are required and sufficient to mediate its interaction with Ptch1. In addition, the Boc FNIIIc domain, which is necessary for Shh binding, is not required for the Boc-Ptch1 interaction, further supporting a Shh-independent interaction between Boc and Ptch1.

We next tested whether Boc interacts with Gas1 and did not detect an interaction between Boc and Gas1 either in the absence or presence of Ptch1 (Fig. 5E, top panel, lanes 5–6), despite detecting a strong interaction between Boc and Ptch1 (Fig. 5E, middle panel, lane 6). These experiments suggest that Boc/Ptch1 complexes do not contain detectable amounts of Gas1 and that Boc, Ptch1 and Gas1 are unlikely to form a tripartite complex.

To further confirm these results, we performed the complementary experiment and looked for the presence of Boc in Gas1/Ptch1 complexes. Lysates of cells transfected with Ptch1-GFP, Boc-Flag and Gas1 were first immunoprecipitated with anti-Gas1 antibodies and, despite detecting a strong interaction between Gas1 and Ptch1 (Fig. 5F, IP#1 middle panel, lane 6), we did not detect an interaction between Boc and Gas1 in the absence nor presence of Ptch1 (Fig. 5F, top panel lanes 5–6). To confirm that Boc is indeed able to interact with Ptch1 in these lysates and test whether both Boc/Ptch1 and Gas1/Ptch1 complexes are present in the same cell lysates, we recovered the supernatants from the anti-Gas1 immunoprecipitation (IP#1) and subjected them to a second immunoprecipitation, this time with anti-Flag antibodies to immunoprecipitate Boc (Fig. 5F; see Fig. S4 for a schematic). We found that the Ptch1-GFP remaining in the supernatant efficiently co-immunoprecipitated with Boc (Fig. 5F, IP#2 middle panels, lane 6). Together, our data indicates that while Boc and Gas1 can both interact with Ptch1, it is unlikely that Boc, Gas1 and Ptch1 form a tripartite complex. Moreover, these results suggest that the Boc-Ptch1 and the Gas1-Ptch1 complexes are distinct molecular entities.

Binding of Shh to Ptch1 is not sufficient to activate Shh signaling

Our results indicate that Boc and Gas1 are required for Shh-mediated CGNP proliferation and that they form independent complexes with Ptch1. While Boc and Gas1 are essential

components of these receptor complexes, they could function as partners of Ptch1, but not necessarily as receptors that bind to Shh. To determine whether the binding of Shh to Gas1 and/or Boc (and Cdon) is required for a Shh response, we generated a mutant Shh protein unable to bind Boc/Cdon/Gas1 but retaining the ability to bind Ptch1. If this mutant Shh molecule with altered specificity no longer activates signaling, it would suggest that Shh binding to Boc/Cdon/Gas1 is required for pathway activation. Conversely, if this mutant form of Shh activates the pathway, it would support a model where binding to Ptch1 alone is sufficient for Shh signaling.

The amino acids responsible for mediating the interaction between Shh and Boc/Cdon have been identified from co-crystal structures of Shh and the third FNIII domain of Cdon and Boc (Fig. 6A,B) (Kavran et al., 2010; McLellan et al., 2008). Although similar structural data is unavailable for Shh in complex with Ptch1, mutagenesis of Shh surface amino acids has identified residues required and residues dispensable for the binding of Shh to Ptch1 (Fig. 6C) (Bosanac et al., 2009). Since Shh E90 is a contact amino acid between Shh and Cdon/Boc (McLellan et al., 2008) that is not required for binding to Ptch1 (Fig. 6A,C) (Bosanac et al., 2009), we predicted that a mutation at this site might affect binding to Boc and Cdon, but not to Ptch1. In contrast, Shh R154 is a contact amino acid between Shh and Boc/Cdon (McLellan et al., 2008) that is also required for Ptch1 binding (Fig. 6B,C) (Bosanac et al., 2009); thus, a R154 mutation is expected to affect binding of Shh to Boc, Cdon and Ptch1.

We introduced mutations of these residues into alkaline-phosphatase (AP)-tagged ShhN (ShhN-AP) and tested their binding to Boc, Cdon, Gas1, and Ptch1 (Fig. 6D,H and Table S1). Consistent with our structural predictions, ShhN-AP R154E was unable to bind to Boc, Cdon and Ptch1. Also in agreement with our predictions, ShhN-AP E90A did not bind to Boc and Cdon, but retained the ability to bind Ptch1, with a dissociation constant not significantly different ($p > 0.05$) from that of WT ShhN (Fig. 6H and Table S1). We also assessed the binding of our Shh mutants to Gas1 and found that they behaved similarly towards Gas1 as they did with Boc and Cdon: ShhN-AP E90A and R154E were both unable to bind Gas1. Thus, according to the binding characteristics of our Shh mutants, some common amino acids may mediate the interaction of Shh with Gas1, Boc, and Cdon, a finding consistent with previous reports (Kavran et al., 2010; McLellan et al., 2008)

We next examined the effect of the E90A and R154E mutations on Shh signal transduction. We introduced these mutations into untagged ShhN and recombinant proteins were purified (Fig. 6D). To measure the signaling activity of the ShhN E90A and R154E mutants, we performed transcription reporter assays using cells stably transfected with a Gli-luciferase reporter plasmid. While WT ShhN activated Shh-mediated transcription in a concentration-dependent manner, ShhN E90A and R154E mutants were unable to do so (Fig. 6E). We next tested the ability of our Shh mutants to promote the osteoblastic differentiation of C3H 10T $\frac{1}{2}$ cells and neither ShhN E90A nor R154E were able to induce alkaline phosphatase expression, a marker of differentiation (Fig. 6F). Finally, we assayed the ability of these altered-specificity Shh ligands to induce CGNP proliferation. We found that while WT ShhN activated Shh-mediated proliferation in a dose-dependent manner, both ShhN E90A and R154E mutants were unable to induce proliferation (Fig. 6G). Together, these data show that ShhN E90A, which interacts with Ptch1 but not with Boc, Cdon, and Gas1, fails to induce Shh signaling and Shh-dependent cellular responses. This indicates that binding of Shh to Ptch1 alone is not sufficient to activate Shh signaling, suggesting that binding to Boc, Cdon, or Gas1 cell surface proteins is absolutely required for Shh-dependent signal transduction to occur.

