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Genes Dev. 2007 21: 1244-1257
Access the most recent version at doi:10.1101/gad.1543607

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The Hedgehog-binding proteins Gas1 and Cdo cooperate to positively regulate Shh signaling during mouse development

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Hedgehog (Hh) signaling is critical for patterning and growth during mammalian embryogenesis. Transcriptional profiling identified Growth-arrest-specific 1 (Gas1) as a general negative target of Shh signaling. Data presented here define Gas1 as a novel positive component of the Shh signaling cascade. Removal of Gas1 results in a Shh dose-dependent loss of cell identities in the ventral neural tube and facial and skeletal defects, also consistent with reduced Shh signaling. In contrast, ectopic Gas1 expression results in Shh-dependent cell-autonomous promotion of ventral cell identities. These properties mirror those of Cdo, an unrelated, cell surface Shh-binding protein. We show that Gas1 and Cdo cooperate to promote Shh signaling during neural tube patterning, craniofacial, and vertebral development. Overall, these data support a new paradigm in Shh signaling whereby positively acting ligand-binding components, which are initially expressed in responding tissues to promote signaling, are then down-regulated by active Hh signaling, thereby modulating responses to ligand input.

[Keywords: Mouse; Hedgehog; neural tube; development; Gas1; Cdo]

Supplemental material is available at http://www.genesdev.org.

Received February 20, 2007; revised version accepted April 3, 2007.

Nearly all developmental decisions during embryogenesis are regulated by a relatively small number of families of secreted growth factors and morphogens, including fibroblast growth factors [Bottcher and Niehrs 2005], Wnts [Logan and Nusse 2004], transforming growth factor-β family members [Massague 1998], and Hedgehog (Hh) proteins [McMahon et al. 2003]. Importantly, these secreted ligands often act on cells at a significant distance from their source [Ashe and Briscoe 2006], and, in the case of Wnts and Hh, these ligands also undergo various lipid modifications that regulate both their range and level of activity [Miura and Treisman 2006]. Understanding how the trafficking, turnover, and signaling levels of these factors are regulated in the extracellular matrix and at the cell surface are critical for a complete mechanistic understanding of their actions.

Hh proteins in the mouse are initially generated as 45-kDa precursor proteins that subsequently undergo autocatalytic cleavage and concomitant cholesterol modification and palmitoylation. The resulting N-terminal 19 kDa, dually lipidated, secreted molecule is responsible for all known Hh signaling activity [Ingham and McMahon 2001]. Of the three mammalian Hh family members [Indian, Desert, and Sonic], Sonic Hedgehog (Shh) has been the most widely studied, in large part because of its role as a morphogen in two key developmental events—the regulation of digit number and polarity, and the specification of ventral cell identities in the developing CNS [for review, see McMahon et al. 2003].

In the developing neural tube, Shh is initially expressed in the notochord underlying the ventral neural tube; as development progresses, Shh autoinduces a secondary domain of Shh production within the floor plate (FP) of the neural tube at the ventral midline [Echelard et al. 1993]. Several lines of evidence indicate that Shh acts in a concentration-dependent manner to specify all ventral cell types of the developing neural tube [for review, see Jessell 2000; Briscoe and Ericson 2001; McMahon et al. 2003]. Specifically, Shh represses [Class I genes; e.g., Pax6, Pax7] or induces [Class II genes; e.g., Nkx2.2, Olig2] the expression of several transcription factors at distinct concentration thresholds. Subsequent cross-repressive interactions between these regulatory factors sharpen the boundaries between different progenitor domains within the ventral neural tube [Briscoe et al. 2000]. Importantly, even relatively small [approximately twofold] changes in Shh concentration result in the specification of distinct cell types [Ericson et al. 1997]. Such strict requirements for the level of Shh protein...
raises the question of how the levels and activity of Shh ligand are regulated such that each ventral cell type is specified at the correct position and in the appropriate numbers within the developing neural tube. One answer lies in mechanisms that exist at the cell surface to regulate the distribution of Shh. Pioneering studies in Drosophila demonstrated that Patched (Ptc), the Hh receptor, acts not only to transduce a Hh signal, but is also a target of Hh signaling that acts as a negative feedback regulator. The up-regulation of Ptc in response to a Hh signal sequesters ligand, limiting its spread in responding tissues and modifying the response at a given position in the target field [Chen and Struhl 1996]. In vertebrates, both Patched1 [Ptch1] [Goodrich et al. 1997] and Hedgehog-interacting protein-1 [Hhip1], which encodes a vertebrate-specific Shh-binding protein [Chuang and McMahon 1999], are up-regulated in response to Shh signaling. Their combined activities restrict the distribution of Shh ligand during neural tube patterning, ensuring the correct specification of all ventral cell identities in their appropriate position [Jeong and McMahon 2005]. In opposition to the above-mentioned negative feedback mechanisms, recent work has identified two additional Shh-binding cell surface proteins, Cdo and Boc, as negative targets of Shh signaling that function to positively regulate Shh signaling [Okada et al. 2006; Tenzen et al. 2006; Yao et al. 2006; Zhang et al. 2006].

One hypothesis that emerges from these reports is that the levels of Shh protein at the cell surface are controlled by transcriptional up-regulation of negative feedback components such as Ptc1 and Hip1, and concomitant down-regulation of positively acting Shh-binding proteins such as Cdo and Boc. While previous mutational analyses have established the importance of Ptc1 and Hip1 in the general negative regulation of Hh signaling [Goodrich et al. 1997; Chuang and McMahon 1999; Milenkovic et al. 1999; Chuang et al. 2003; Jeong and McMahon 2005], genetic analysis of Cdo and Boc have revealed only limited, tissue-specific roles for these structurally related proteins in the promotion of Shh signaling [Cole and Krauss 2003; Okada et al. 2006; Tenzen et al. 2006; Zhang et al. 2006]. Although it is possible that semiredundant functions of Cdo and Boc are responsible for the relatively mild effects on Shh signaling, another possibility is that other, unidentified components compensate for their loss of function. Interestingly, transcriptional profiling experiments identified Growth-arrest-specific 1 (Gas1) as a gene commonly down-regulated in response to Shh signaling in multiple tissues, a transcriptional signature shared with Cdo and Boc [T. Tenzen and A.P. McMahon, in prep.].

