Gas1 is a modifier for holoprosencephaly and genetically interacts with sonic hedgehog

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Holoprosencephaly (HPE) is a clinically heterogeneous developmental anomaly affecting the CNS and face, in which the embryonic forebrain fails to divide into distinct halves. Numerous genetic loci and environmental factors are implicated in HPE, but mutation in the sonic hedgehog (Shh) gene is an established cause in both humans and mice. As growth arrest–specific 1 (Gas1) encodes a membrane glycoprotein previously identified as a Shh antagonist in the somite, we analyzed the craniofacial phenotype of mice harboring a targeted Gas1 deletion. Gas1−/− mice exhibited microform HPE, including midfacial hypoplasia, premaxillary incisor fusion, and cleft palate, in addition to severe ear defects; however, gross integrity of the forebrain remained intact. These defects were associated with partial loss of Shh signaling in cells at a distance from the source of transcription, suggesting that Gas1 can potentiate hedgehog signaling in the early face. Loss of a single Shh allele in a Gas1−/− background significantly exacerbated the midline craniofacial phenotype, providing genetic evidence that Shh and Gas1 interact. As human GAS1 maps to chromosome 9q21.3–q22, a region previously associated with nonsyndromic cleft palate and congenital deafness, our results establish GAS1 as a potential locus for several human craniofacial malformations.

Introduction

Holoprosencephaly (HPE) is a clinically heterogeneous developmental field defect of the CNS, in which the embryonic forebrain or prosencephalon fails to divide into distinct halves (1, 2). The underlying brain malformation can have a profound affect upon midline development of the face. In the most severe form of HPE, the forebrain remains as a single undivided vesicle and there is cyclopia, with a single midline eye situated below a rudimentary nose or proboscis and midline clefting of the lip and palate. However, the severity varies in both the brain malformation and the craniofacial features, even among members of the same pedigree. In microform HPE, milder craniofacial features such as ocular hypertelorism, premaxillary agenesis, and solitary median maxillary central incisor (SMMCI) can occur in the absence of defects within the CNS (3). The etiology of HPE is complex, with both environmental and genetic factors being implicated (4). Maternal diabetes, alcohol or drug ingestion, and defects in cholesterol metabolism have all been associated with HPE (5, 6), while a number of candidate genes have also been identified in humans, including sonic hedgehog (SHH) (7, 8).

Shh signaling is essential for normal development of the early forebrain, as transcripts are expressed in mesendoderm of the prechordal plate (9, 10) and are required for formation of ventral midline structures. Shh−/− mice have otocyst, with only a single forebrain vesicle and cyclopia (11). Moreover, mutations in human SHH that have been identified suggest a role in both familial and sporadic HPE (7, 8) and represent a substantial proportion of cases demonstrating autosomal-dominant inheritance of this condition (12). Interestingly, the spectrum of phenotypic severity characteristic of HPE can be seen in association with identical mutations in SHH, even among members of the same pedigree (8). SHH mutation can also lead to SMMCI in the primary or secondary dentition, occurring as an isolated syndrome (12, 13) or as a manifestation of HPE (14).

Shh is a member of the hedgehog family of vertebrate signaling molecules and is essential for normal development of many regions within the embryo (15). The versatility of Shh is reflected in a complex signaling pathway, with unique mechanisms of generation, distribution, reception, and intracellular transduction of the signal (16). Reception at target cells is mediated via direct physical interaction with the Patched1 (Ptc1) transmembrane domain protein (17); in the resting state, Ptc1 inhibits transduction, which is only relieved by the binding of ligand (18). This inhibitory activity is indirect, via Ptc1-mediated membrane localization and phosphorylation of Smoothened (Smo), a G protein–coupled receptor whose activity is essential for signal transduction (19). Because Ptc1 is also a direct transcriptional target of signaling, pathway activity is therefore buffered by the relative availability of bound and unbound receptor at the cell surface (20, 21). In vertebrates, signaling is mediated and interpreted principally via Gli transcription factors (Gli1–Gli3) through a large complex of intracellular mediators. Gli2 and Gli3 function as both activators and repressors and are the principal transducers of Shh signaling activity (22–24). Gli1 is a sole activator and specific target of Shh transduction, but plays a secondary role in signaling (23, 25). The precise events that mediate reception of Shh on the surface of receiving cells are not fully understood. However, several plasma membrane–associated proteins have been identified that can bind Shh and regulate pathway activity in vertebrates. These include Megalin, a low-density lipoprotein receptor (26), the Ig/fibronectin type III repeat transmembrane domain proteins Cdo and Boc (27, 28), and the membrane glycoproteins hedgehog-interacting protein (29, 30) and growth arrest–specific 1 (Gas1; ref. 31).