DISCUSSION

In this study, we used three complementary approaches to establish the receptor requirements for Shh signaling in vertebrates. In one approach, we generated an altered-specificity Shh ligand (ShhN E90A) that cannot bind Boc, Cdon, and Gas1, but still binds Ptch1. This ligand was unable to induce Shh-dependent signaling, Shh-dependent osteoblastic differentiation, and CGNP proliferation, indicating that binding to Ptch1 alone is not sufficient to induce Shh signaling. In a second approach, we used gene inactivation of *Boc* and/or *Gas1*, and examined the ability of CGNPs, which already do not express Cdon, to respond to Shh. While inactivation of either *Boc* or *Gas1* caused a partial decrease in the ability of CGNPs to respond to Shh, the inactivation of both completely abolished the response. Thus, Boc and Gas1 are absolutely required for CGNPs to respond to Shh, consistent with our finding that a Shh mutant unable to bind Boc and Gas1 cannot activate Shh signaling in CGNPs. Further supporting the idea that Boc and Gas1 are Shh receptors functioning upstream of Smo, we showed that a Smo agonist rescued the proliferation defect of *Gas1;Boc* mutant CGNPs. Thirdly, we used a biochemical approach to show that Boc and Gas1 interact with Ptch1. The interaction between Boc and Ptch1 appears to be constitutive as it is not modulated by Shh and it occurs independently of the Shh-binding domains in Boc and Ptch1. We also observed that Boc/Ptch1 complexes do not contain detectable amounts of Gas1 (and that Gas1/Ptch1 complexes do not contain detectable amounts of Boc), suggesting that Boc/Ptch1 and Gas1/Ptch1 complexes are distinct entities.

Together, these results lead us to propose the following model. Boc, Cdon, and Gas1 interact with Ptch1 to form Boc/Ptch1, Cdon/Ptch1 or Gas1/Ptch1 complexes (Fig. 7), with the Boc/Ptch1 interaction being mediated by the FNIIIa and FNIIIb domains of Boc. The combination of receptor complexes present in a cell would depend on the expression of Boc, Cdon, and Gas1. In the presence of Shh, the ligand binds to Boc/Ptch1, Cdon/Ptch1, and Gas1/Ptch1 receptor complexes, leading to the de-repression of Smo, which in turn activates a series of signal transduction events that result in Gli-mediated transcription. In the case of Boc and Cdon, the interaction with Shh is direct and is mediated by the third FNIII (FNIIIc) domain of Boc and Cdon (McLellan et al., 2008; Okada et al., 2006; Tenzen et al., 2006; Yao et al., 2006).

Gas1 is an essential vertebrate Shh binding protein

Previous studies have shown that forced expression of Boc, Cdon, or Gas1 can potentiate Shh signaling and that inactivation of *Cdon* or *Gas1* can decrease Shh signaling in the neural tube (Allen et al., 2007; Martinelli and Fan, 2007a; Tenzen et al., 2006). Although these studies suggested a modulatory role for Boc, Cdon, and Gas1 in Shh signaling, whether Ptch1 alone is sufficient in the absence of any of these receptors remained an open question. Our study, together with a companion study by Allen et al. showing that embryos mutant for *Boc*, *Cdon*, and *Gas1* completely lack Shh signaling in the neural tube, demonstrate that in vertebrates these receptors do not function solely as auxiliary Shh receptors but are absolutely required for Shh signaling *in vivo*.

In *Drosophila*, Ihog and Boi, the orthologs of Boc and Cdon, are absolutely required for Hh signaling (Camp et al., 2010; Zheng et al., 2010). If Hh receptor requirements were entirely conserved, it would have been expected that vertebrate cells lacking Boc and Cdon would not be able to respond to Shh. However, the inactivation of *Boc* in CGNPs (which do not express Cdon) leads to a partial decrease in their proliferative response to Shh. Moreover, inactivation of *Cdon* in *Boc*^{-/-} cerebella did not further decrease CGNP proliferation compared to inactivation of *Boc* alone (Fig. S1). Hence, unlike *Drosophila* which absolutely require either Ihog or Boi for Hh signaling, vertebrate cells still respond to Shh in absence of Boc and Cdon. These results highlight a fundamental difference between *Drosophila* and

vertebrate Hh signaling and raised two possible mechanisms for Hh reception in vertebrates: Either Ptch1 is sufficient for Shh signaling or, unlike *Drosophila*, additional Shh binding molecules enable vertebrate cells to respond to Shh. Our data, together with results from Allen et al. (Allen et al., submitted), support a model where Gas1 acts with Boc and Cdon as essential Shh receptors. We showed that the combined inactivation of *Boc*, together with *Gas1*, completely abolishes the ability of CGNPs to respond to Shh, and that a mutant Shh ligand which binds Ptch1 (but not Boc, Cdon nor Gas1) is insufficient to activate Shh signaling. Thus, Gas1 – which is not present in the *Drosophila* genome – is an essential vertebrate Shh binding protein.

Other differences also exist between Hh signaling in invertebrates and vertebrates. The crystal structure of Hh in complex with Ihog, and Shh in complex with Cdon show that while Shh interacts with the third FNIII domain of Cdon and Boc (McLellan et al., 2008; Okada et al., 2006; Tenzen et al., 2006), Hh interacts with the non-orthologous first FNIII domain of Ihog and Boi (McLellan et al., 2006; Yao et al., 2006). In addition, the mode of Hh binding by the FNIII domains is not conserved between *Drosophila* and vertebrates (McLellan et al., 2008).

Previous studies in *Drosophila* S2 cells have shown that expression of Ihog results in a dramatic relocalization of Ptc to the cell surface (Zheng et al., 2010). Surprisingly, neither Boc nor Gas1 expression resulted in an increased relocalization of Ptch1 to the surface in at least two mammalian cell types (Fig. S5A,B). Surface biotinylation experiments also led to the same conclusion (Fig. S5C). While it is possible that tagging Ptch1 with GFP may interfere with its relocalization, we think that this is unlikely to occur, as Ptch1-GFP appears properly targeted to the cell surface given that we were able to detect an interaction between Boc and Ptch1-GFP in our cell surface binding assays (Fig. 5G). Thus, in contrast to what was observed for Ihog and Ptc, Boc and Gas1 do not appear to play a major role in relocalizing Ptch1 to the cell surface. Taken together, these results highlight important differences between *Drosophila* and vertebrate Hh signal reception.

Another fundamental difference is the requirement for the primary cilium for vertebrate Hh signaling (Eggenschwiler and Anderson, 2007). While Boc and Gas1 do not relocalize Ptch1 to the cell surface, it will be interesting to determine whether they contribute to its localization at the primary cilium (Corbit et al., 2005; Rohatgi et al., 2007).

Another interesting subject for future investigation is whether posttranslational modifications and multimerization of Shh (Dessaud et al., 2008) affect its interaction with the receptor complexes described in this study.

Are there specialized functions for Boc, Cdon, and Gas1?