Gas1 encodes a 45-kDa GPI-anchored cell surface protein that binds Shh with high affinity [Kd ~ 6 nM] [C.S. Lee et al. 2001a]. Gas1 was initially described as an antagonist of Shh signaling, based on ectopic expression studies in the developing somite [C.S. Lee et al. 2001a] and tooth [Cobourne et al. 2004]. Paradoxically, the phenotypes reported for Gas1 mutant mice reveal eye (C.S. Lee et al. 2001b), cerebellar [Liu et al. 2001], and limb deficiencies [Liu et al. 2002] that are more consistent with reduced Shh signaling [Wang et al. 2002; Harfe et al. 2004; Lewis et al. 2004].

To address whether Gas1 functions to promote or antagonize Shh signaling, we examined the role of Gas1 in the Shh-mediated specification of ventral cell types and other Shh-dependent patterning events. This study establishes that Gas1 functions in vivo to promote Shh signaling during embryogenesis. Additionally, we demonstrate overlapping roles for Gas1 and Cdo in the positive regulation of an appropriate transcriptional response to Shh signaling in Shh target fields. Overall, these findings suggest a new paradigm of Shh signaling where the negative transcriptional regulation of positively acting, cooperative Shh-binding components constitutes part of the dynamic response to a Shh morphogen.

Results

Gas1 is a negative target of Shh signaling that is initially expressed in Shh-responsive tissues

Multiple transcriptional profiling analyses were performed at several stages of early mouse development [embryonic days 8.5–10.5 [E8.5–E10.5]] in distinct Shh target fields. These data, which will be presented in detail elsewhere [T. Tenzen and A.P. McMahon, in prep.], identified a number of genes with common, tissue-independent signatures of Shh signaling activity. Of those genes commonly repressed by Shh signaling, Gas1 stood out as a general negative target of Shh regulation, a result consistent with the original description of Gas1 expression [C.S. Lee et al. 2001a]. To confirm that Gas1 is, in fact, a general negative target of Shh, in situ hybridization analysis of Gas1 expression was performed at E8.5 on wild-type, smo−/−, and Ptch1−/− embryos [Fig. 1]. Gas1, which is normally strongly expressed in surface ectoderm of the headfold region and somites [Fig. 1A], is up-regulated in Hh loss-of-function smo−/− embryos [Fig. 1B], while its expression is almost completely abolished in Hh gain-of-function Ptc1−/− embryos [Fig. 1C], as expected for a general negative target of Shh signaling.

To more closely examine the expression of Gas1 in Shh-responsive tissues in conjunction with Shh-mediated patterning, we used a novel Gas1LacZ allele [Martelli and Fan 2007] in which the entire coding region of Gas1 is replaced by a tau-LacZ fusion protein [Callahan and Thomas 1994]. Whole-mount and section views of β-galactosidase activity [Fig. 1D–AA] reveal that Gas1 is present throughout the neural tube at E8.5, including low levels of notochord expression [arrows in Fig. 1F,J]. Additionally, Gas1 expression correlates temporally with the Shh-dependent specification of ventral neural cell fates, as assayed by expression of Nkx6.1, a marker of the vp2, vpMN, and vp3 neural progenitor domains [Fig. 1K]. One day later, in E9.5 embryos, Gas1 is restricted to more dorsal regions, although expression still overlaps the dorsal-most subset of Shh-responsive, Nkx6.1+ cells [Fig. 1L–S]. At E10.5, Gas1 expression remains dorsally restricted, and includes an additional domain of expression in commissural axons that project...
ventrally from the dorsal neural tube to cross the FP [Fig. 1Z, arrowhead] via a Shh-dependent guidance process (Charron et al. 2003; Okada et al. 2006). These results demonstrate that in the neural tube Gas1 is initially present in all Shh-responsive cells at the outset of Shh signaling, but gradually becomes more dorsally restricted, as the levels of Shh increase and the Shh signaling domain expands, consistent with Gas1 being a negative target of Shh regulation.

**Craniofacial and skeletal defects in Gas1−/− and Gas1−/−; Shh+/− embryos**

To address the potential involvement of Gas1 in Shh signaling, Gas1−/− embryos were analyzed. At E18.5, Gas1 mutants are easily identified by their small eyes (microphthalmia) (C.S. Lee et al. 2001b) and generally reduced body size. Skeletal analysis of the heads of Gas1−/−; E18.5 embryos indicates several defects consistent with reduced Hh signaling (Jeong et al. 2004), including a truncated maxilla, reduced parietal bone, and disrupted tympanic bone [Fig. 2A–C].

If the skeletal defects observed in Gas1−/− embryos reflect reduced levels of Shh signaling, then lowering the dosage of Shh would be expected to enhance these phenotypes. To test this prediction, Gas1; Shh compound mutants were analyzed. While Gas1−/−; Shh−/− embryos appear phenotypically normal [Fig. 2D], Gas1−/−; Shh−/− embryos are severely reduced in overall body size [data not shown] and display pronounced skeletal defects [Fig. 2E] that are significantly more severe than those seen in Gas1−/− embryos. Additionally, Gas1−/−; Shh+/− embryos display other defects not seen in Gas1−/− embryos, the most obvious of which is a profound truncation of the mandible [Fig. 2E] and axial skeletal deficiencies that include severely reduced ossification centers in vertebral bodies, and partial fusion of the intervertebral discs [data not shown]. These phenotypes are reminiscent of mice that lack the Hh-specific transcriptional effector Gli2 (Mo et al. 1997).

Examination of the limbs of Gas1−/− embryos also reveals an apparent reduction in Shh signaling, a phenotype first observed by Martineilli and Fan (2007). In the limb, digit 1 is Shh-independent, while all other digits are Shh-dependent (Chiang et al. 2001; Lewis et al. 2001). Of these, only digit 2 is completely dependent on secreted Shh; digit 3 is a mosaic of cells, a subset of which originate from Shh-expressing cells, while digits 4 and 5 are wholly derived from Shh-producing cells (Harfe et al. 2004). Importantly, Gas1 is expressed in the anterior two-thirds of the developing limb bud mesenchyme starting at E9.0 (Liu et al. 2002). In Gas1−/− embryos, forelimb digits 2 and 3 are fused, while digit 2 or 3 is completely absent from the hindlimbs of Gas1−/− embryos [Supplementary Fig. 1]. Reduction of Shh dosage in Gas1−/−; Shh−/− embryos enhances the forelimb defect such that now one digit [2 or 3] is completely absent. In contrast to the digits, the long bones of E18.5 Gas1−/− embryos are overtly normal [data not shown], suggesting that there is not a significant effect on Ihh-dependent long bone growth in Gas1 mutants at this stage.

Given the severe craniofacial defects observed at E18.5, Gas1−/− and Gas1−/−; Shh+/− embryos were exam-
ined at earlier developmental time points to determine when these defects first manifest themselves. At E10.5, Gas1\(^{-/-}\) embryos display partial fusion of the medial nasal processes (Fig. 2F–H), a phenotype similar to that of Cdo\(^{-/-}\) embryos (Tenzen et al. 2006; Zhang et al. 2006). Consistent with the increased severity of the facial phenotypes at E18.5, this phenotype is enhanced in Gas1\(^{-/-}\); Shh\(^{+/-}\) compound mutants, leading to a complete fusion of the medial nasal processes (Fig. 2I,J). Interestingly, a similar genetic interaction is observed between Cdo and Shh (Tenzen et al. 2006). These early facial phenotypes likely represent a secondary outcome stemming from an initial failure of Shh patterning of the rostral forebrain (Jeong et al. 2004). The reduced expression of the Shh-dependent transcriptional regulator Nkx2.1 (Pabst et al. 2000) in the ventral telencephalon of Gas1\(^{-/-}\) embryos (Fig. 2K–M) and the further diminished expression in Gas1\(^{-/-}\); Shh\(^{+/-}\) embryos (Fig. 2N,O) supports this view.

**Loss of Gas1 results in a Shh dosage-dependent loss of ventral cell identities in the ventral neural tube**

Shh signaling during development is best understood with respect to its role in patterning of the ventral neural tube. To explore the effects of Gas1 on Shh-dependent neural tube patterning we initially examined presumptive spinal cord regions at the forelimb level in E10.5 embryos. At the ventral midline, specification of FP cells requires the highest level of Shh signaling (Roelink et al. 1995) for the localized expression of FoxA2, itself a direct transcriptional regulator of Shh (Epstein et al. 1999; Jeong and Epstein 2003). When FoxA2 is first activated at the ventral midline, its expression overlaps with Nkx2.2, a determinant of ventrolateral v3 interneuron progenitors (Jeong and McMahon 2005). Elevated FoxA2 levels and loss of Nkx2.2 within FP progenitors correlates with cells assuming a typical polarized FP morphology and transcriptional activation of Shh. Thus, FP induction is a dynamic process wherein a mature FP identity is Nkx2.2\(^{-}\), FoxA2\(^{+}\), Shh\(^{+}\). Initial examination showed that FoxA2 is present in Gas1\(^{-/-}\) embryos (Fig. 3A,C,E). Analysis of FoxA2 and Nkx2.2, however, revealed that their expression is almost completely overlapping at a time when Nkx2.2 is normally ventrolaterally restricted (Fig. 3K–S), suggesting that FP specification is incomplete. Quantitation of FoxA2\(^{+}\), Nkx2.2\(^{+}\) cell number revealed a highly significant difference in the number of double-positive cells between wild-type and Gas1\(^{-/-}\) embryos (Fig. 3T). Consistent with this view, Shh is also variably reduced or entirely absent from midline cells [Fig. 3, cf. F and B,D] of Gas1\(^{-/-}\) embryos. In addition, while reduction of Shh dosage has no effect on FoxA2 expression in Gas1\(^{-/-}\); Shh\(^{+/-}\) embryos [Fig. 3G], Gas1\(^{-/-}\); Shh\(^{+/-}\) embryos exhibit a complete loss of FoxA2\(^{+}\) cells [Fig. 3I]. Importantly, Shh expression at the midline is also lost in all Gas1\(^{-/-}\); Shh\(^{+/-}\) embryos [Fig. 3H,J]. Thus, FP specification is dependent on Gas1 action in a Shh dosage-dependent manner.
To explore more fully the role of Gas1 in ventral neural tube patterning, specification of vp3 \( (\text{Nkx2.2}^+) \) interneuron progenitors and pMN \( (\text{Olig2}^+) \) motorneuron progenitors was examined in Gas1\(^{-/-}\) and Gas1\(^{-/-}\); Shh\(^{+/+}\) embryos (Fig. 4). Specification of vp3 progenitors requires a significantly higher level of Shh signal than pMN progenitors (Ericson et al. 1997), in agreement with the more dorsal position of the pMN progenitor pool. Nkx2.2\(^+\) vp3 progenitors are significantly reduced in Gas1\(^{-/-}\) embryos (Fig. 4A–L), and further reduced in Gas1\(^{-/-}\); Shh\(^{-/-}\) embryos (Fig. 4M–U). In contrast, while Olig2\(^+\) pMN progenitors are not significantly affected in Gas1\(^{-/-}\) embryos (Fig. 4K), they are dramatically reduced in Gas1\(^{-/-}\); Shh\(^{-/-}\) embryos (Fig. 4S,V), though their relative position dorsal to Nkx2.2\(^+\) progenitors is preserved (Fig. 4L,T). Surprisingly, Olig2\(^+\) cell numbers are increased in Gas1\(^{-/-}\); Shh\(^{-/-}\) embryos [Fig. 4O] compared with wild-type littermates [Fig. 4C], suggesting that both Gas1 and Shh levels are critical for proper specification of ventral cell identities. Overall, these data suggest that although cells may be exposed to reduced levels of Shh, or are less able to respond to Shh, the graded response to Shh appears to be maintained. Additionally, examination of other markers of neural progenitor cell specification that are positively \( (\text{Isl1}^+\text{pMN}, \text{En1}^+\text{v1}, \text{or Nkx6.1}^+\text{vp2, pMN, vp3}) \) or negatively \( (\text{Pax6, Pax7}) \) regulated also show modified expression consistent with reduced Shh signaling [Supplementary Fig. 2; data not shown]. Importantly, despite the strong expression of Gas1 in dorsal domains, specification of general dorsal cell identities \( (\text{Pax6}^+, \text{Pax7}^+) \) and specific Msx1\(^+\) roof plate \( [\text{data not shown}] \) and Math1\(^+\) dpl progenitors [Supplementary Fig. 2] is normal in both Gas1\(^{-/-}\) and Gas1\(^{-/-}\); Shh\(^{-/-}\) embryos. Overall, these results are consistent with Gas1 functioning to specifically modulate the level of Shh signal that cells are exposed to during neural tube patterning.