Although Boc and Gas1 seemed to act redundantly in our CGNP proliferation experiments, it is possible that, in other cellular contexts, Boc, Gas1, or Cdon might impart additional non-redundant functions to the Shh signaling pathway. For example, Boc is required for Shh-mediated axon guidance (Fabre et al., 2010; Okada et al., 2006). Shh guides axons through a Src-family kinase (SFKs) dependent and Gli-independent pathway, where SFKs couple Shh signaling to cytoskeletal changes that elicit axon turning (Charron et al., 2003; Yam et al., 2009). It is possible that axon guidance by Shh is a specific function of Boc, since inactivation of Cdon did not disrupt axon guidance (Fabre et al., 2010; Okada et al., 2006). Thus, it is likely that different Shh receptor complexes, besides eliciting a canonical Shh signal transduction cascade, also impart additional functions. We speculate that the acquisition of novel functions for the vertebrate Shh signaling pathway during evolution might have paralleled the appearance of additional Shh binding proteins, such as Gas1.

A role for Boc, Cdon and Gas1 receptors in Hh signaling pathway-dependent pathologies

Many lines of evidence suggest that disruption of Hh proteins binding to Boc, Cdon, and Gas1 have pathological outcomes in humans. It is noteworthy that mutations of Shh amino acids important for binding to Boc, Cdon, and Gas1 have been identified in holoprosencephaly (HPE) and in brachydactyly (McLellan et al., 2008). Moreover, mutations in GAS1 have been identified in HPE patients (Ribeiro et al., 2010) and, in mouse, inactivation of *Cdon* and *Gas1* lead to HPE (Seppala et al., 2007; Zhang et al., 2006). In addition, while *Boc*^{-/-} mice do not display HPE, inactivation of *Boc* in a *Cdon* mutant background enhances the severity of the HPE phenotype in a dosage-dependent manner (Zhang et al., 2010). Thus, the identification and molecular understanding of the exact components and interactions found in the vertebrate Shh receptor complexes might help our understanding of pathologies associated with defective Shh signaling. Additionally, because we show that, in addition to cell fate specification, these complexes are also involved in the control of cellular proliferation, our results provide new avenues for the treatment of Hh pathway-dependent cancers (Scales and de Sauvage, 2009).

EXPERIMENTAL PROCEDURES

See Supplementary material for additional procedures.

Mice

Boc and *Cdon* (Okada et al., 2006; Zhang et al., 2010) and *Gas1* (Martinelli and Fan, 2007a) mice were described previously.

Plasmids and reagents

Recombinant ShhN C24II and IGF-I were from R&D Systems. pEGFP-mCdon, pEGFP-mBoc and pCA-gap-EGFP were previously described (Okada et al., 2006). pEGFP-mPtc1, mGli1 and pcDNA3-mGas1 were kindly provided by C.C. Hui and V. Wallace. pEGFP-Smo was kindly provided by P. Beachy. (Okada et al., 2006)

Histology and immunohistochemistry

β -gal activity detection and immunohistochemistry on sections were performed according to protocols described previously (Charron et al., 2003; Fabre et al., 2010; Okada et al., 2006). Antibody dilutions: rabbit anti-mouse Lim1 (1:500, T. Jessell lab), rabbit anti-mouse Pax6 (1:100, Chemicon), rabbit anti-mouse Calbindin (1:200, Chemicon), goat anti-mouse TAG1 (1:400), rabbit anti-GFP (1:1000, Invitrogen), goat anti-mouse Boc (1:100, R&D), goat anti-mouse Cdon (1:250, R&D), goat anti-mouse Gas1 (1:200, R&D). Prior to performing immunostainings with anti-Boc antibody, sections were subjected to antigen retrieval for 1hr at 98°C in a sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0), cooled at RT for 20 minutes and washed extensively with PBS.

Isolation of CGNPs and in vitro proliferation assays

CGNPs were isolated from either E18.5 or P4 mouse or rat cerebella using a modified protocol previously described (Wechsler-Reya and Scott, 1999). Briefly, isolated cerebella were cut in small pieces and treated with 0.25% trypsin and DNaseI. Following trituration, single cell suspensions were centrifuged through a 30% to 65% Percoll step gradient. Cells harvested at the 30% interphase were resuspended in Neurobasal supplemented with B27, 0.5 mM L-Glutamine and Pen/Strep and plated in 96-well plates pre-coated with 100 μ g/ml poly-D-Lysine. For CGNP proliferation assays with IGF-I, cells were resuspended in Neurobasal supplemented with 0.06% D-glucose, 100 μ g/ml apo-transferrin, 16 μ g/ml putrescine, 30 nM sodium selenite, 20 ng/ml progesterone and 1 mg/ml BSA. For ³H-

thymidine incorporation assays, cells were seeded at 4×10^5 cells/well in a 96-well plate in triplicate and treated with ShhN C24II for 48h. CGNPs were pulsed with $1 \mu\text{Ci/ml}$ ^3H -thymidine (PerkinElmer) for the last 12h. Incorporation was measured using the Filtermate harvester (PerkinElmer) and TopCount NXT beta counter (Packard). Alternatively, CGNPs were seeded at 2.5×10^4 cells/well of a 96 well/plate and cultured as described above. Following 48h in culture, cells were fixed with 4% PFA, blocked with 10% PHT and immunostained with mouse anti-Ki67 antibody (1:100, Becton-Dickinson).