The reduction of vp3 progenitors in Gas1\(^{-/-}\); Shh\(^{-/-}\) embryos is exacerbated by reducing Shh dosage. Antibody detection of FoxA2 [red; A,C,E,G,I] and Shh [green; B,D,F,H,J] in forelimb-level sections of E10.5 Gas1; Shh embryos. Inset in F denotes variable FP expression of Shh seen in Gas1\(^{-/-}\) embryos. Double staining of wild-type [K,L,M], Gas1\(^{+/+}\) [N,O,P], and Gas1\(^{-/-}\) [Q,R,S] embryos with FoxA2 [red] and Nkx2.2 [green]. [T] Quantitation of FoxA2, Nkx2.2 double-positive cells. Error bars represent the mean ± SD of three different embryos. Error bars represent the mean ± SD of three different embryos. P-values calculated from comparison of wild-type and Gas1\(^{-/-}\) data by two-tailed Student’s t-test are listed. [N.S.] Not significant \( (p > 0.5) \). Bar: A, 50 µm.
embryos is consistent with the phenotype of Gli2−/− mice (Ding et al. 1998; Matise et al. 1998) that also fail to specify a Shh-expressing FP. However, Gas1−/−; Shh+/- embryos display an additional phenotype, a dramatic reduction in Olig2+ pMN progenitors at E10.5. To determine whether the loss of Olig2+ cells results from an initial failure in pMN specification, or in the later proliferation or maintenance of progenitors, we examined Gas1−/−; Shh+/- embryos at E9.5 [Fig. 5]. Examination of the FP marker FoxA2 in Gas1−/− and Gas1−/−; Shh+/- embryos at E9.5 suggested that FP specification initiated relatively normally. However, only a few, weakly positive FoxA2+ cells were detected in Gas1−/−; Shh+/- embryos [Fig. 5A,D,G]. Decreased Nkx2.2 expression was detected in Gas1−/−; Shh+/- embryos [Fig. 5B,E]; this phenotype was also enhanced by reducing Shh dosage in Gas1−/−; Shh+/- embryos [Fig. 5H]. In contrast, Olig2 specification did not appear to be dramatically altered in Gas1−/−; Shh+/- embryos at E9.5 [Fig. 5C,F,I]. Together these data suggest that Gas1 promotion of Shh signaling is required

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**Figure 4.** Reduced Olig2+ and Nkx2.2+ cell specification in E10.5 Gas1; Shh compound mutants. DAPI (A,E,I,M,Q), Nkx2.2 (red; B,F,J,N,R), and Olig2 (green; C,G,K,O,S) detection in forelimb-level E10.5 sections of Gas1, Shh embryos. (D,H,L,P,T) Nkx2.2 and Olig2 merged images are shown. Quantitation of numbers of Nkx2.2+ (U) and Olig2+ (V) cells in Gas1−/− [dark-gray bars], Gas1−/− [light-gray bars], and Gas1−/−; Shh+/- (white bars) E10.5 embryos. Error bars represent the mean ± SD of three different embryos. *P*-values calculated from comparison with Gas1−/− data by two-tailed Student’s *t*-test are listed. (N.S.) Not significant (*p* > 0.1). Bar: B, 50 µm.
for initial specification of FP and vp3 cells functions while attenuation of Shh signaling in a Gas1−/− background argues for an ongoing Gas1-Shh dependence beyond initial specification for the proliferation or maintenance of ventral progenitor domains.

Ectopic Gas1 expression in the chick neural tube results in Shh-dependent cell-autonomous promotion of ventral cell identities

To directly test the ability of Gas1 to promote Shh signaling, a full-length Gas1 construct was electroporated into developing chick neural tubes (Fig. 6). In contrast to electroporation of a control vector [Fig. 6A–D], electroporation of Gas1 results in a significant cell-autonomous dorsal expansion of Nkx6.1+ and Nkx2.2+ progenitors [Fig. 6G–J; data not shown]. Thus, Gas1 overexpression induces ectopic, Shh-dependent cell fates in the developing neural tube. Further, examination of Nkx2.2 and Olig2 in the same section revealed cell-autonomous dorsal expansion of both cell types in Gas1 electroporated neural progenitors [Fig. 6C,D,I,J]. Importantly, the positions of ectopic Nkx2.2+ and Olig2+ cell identities relative to a ventral Shh signaling source are maintained [arrows in Fig. 6I,J]. These data suggest that a graded response to Shh is still maintained, even in ectopic positions, when cells overexpress Gas1. Ectopic FoxA2 [Fig. 6, cf. K,L and E,F] in Gas1 electroporated cells confirms that Gas1 is able to promote the Shh-dependent expansion of even the most ventral cell identities.

In addition to the dorsal expansion of Class II genes [e.g., Nkx2.2, Nkx6.1] that are normally activated in response to Shh signaling, Class I targets [e.g., Pax6, Pax7] normally repressed at distinct Shh thresholds [Briscoe et al. 2000] are also repressed at relatively more dorsal positions in cells ectopically expressing Gas1 [Fig. 6M–T]. The cell-autonomous repression of Pax6 [Fig. 6Q,R] and Pax7 [Fig. 6S,T] at the dorsal–ventral intersect, the dorsal limit of Shh signaling [Wijsgerde et al. 2002], but not at significantly more dorsal positions, confirms the Shh-dependent specificity of Gas1 action. Finally, similar to the effects of overexpression of the cell surface, Shh-binding proteins Cdo and Boc [Tenzen et al. 2006], non-cell-autonomous ventral expansion of Pax7 [Fig. 6S,T, arrowhead] is also detected when a significant population of Gas1 electroporated cells are positioned just ventral to the normal Pax7 domain, a result consistent with Gas1 sequestration of Shh ligand.

The promotion of Shh-dependent cell fates in the chick neural tube following ectopic Gas1 expression, taken together with the high-affinity interaction between these two proteins [C.S. Lee et al. 2001a], strongly suggests that Gas1 functions at the level of Shh ligand to promote Shh signaling. To directly test this idea, coelectroporation experiments were performed with Gas1 and Pch1Δloop2, a variant of Pch1 that lacks Shh binding,
but retains the ability to inhibit Smo (Briscoe et al. 2001; Tenzen et al. 2006). If Gas1 functions at the level of ligand, then its effects on Shh-mediated patterning should be blocked by coexpression with Ptch1\(^{\Delta}\)loop2. As expected, coelectroporation of Gas1 and a control vector resulted in the cell-autonomous promotion of Class II genes (e.g., Nkx6.1) (Fig. 7A–D), the cell-autonomous inhibition of Class I genes at the ventral limit of their normal expression domains (e.g., Pax6, Pax7) (arrows in Fig. 7G–J, K, and L) and the non-cell-autonomous expansion of Class I genes due to ligand sequestration (arrowheads in Fig. 7S and T) consistent with the reduced Shh signaling that results from Ptch1\(^{\Delta}\)loop2 expression. These data support a model where Gas1 promotes Shh-dependent cell fates through a Shh ligand-binding-based mechanism (see Discussion).

**Figure 6.** Ectopic expression of Gas1 promotes Shh-dependent cell fate specification in the developing chick neural tube. HH stage 19–22 chick neural tubes electroporated with pCIG [A–F,M–P] or Gas1–pCIG [G–L,Q–T] were sectioned at the forelimb level and stained with antibodies raised against Nkx6.1 [red, A,B,G,H], Nkx2.2 and Olig2 [red and blue, respectively, C,D,I,J], Nkx2.2 and FoxA2 [red and blue, respectively, E,F,K,L], Pax 6 [M,N,Q,R], and Pax7 [O,P,S,T]. Arrows in G, H, I, J, K, and L indicate ectopic expression of the indicated markers, while arrows in Q, R, S, and T denote repressed marker expression. Arrowheads in S and T identify non-cell-autonomous ventral expansion of Pax7 expression. Asterisks indicate nonspecific antibody background present in the FP of some sections. The results are representative of nine pCIG-electroporated embryos and 15 Gas1–pCIG electroporated embryos. Bar: A, 50 µm.

Gas1 and Cdo cooperate to promote Shh signaling

Gas1 promotion of Shh signaling in target cells in a Shh dosage-dependent manner is similar to recent findings on the roles of the structurally unrelated, Shh-binding membrane proteins Cdo and Boc (Tenzen et al. 2006).
Additionally, a recent study has identified Boc as a receptor for Shh in commissural axon guidance (Okada et al. 2006). Given that Gas1 is also expressed in commissural axons (Fig. 1Z), we examined Gas1 mutants for a possible role in axon guidance. While Gas1-expressing axons project normally at E11.5 in Gas1−/− embryos (Supplementary Fig. 3B,E), aberrant axonal projections, visualized with anti-β-galactosidase antibody, are apparent in Gas1−/− embryos that are misrouted through the Isl1/2 motor column (Fig. 1A–H). It is difficult at present to determine whether these projection defects are due directly to a loss of a Gas1-Shh-based mechanism of axon guidance or are secondary to deficiencies in the specification of ventral populations—for example, the FP—that are known to have Shh-independent actions on commissural axon guidance.