Statistical Methods

Unless otherwise noted, all data are expressed as mean \pm SEM. The statistical tests used to measure differences are indicated in the appropriate legends. Statistical significances are indicated as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; n.s., not significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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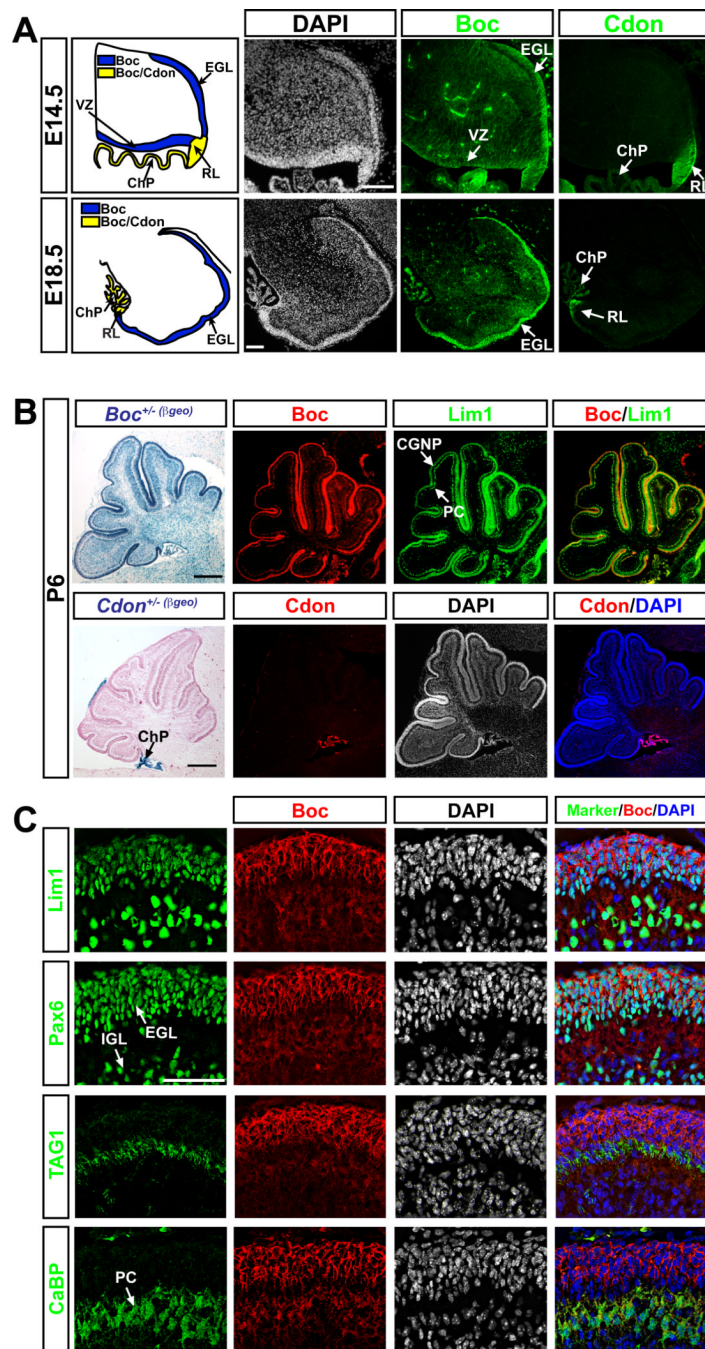


Figure 1. Boc and Cdon expression in the developing cerebellum

(A) Diagram of the developing cerebellum at E14.5 and E18.5. Immunostaining of Boc and Cdon in sagittal sections of the developing mouse cerebellum shows that Boc is expressed in the EGL, ChP and RL at E14.5 and expression is maintained in the EGL and ChP at E18.5. Cdon is expressed in the RL and ChP at E14.5 and E18.5. (B) At P6, Xgal staining (*left*, counterstained with nuclear fast red) and immunofluorescence on sagittal cerebellum sections from *Boc*^{+/-} and *Cdon*^{+/-} βgeo (β-galactosidase-neomycin) mice reveal Boc and Cdon expression. Boc is expressed in Lim1⁺ cells (B,C) and Cdon in the ChP (B). (C) P3 WT mouse cerebellum sections co-immunolabeled with Boc (red) and various cerebellar cell markers (green) showing high Boc expression in proliferating CGNPs (Lim1⁺, Pax6⁺) and

lower expression in PC (CaBP⁺, Lim1⁺) and differentiated granule cells (Pax6⁺ in the IGL). *Scale bars:* (A) 100 μm (B) 250 μm , (C) 50 μm . EGL, external germinal layer; IGL, internal granular layer; ChP, choroid plexus; RL, rhombic lip; PC, Purkinje cells; CGNP, cerebellar granule neurons; CaBP, Calbindin; VZ, ventricular zone.