Together, the above data raise the question of whether Gas1, Cdo, and Boc might cooperate to augment Shh signaling. To address this issue, Gas1−/−; Cdo−/− double mutants (Fig. 8). Remarkably, an initial examination of facial development revealed a progressive increase in the severity of nasal process fusion as Gas1 and Cdo activity are removed (Fig. 8A–G), such that Gas1−/−;
Cdo−/− embryos completely lack medial facial structures and exhibit a marked holoprosencephaly, phenotypes shared by Shh-null embryos (cf. Fig. 8H). Molecular analysis of Shh, FoxA2, Nkx2.2 and Olig2 expression also revealed a progressive decrease in the proportion of these cell types such that no cells expressing any of these markers are detected in Gas1−/−; Cdo−/− embryos (Fig. 8I–FF). Strikingly, and distinct from Gas1−/−; Shh +/− and Cdo−/−; Shh +/− embryos, Gas1−/−; Cdo−/− embryos also display loss of Shh expression from the notochord (Fig. 8Y–EE). While these data are consistent with a Shh-independent loss of notochord integrity, it is also possible that severely reduced Shh signaling is responsible for this phenotype, since both Shh−/− embryos and Dispatched 1 (Disp1) mutants display defects in notochord maintenance (Chiang et al. 1996; Kawakami et al. 2002; Ma et al. 2002). To test this possibility, the notochord-specific marker carbonic anhydrase III (CAIII) (Lyons et al. 1991) was used to examine notochord integrity in Gas1; Cdo embryos at E9.5 (Fig. 8GG–MM). Importantly, the notochord is intact in E9.5 Gas1−/−; Cdo−/− embryos, suggesting that notochord formation and maintenance is not affected in these mutants. In contrast, examination of CAIII expression in Shh−/− mutants at E9.5 indicates a degenerating notochord (Fig. 8NN). Thus, although Gas1−/−; Cdo−/− embryos display quite severe defects, they do not recapitulate a complete loss of Shh activity. This conclusion was confirmed by the examination of craniofacial and vertebral defects at E18.5 by skeletal analysis (Supplementary Fig. 4).

Gas1−/−; Cdo−/− embryos display significantly more severe craniofacial defects than Gas1−/−; Shh−/− mutants, with a marked loss of both mandibular and maxillary components [Supplementary Fig. 4M]. Additionally, Gas1−/−; Cdo−/− embryos show fusion of cervical vertebrae [Supplementary Fig. 4N], similar to loss of the Hh-specific transcription factor Gli3 (Mo et al. 1997), though the specification of vertebral components is distinct from Shh−/− embryos (Chiang et al. 1996). In Shh−/− embryos, all ventral medial components are absent, whereas only ventral medial components are absent from Gas1−/−; Cdo−/− compound mutants. Further, in the

Figure 8. Gas1; Cdo compound mutants display severely reduced Shh signaling. (A–G) Nasal process defects in E10.5 Gas1; Cdo embryos are shown. Brackets indicate the distance between nasal pits. (H) A Shh−/− E10.5 embryo is shown for comparison. Examination of Nkx2.2 (red) and Olig2 (green) expression in E10.5 Gas1; Cdo (I–O) and Shh−/− (P) forelimb-level sections. Forelimb-level expression of FoxA2 (red; Q–X) and Shh (green; Y–Z, AA–FF) in E10.5 Gas1; Cdo and Shh−/− embryos. In situ hybridization analysis of the notochord marker CAIII in E9.5 Gas1; Cdo embryos [GG–MM]. Discontinuous CAIII expression is detected in a Shh−/− E9.5 embryo [NN], indicative of notochord degeneration. Arrows in NN highlight the broken CAIII expression. Bars: A, 1 mm; I, 50 μm; GG, 1 mm. For Gas1−/−; Cdo−/− embryos, a total of five embryos were examined with similar results.
Gas1 cooperates with Cdo to promote Shh signaling

In addition to the identification of Gas1 as a positive component of Shh signaling, data presented here suggest that Gas1 cooperates with Cdo, a structurally unrelated, cell surface Shh-binding protein, to promote Shh signaling. How this occurs at the cellular level remains to be determined. As both factors bind Shh, one attractive hypothesis is that Gas1 and Cdo may form a physical complex together through Shh binding, and that this complex promotes Shh signaling, possibly through ligand presentation to the Shh receptor Ptch1. Future biochemical analyses examining whether such a complex is assembled and if so, determining the nature of such a complex will be critical next steps in understanding mechanistically how these proteins function. Additionally, given the recent report that the ciliary localization of the Hh signaling molecule Smo is critical for its function (Hacker et al. 2005), an examination of the subcellular localizations of these proteins may yield significant insight into their function. Considering that Gas1 is a GPI-anchored protein (Stebel et al. 2000), and that Cdo is a transmembrane protein (Kang et al. 1997), an intriguing possibility is that these proteins display distinct membrane localizations in the absence of Shh, but that following ligand binding these proteins redistribute in order to promote Shh signaling through Ptch1.

Surprisingly, despite the strong cooperation seen between Gas1 and Cdo in the promotion of Shh signaling during craniofacial and neural tube development, there appear to be no such cooperative interactions in the limb. This result is especially striking given the Shh-specific limb defects seen in both Gas1−/− and Cdo−/−; Shh−/− embryos, and that Gas1 and Cdo are expressed in overlapping domains in the limb (Lee and Fan 2001; Tenzen et al. 2006). One explanation is that other molecules with similar expression patterns and activity, notably Boc, may compensate for the loss of Gas1 and Cdo in the limb. Alternatively, inherent differences may exist in the reception and interpretation of Shh signals during limb and ventral neural tube development that underlie the contrasting phenotypes. Recent data from the limb suggest that both the level and duration of Shh signal exposure are critical for proper digit specification (Ahn and Joyner 2004; Harfe et al. 2004). Importantly, in the developing limb bud, Shh-expressing descendants contribute to the majority of Shh-dependent digits, while in the neural tube only FP cells ever express Shh and all ventral neural progenitors are initially specified by a notochord-derived Shh signal. In this regard, patterning of the neural tube is clearly more reliant on a secreted Shh signal. Thus, if Gas1 and Cdo function to regulate cellular responses to secreted Shh ligand, then the mild digit specification defects and severe ventral neural tube patterning phenotypes seen in Gas1; Cdo compound mutants are entirely consistent.