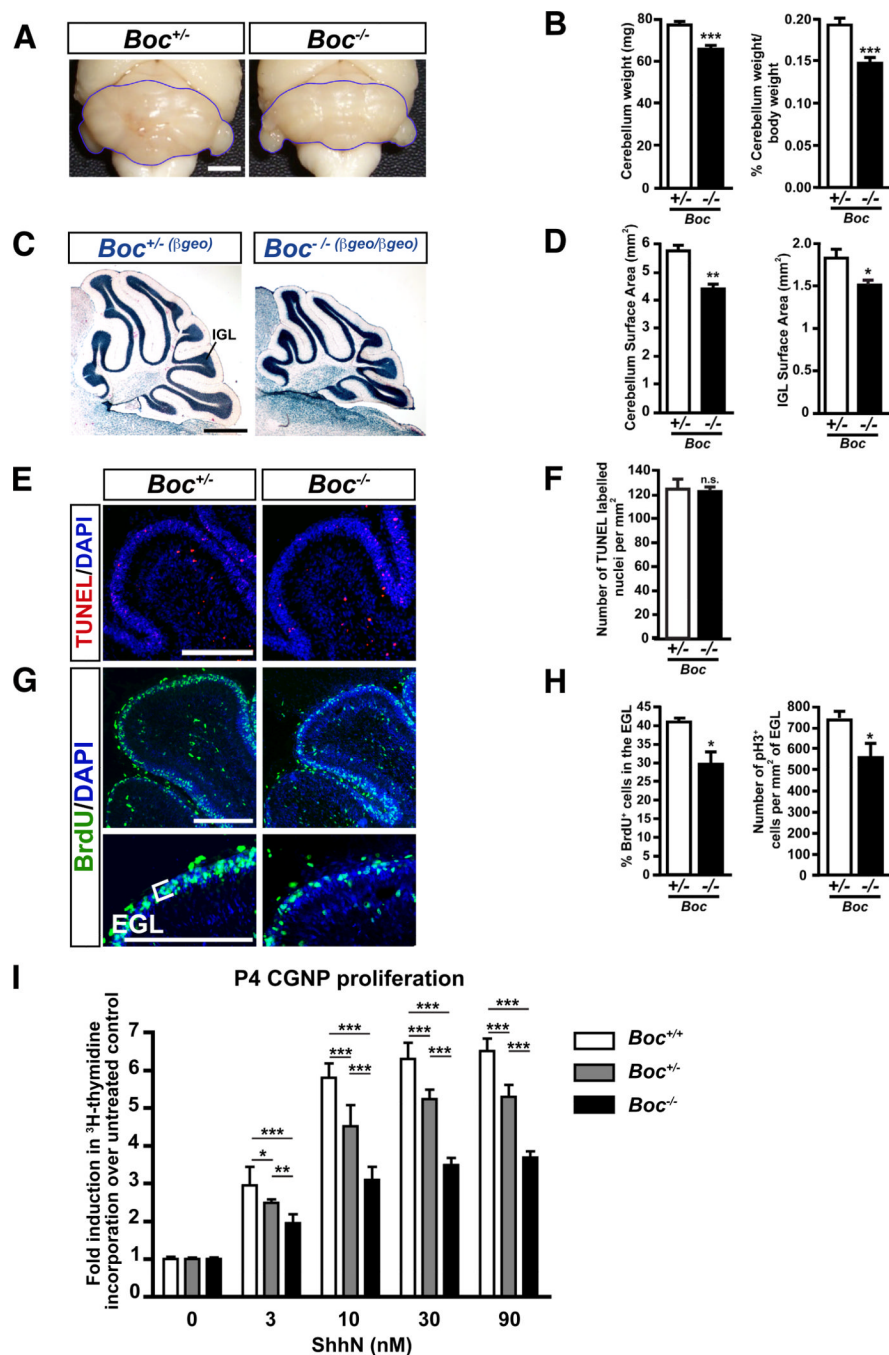


Figure 2. *Boc*^{-/-} mice have a smaller cerebellum than control mice

(A) Comparison of whole cerebella and (C) sagittal sections from adult *Boc*^{+/-} and *Boc*^{-/-} mice. (B) Cerebellum weight and normalized cerebellum weight relative to body weight. n=11 cerebella/group. (D) Cerebellum surface area (left) and IGL surface area (right) measured from three medio-lateral matching levels. n=4 cerebella/group. (E) Apoptotic CGNPs from P3 *Boc*^{+/-} and *Boc*^{-/-} mice visualized by TUNEL staining. (F) Number of apoptotic cells/mm² in the EGL from TUNEL-stained sections from 5 *Boc*^{+/-} and 4 *Boc*^{-/-} mice. (G) Proliferating CGNPs in the EGL from P3 *Boc*^{+/-} and *Boc*^{-/-} mice visualized by anti-BrdU staining. (H) (left) Percentage of BrdU⁺ cells in the EGL. n=4 animals/group. (right) Number of pH3⁺ cells/mm² in the EGL of 5 *Boc*^{+/-} and 4 *Boc*^{-/-} P3 mice. (I) CGNPs

purified from *Boc^{+/+}*, *Boc^{+/-}* and *Boc^{-/-}* mice cerebella at P4 were cultured in the presence of 0–90 nM ShhN. Proliferating cells were measured by ³H-thymidine incorporation. Data is represented as fold induction in CGNP proliferation compared to untreated cells. *Scale bars*: (A) 2mm, (C) 1mm, (E,G) 250 μ m. IGL, internal granular layer; EGL, external germinal layer. p values measured from Student's t-test (B,D,F,H) and two-way ANOVA (I).

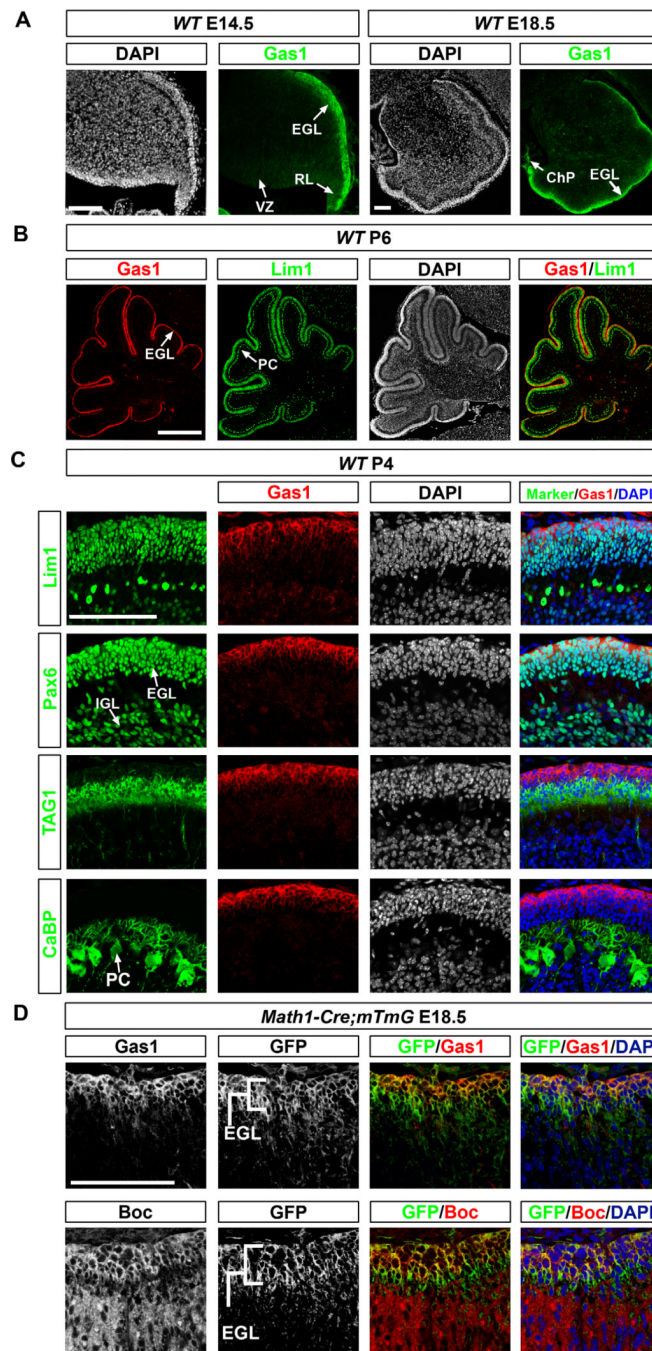


Figure 3. Gas1 expression in the developing cerebellum

(A) Immunostaining of sagittal sections showing Gas1 expression in the RL and EGL of the developing cerebellum at E14.5 and E18.5. (B,C) P4-6 WT cerebellum sections immunostained for Gas1 (red) and various cerebellar cell markers (green). Gas1 is highly expressed by proliferating CGNPs (Lim1⁺ cells; Pax6⁺ in EGL). (D) Immunostaining of E18.5 cerebellum sections from Math1-Cre;mTmG mice show that Boc and Gas1 are expressed in Math1⁺,GFP⁺ CGNPs in the EGL. EGL, external germinal layer; IGL, internal granular layer; PC, Purkinje cells; RL, rhombic lip; VZ, ventricular zone; ChP, choroid plexus. *Scale bars:* (A, C and D) 100 μ m, (B) 500 μ m.

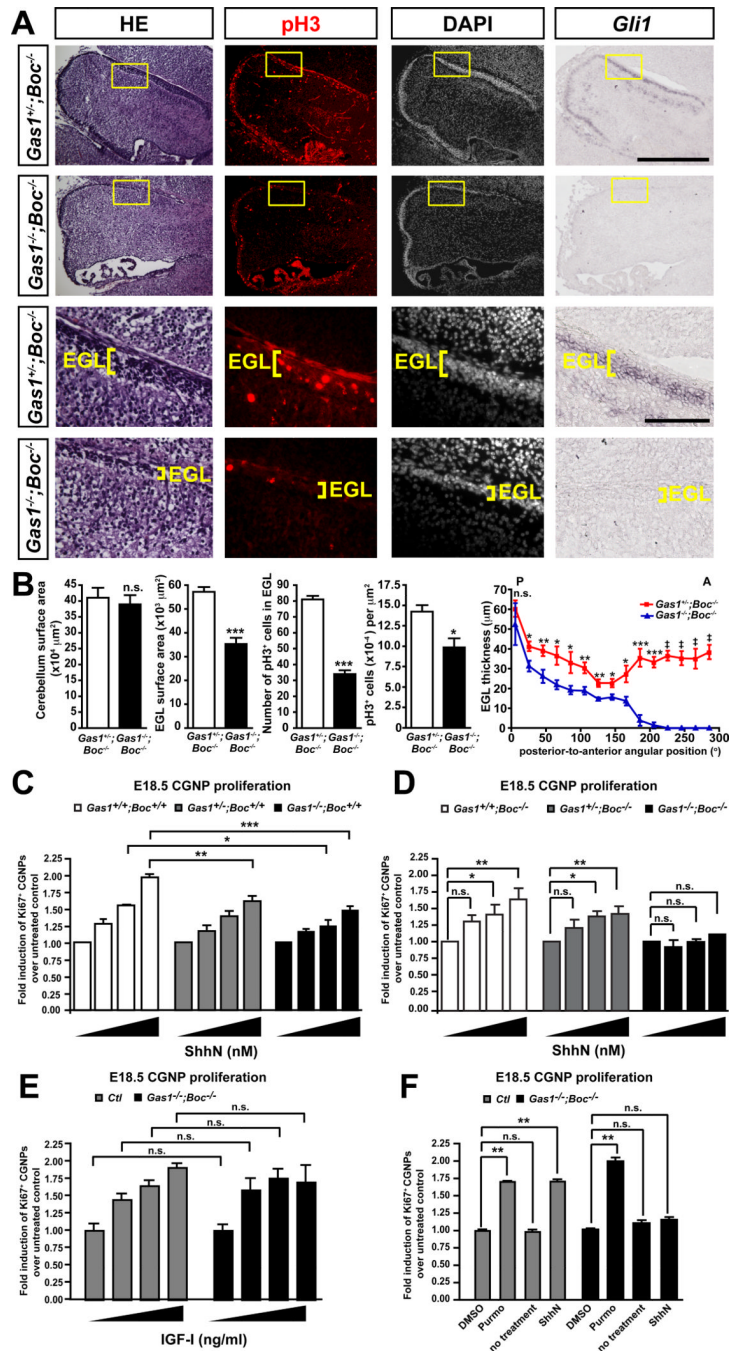


Figure 4. Shh-dependent proliferation is completely lost in *Gas1^{-/-};Boc^{-/-}* CGNPs
 (A) Haematoxylin-eosin staining on sagittal sections of E18.5 cerebellum revealing a thinner EGL in *Gas1^{-/-};Boc^{-/-}* cerebella than control. Anti-pH3 immunostaining of sagittal sections of *Gas1^{+/+};Boc^{-/-}* and *Gas1^{-/-};Boc^{-/-}* cerebella counterstained with DAPI. RNA *in situ* hybridization showing the loss of expression of the Shh transcriptional target *Gli1* in *Gas1^{-/-};Boc^{-/-}* cerebella at E18.5. (B) Quantification of: cerebellum surface area, EGL surface area, pH3⁺ cells in EGL, pH3⁺ cells per μm², and EGL thickness along the postero-anterior axis, n=4 animals/group. (C) CGNPs purified from *Gas1^{+/+};Boc^{+/+}* (n=3), *Gas1^{+/-};Boc^{+/+}* (n=4) and *Gas1^{-/-};Boc^{+/+}* (n=4) mice cerebella at E18.5 were cultured with 0, 3, 10, 30 nM ShhN. Proliferating cells were visualized by immunostaining with an anti-

Ki67 antibody. Data is represented as fold CGNP proliferation over untreated control (C, D and E) or DMSO control (F). (D) Similar to (C) but CGNPs were purified from *Gas1^{+/+};Boc^{-/-}* (n=3), *Gas1^{+/-};Boc^{-/-}* (n=3) and *Gas1^{-/-};Boc^{-/-}* (n=3) mice cerebella at E18.5. (E) CGNPs were purified from control (*Ctrl; Gas1^{+/+};Boc^{-/-}* and *Gas1^{+/-};Boc^{-/-}*) (n=3) and *Gas1^{-/-};Boc^{-/-}* (n=3) mice cerebella at E18.5 and treated with 0, 20, 50 or 100 ng/ml of IGF-I. (F) Similar to (E) but CGNPs were treated with either DMSO, 0.150 μ M purmorphamine, or 30 nM ShhN. p values measured from Student's t-test (B), two-way ANOVA (C, E), and ANOVA (D, F). EGL, external germinal layer. *Scale bars*: top two rows=500 μ m, bottom two rows=100 μ m.