The data presented here, however, do suggest an important similarity between digit specification and ventral neural tube patterning that has not been fully appreciated previously: time. Comparison of Gas1 and Cdo expression patterns indicates that they overlap only briefly in the ventral neural tube and notochord during early stages of neural patterning, yet analysis of Gas1−/−; Cdo−/− double mutants demonstrates a complete loss of FP, vp3, and pMN progenitors, three cell types that de-
pend critically on Shh for proper specification. Additionally, examination of pMN (Olig2⁺) cell specification at different time points during neural tube patterning of Gas1⁺/−; Shh−/− embryos suggests that these cells depend on Shh signaling not only for initial specification signals, but also for maintenance or expansion of cell fates post-initial patterning. These data suggest strongly that time is a critical factor controlling Shh-dependent patterning of the ventral neural tube. Thus, there is a brief, but important temporal window during ventral neural tube patterning where coexpression of Gas1 and Cdo is required for proper transduction of the Shh signal. This temporal dependence contrasts with current models of ventral neural tube patterning, where the level of Shh exposure is of primary importance (Hooper and Scott 2005).

A model for cell surface regulation of Shh signaling

A critical aspect of Gas1 promotion of Shh signaling is that Gas1 expression is down-regulated as Shh signaling levels increase. The same is true for Cdo and Boc, which are also general negative targets of Shh (Tenzen et al. 2006). Importantly, these expression patterns are in direct contrast to the transcriptional up-regulation of the negative Shh signaling components Ptc1 and Hhip1, which sequester Shh ligand and block signaling (Jeong and McMahon 2005). A synthesis of these data suggests the following model: Cell surface molecules that promote Shh signaling are initially expressed on Shh-responsive cells, sensitizing cells to even low levels of Shh ligand, as the level of Shh signaling increases, there is a transcriptional down-regulation of these positive components, and a concomitant up-regulation in the expression of negative feedback components, thus providing multiple mechanisms to tightly control both the range and level of Shh signal that is necessary for proper neural cell specification.

Importantly, the transcriptional regulation of these components is not an all or nothing response; instead, it is dynamically modified within the target field. For example, while Gas1 expression is lost in the most ventral cell types as development proceeds, its expression is maintained in Shh-responsive cells more dorsally that require lower levels of Shh signal for proper specification. Here, continued expression of Gas1 is clearly critical for mediating a robust response to the normal levels of Shh ligand that regulate cell identities in this position. This is evident from the dramatic loss of progenitor cell numbers when Shh dosage is decreased on a Gas1 mutant background. Additionally, an examination of Boc and Cdo expression demonstrates that although their transcripts are dorsally restricted during neural tube specification, Cdo expression is preserved within the FP and its activity there is required at a late stage for maintenance of FP integrity (Tenzen et al. 2006), suggesting an ongoing role for these Shh signaling components in maintaining Shh expression in midline cells even after the initial establishment of Shh signaling. Overall, these data suggest that patterning of the ventral neural tube depends critically on both the level and duration of Shh action, and that Gas1 and Cdo comprise two key components that cooperate to regulate both aspects of this vital developmental process.

Materials and methods

Mice

The Gas1lacz allele (referred to here as Gas1) was generated by Dr. C.M. Fan’s laboratory (Carnegie Institution of Washington, Baltimore, MD). For details of the allele, please see the accompanying paper by Martinelli and Fan (2007). Cdo (Cole and Krauss 2003), Ptc1 (Goodrich et al. 1997), Shh (St-Jacques et al. 1998), and Smo (Zhang et al. 2001) mutant mice have all been described previously. Cdo mice were maintained on a 129/Sv; C57BL6/J background, while Gas1 and Shh mice were maintained predominantly on a C57BL6/J background. Noon of the day on which a vaginal plug was detected was considered E0.5.

Chick electroporation

Gas1 was cloned into the pCIG vector (Megason and McMahon 2002) to enable coexpression of Gas1 with GFP to visualize electroporated cells. Ptc1loop2 constructs have been described previously (Tenzen et al. 2006). Electroporations were performed essentially as described previously (Tenzen et al. 2006). Gas1–pCIG and pCIG were injected into the neural tubes of Hamburger-Hamilton (HH) stage 10–12 chicken embryos at concentrations of 1.0 µg/µL in PBS with 50 ng/µL Fast Green. For coelectroporation experiments, either Gas1–pCIG and pCIR or Gas1–pCIG and Ptc1loop2–pCIR were injected at concentrations of 0.75 µg/µL for each construct. Approximately 48 h following electroporation, embryos were recovered and fixed in 4% paraformaldehyde for subsequent immunofluorescent analysis.