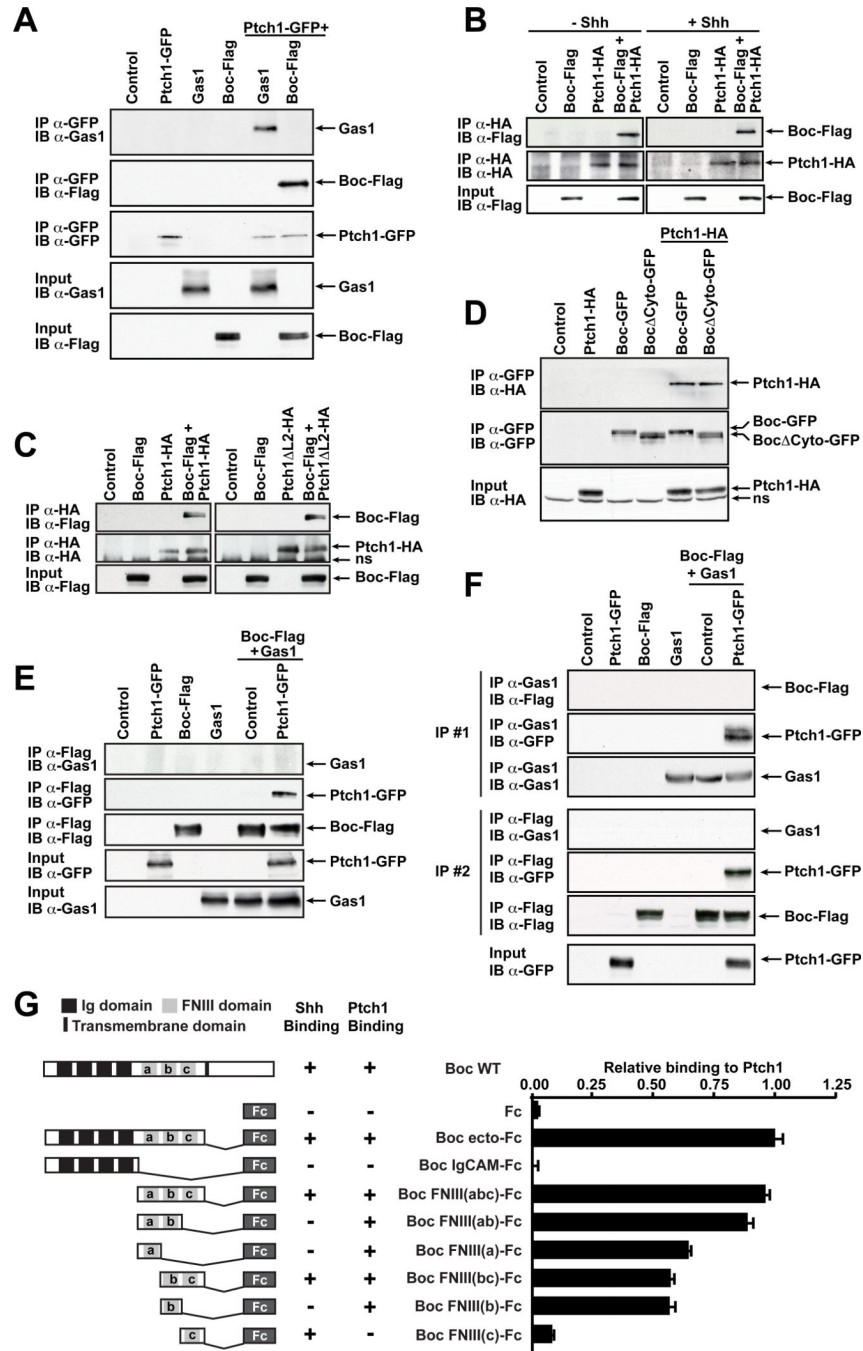


Figure 5. Gas1 and Boc interact with Ptch1

(A) Boc and Gas1 interact with Ptch1. COS7 cells were transfected with the indicated constructs and lysates were immunoprecipitated (IP) with an anti-GFP antibody and immunoblotted (IB) with anti-Gas1, anti-Flag or anti-GFP antibodies. (B) Boc interacts with Ptch1 in a constitutive manner. COS7 cells expressing Ptch1-HA and Boc-Flag were treated with ShhN and subjected to anti-HA IP and either anti-Flag or anti-HA IB. (C) The Ptch1 L2 region is not required for the Boc-Ptch1 interaction. Anti-HA IP was performed on COS7 lysates expressing Boc-Flag and Ptch1-HA or Ptch1 Δ L2 followed by anti-Flag or anti-HA IB. (D) The Boc cytoplasmic tail is not required for the Boc-Ptch1 interaction. COS7 cells expressing Ptch1-HA and Boc-GFP or Boc Δ cyto-GFP were subjected to anti-GFP IP and

either anti-HA or anti-GFP IB. (E, F) Ptch1 forms receptor complexes with either Boc or Gas1 but not both. Boc-Flag was co-transfected with Gas1 with or without Ptch1-GFP in COS7 cells. (E) Lysates were IP with anti-Flag antibodies, followed by anti-Gas1 or anti-GFP IB. (F) Lysates were first immunoprecipitated (IP #1) with anti-Gas1 antibodies. Supernatants from IP#1 were subjected to a second immunoprecipitation (IP #2) with anti-Flag antibodies. Both anti-Gas1 (IP #1) and anti-Flag (IP #2) immunoprecipitates were IB with anti-Gas1, anti-Flag and anti-GFP antibodies. See Fig. S4 for a schematic of this experiment. (A–F) Protein expression inputs were verified by IB with the indicated antibodies. ns, non-specific. (G, left) Diagram of WT Boc, Boc-Fc and Boc-Fc mutant proteins. (G, right) The Boc-Ptch1 interaction is mediated by the Boc FNIII(ab) domains. COS7 cells expressing Ptch1-GFP were incubated with conditioned medium containing Boc-Fc proteins. Bound proteins were labeled with HRP-conjugated anti-Fc antibody and peroxidase activity measured.

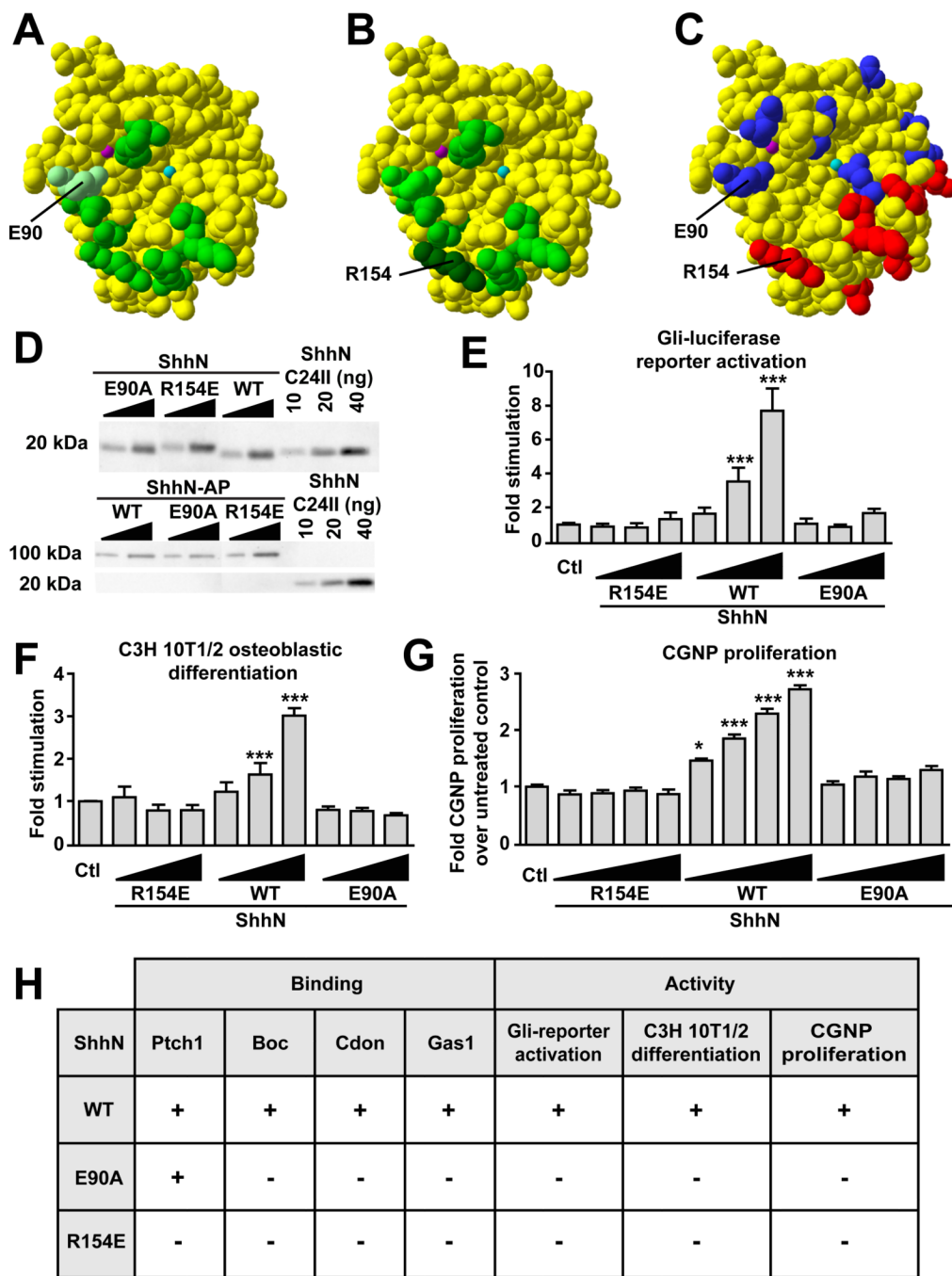


Figure 6. A Shh mutant which binds Ptch1 but which fails to bind Boc, Cdon and Gas1 does not induce Shh signaling

(A, B, C) Structural representation of ShhN. Residues colored in light, medium and dark green represent amino acids contacting Boc/Cdon FNIII(c) domain. E90 is represented in light green (A). R154 is represented in dark green (B). (C) Amino acids important for Ptch1 binding are colored in red while residues dispensable for the Ptch1-Shh interaction are illustrated in blue. (D) Anti-ShhN immunoblots showing the expression of purified ShhN WT, E90A, and R154E in comparison to ShhN C24II (top panel). Anti-ShhN immunoblot showing conditioned medium (CM) containing ShhN-AP WT, E90A, and R154E in comparison to ShhN C24II (bottom panel). (E) Stimulation of Gli-luciferase reporter activity

in C3H 10T1/2 cells treated with 17, 50 and 150 nM ShhN WT, E90A or R154E mutants. (F) Differentiation of C3H 10T1/2 cells treated with 12.5, 25 and 50 nM ShhN WT, E90A or R154E mutants. (E–F) Each condition was performed in duplicate, n=3. (G) Induction in rat P4 CGNP proliferation upon treatment with 6.25, 12.5, 25 and 50 nM of ShhN WT, E90A or R154E mutants. Proliferation was measured by ³H-thymidine incorporation. Each condition was performed in triplicate, n=3. (E–G) Two-way ANOVA comparing the activity of ShhN E90A to ShhN WT was performed. (H) Table summarizing binding of ShhN mutants to Ptch1, Boc, Cdon and Gas1 and their functional activity.

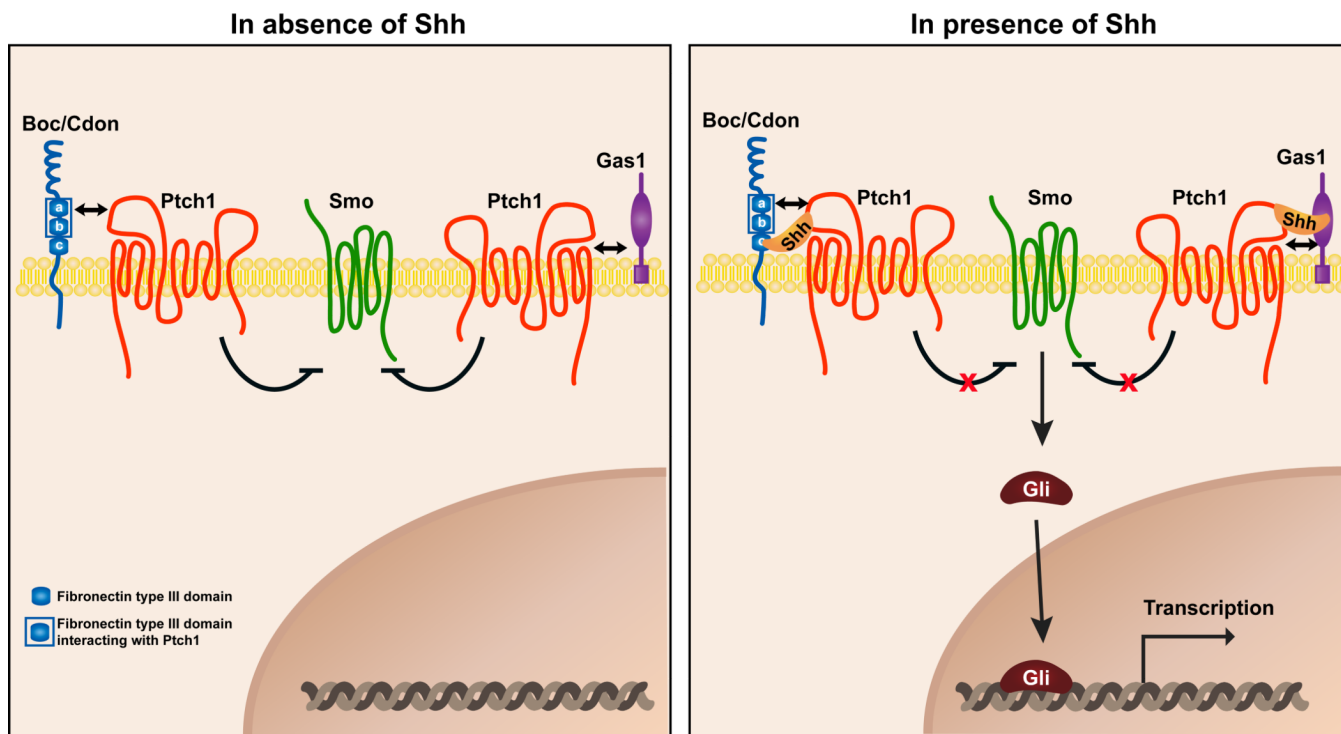


Figure 7. Shh signaling absolutely requires the Boc, Cdon and Gas1 receptors

(Left) In the absence of Shh, Boc, Cdon and Gas1 interact with Ptch1 to form Boc/Ptch1, Cdon/Ptch1 or Gas1/Ptch1 complexes. The Boc/Ptch1 interaction is mediated by the FNIIIa and FNIIIb domains of Boc. (Right) Shh binding to Boc/Ptch1, Cdon/Ptch1, and Gas1/Ptch1 receptor complexes leads to the de-repression of Smo, which in turn activates signal transduction events that result in Gli-mediated transcription. In the case of Boc and Cdon, the interaction with Shh is direct and is mediated by the FNIIIc domain of Boc and Cdon. Unlike Ihog and Boi which promote the relocalization of Ptc to the surface of *Drosophila* cells, Boc and Gas1 do not seem to relocalize Ptch1 to the surface of mammalian cells (Fig. S5A-C).