In situ hybridization and immunofluorescence

Whole-mount digoxigenin in situ hybridization was performed as described (Wilkinson 1992). For immunofluorescent analysis, specimen collection, processing, and staining were performed essentially as previously described (Wijgerde et al. 2002; Jeong and McMahon 2005). Briefly, embryos were collected, fixed for 90 min in cold 4% paraformaldehyde, washed overnight at 4°C in PBS, cryoprotected overnight at 4°C in PBS containing 30% sucrose, and frozen in OCT (Tissue-Tek). Twelve-micron sections were then cut for subsequent immunofluorescent analysis. During immunostaining, the following antibodies were used: rabbit-anti-β-gal (1:10,000, Cappell), mouse anti-FoxA2 (1:20, Developmental Studies Hybridoma Bank [DSHB]), mouse anti-Shh (1:20, DSHB), mouse anti-Nkx2.2 (1:20, DSHB), mouse anti-Pax6 (1:20, DSHB), mouse anti-Pax7 (1:20, DSHB), rabbit anti-Nkx6.1 (1:600, gift of J. Jensen), mouse anti-Math1 (1:20, DSHB), mouse anti-Nkx6.1 (1:20, DSHB), rabbit anti-Olig2 (1:5000, gift of H. Takebayashi), rabbit anti-Nkx2.2 (1:4000, gift of T. Jessell), mouse anti-Islet1/2 (1:20, DSHB), rabbit anti-DisRed (1:700, Clontech). Nuclei were visualized with DAPI (1:30,000, Molecular Probes). Alexa 488, 568, and 633 secondary antibodies (1:500, Molecular Probes) were visualized on a Zeiss LSM510 confocal microscope. For quantitation of neural cell progenitors, at least two sections from three embryos of each genotype were counted. Statistical analyses were performed using a two-tailed Student’s t-test.
Skeletal analysis and whole-mount LacZ staining

All skeletons were prepared according to a modified Alcian Blue/Alizarin Red staining protocol [Kessel et al. 1990, Wallin et al. 1994]. Whole-mount detection of β-galactosidase activity was performed using X-gal [Shelton Scientific] as described previously [Whiting et al. 1991].

Acknowledgments

We are grateful to Dr. R. Krauss for the Cdo mutant and Dr. M. Scott for the Ptc1 mutant. We thank Drs. T. Jessell, H. Takebayashi, and J. Jensen for antibodies for neural tube analyses. FoxA2, Is11/2, Math1, Nkx2.2, Nkx6.1, Pax6, Pax7, and Shh antibodies were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA. We especially thank Chen-Ming Fan and David Martinelli for their generous sharing of the Gas1-LacZ mouse strain and of key data prepublication. Work in A.P.M.’s laboratory was supported by a grant from the NIH [R37 NS033642]. B.L.A. was supported by post-doctoral fellowship #PF0512501DDC from the American Cancer Society.

References


Gas1 promotes Shh signaling


Figure S1. Loss of Shh concentration-dependent digits in Gas1−/− and Gas1−/−; Shh+/− E18.5 embryos. Skeletal analysis of digit specification in E18.5 forelimbs (A, C, E, G, I) and hindlimbs (B, D, F, H, J) of Gas1; Shh embryos. Numbers identify each individual digit. Note the fusion of digits 2 and 3 in Gas1−/− forelimb (E). Digit loss in Gas1−/− hindlimbs (F) and Gas1−/−; Shh+/− forelimb (I) and hindlimb (J) is indicated by labeling of the remaining digit 2/3. Detailed morphological analysis could not conclusively identify the digit as either 2 or 3. Bar in A, 1mm.

Figure S2. Ventral neural tube-specific defects in cell fate specification in E10.5 Gas1; Shh embryos. Isl1+ motor neuron progenitors (A-C) are detected with anti-Isl1 antibody in forelimb level sections of E10.5 Gas1; Shh embryos. Antibody detection of Pax 6 (D, E, F), Nkx6.1 (red; G, H, I) and Pax7 (green; G, H, I) is shown. Dorsal progenitor 1 (dp1) cells are identified by detection of Math1+ cells (J, K, L). Scale bars in A, D, G, J, 50µm.

Figure S3. Commissural axon guidance defects in E11.5 Gas1−/− embryos. Analysis of β-galactosidase expression in forelimb level sections of E11.5 Gas1+/− (B) and Gas1−/− (D) embryos. DAPI detection of nuclei (A, C). Arrowheads in D mark axonal projections through the motor column. Boxes in B and D denote areas of greater magnification shown in E and G, respectively. Sections from Gas1+/− (F) and Gas1−/− (H) embryos were also double-stained for β-galactosidase (green) and Isl1/2 (red).
expression. Arrowheads in G and H identify abnormal axonal projections through the Isll/2+ motor column. Scale bars in B, F, 50µm.

**Figure S4. Craniofacial and vertebral defects in E18.5 Gas1; Cdo embryos.** Lateral view of heads (A, C, E, G, I, J, K, M) and frontal view of cervical vertebrae (B, D, F, H, J, K, L, N) of Gas1; Cdo E18.5 embryos. Black arrowheads denote mandibular components, while white arrowheads indicate the maxillary process. Black arrows in B, D, F, H, J, L, N highlight ossification of intervertebral discs. Note the lack of ossification in Gas1−/− (H) Gas1−/−; Cdo+−/− (L) and Gas1−/−; Cdo−− (N) embryos. Asterisks (*) indicate cervical vertebrae that are fused in Gas1−/−; Cdo−− embryos (N). Scale bars in A and B, 1mm.

**Figure S5. Skeletal analysis of Gas1; Cdo E18.5 limbs.** Forelimbs (A, C, E, G, I, K, M) and hindlimbs (B, D, F, H, J, L, N) of E18.5 embryos are shown. Numbers denote the appropriate digits. Note the fusion of digits 2 and 3 in Gas1−/− (G), Gas1−/−; Cdo+−/− (K), and Gas1−/−; Cdo−−/− (M) embryos. Missing hindlimb digits in Gas1−/− (H), Gas1−/−; Cdo−−/− (L), and Gas1−/−; Cdo−−/− (N) embryos are noted by labeling of the remaining digit 2/3. It is not possible to conclusively identify the digit as either 2 or 3. Bar in A, 1mm.
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