Nonstandard abbreviations used: Gas1, growth arrest–specific 1; HPE, holoprosencephaly; Ptc1, Patched1; Shh, sonic hedgehog.

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Gas1 encodes a glycosylphosphatidylinositol–linked membrane glycoprotein that is repressed in response to Shh and has previously been demonstrated in vitro to have an antagonistic effect on Shh signaling in the somites (31). The human homolog of mouse Gas1 maps to chromosome 9q21.3–q22 (32), a locus previously associated with a number of human disorders that involve defects within the craniofacial region, including Chiari type I malformation (OMIM 118420; ref. 33), autosomal-dominant and recessive neurosensory deafness (OMIM 606705 and OMIM 600974, respectively; refs. 34, 35), and nonsyndromic cleft lip with or without cleft palate (36). Moreover, mosaic trisomy 9 has been associated with both HPE (37) and defects of the facial midline in the absence of gross brain malformation (38). Therefore, we analyzed the craniofacial phenotype of mice generated with targeted mutation of Gas1 and found that they demonstrated features associated with microform HPE. There was lack of development in the maxillary region, fusion of the premaxillary incisors, cleft secondary palate, and pituitary anomalies; however, the telencephalic ventricles remained intact. Surprisingly, these defects were associated with a lack of Shh transduction in cell populations at a distance from the source of transcription, suggesting that Gas1 function can also potentiate signaling in the early face. Indeed, the loss of a single Shh allele in a Gas1 mutant background significantly worsened the midline craniofacial phenotype, which suggests that Shh and Gas1 genetically interact.

Results

Gas1 mutants have microform HPE associated with multiple craniofacial defects. Newborn Gas1–/– mice exhibit microphthalmia and a lack of retinal pigmentation; despite also being smaller, the gross morphology of Gas1–/– heads appears normal (39). In a mixed CD1-129Sv background these mice are viable until adulthood,
while in a mixed 129Sv-C57BL/6 background the majority die within 3 days of birth. At birth, gross morphology of the brain has been reported to be normal in both mutant lines compared with Gas1+/– WT mice (39, 40).

Because of the putative link between Gas1 function and Shh signaling (31) as well as the pattern of expression (41) and association of the human GAS1 locus with craniofacial anomalies, we analyzed perinatal ontogeny of the craniofacial skeleton in 129Sv-C57BL/6 Gas1+/– mice. Chondrocranial (including both neurocranial and splanchnocranial) and dermatocranial defects were evident, particularly along the neurocranial midline and in the middle and inner ear, but including all 3 primary sensory capsules (nasal, optic, and otic; Figure 1, A and B). These midline defects varied in severity, ranging from bilateral cleft of the secondary palate to absence of elaborated tympani and retroarticular processes of the squamosals (Figure 1, E–L).

Gas1 and Ptc1 are coexpressed in peripheral regions of hedgehog transduction in the early craniofacial region. Shh signaling is known to be important for normal development of the facial midline (7, 8, 42). The findings that lack of Gas1 function is associated with abnormalities in development of the facial midline led us to examine and compare expression domains of Gas1 and members of the Shh signaling pathway during early development of the facial region.

Shh transcripts demonstrated a very specific temporospatial expression domain in the early craniofacial region. At E10.5 and E11.5, Shh was expressed within the CNS, in the floorplate of the mesencephalon and neuroectoderm of the diencephalon, and in the pharyngeal endoderm and facial ectoderm of the frontonasal process (Figure 2, A, B, F, and G) (42). As targets of Shh signaling, Ptc1 and Gli1 were strongly expressed in these regions, showing a graded distribution of transcriptional activity across fields of responding cells (Figure 2, C, D, H, and I). In contrast, Gas1 was expressed at a distance from the source of Shh transcription, in a domain largely reciprocal to that of Ptc1 and Gli1 (Figure 2, E and J). However, examination of serial sections revealed that regions of coexpression existed among Ptc1, Gli1, and Gas1 along the peripheral margins of their respective expression domains. These overlapping regions of transcription were clearly identifiable at known sites of Shh signaling activity in the craniofacial tissues, particularly the neural tube, first branchial arch, and frontonasal region (Figure 2, C–E and H–J). Given that Shh is able to signal over a distance equal to many cell diameters from the source of transcription (43, 44), these findings suggest that Gas1 may interact with Shh in peripheral regions of the signaling domain within the craniofacial region.

Shh signal transduction is reduced in the frontonasal region of Gas1+/– mice. We next compared expression of Shh pathway genes in the craniofacial and midfacial regions of WT and Gas1+/– mice at E10.5 and E11.5. The domains of Shh transcription were normal at both developmental stages within these regions in Gas1+/– embryos (Figure 2, K, L, O, and P) compared with WT embryos (Figure 2, A, B, F, and G). Ptc1 and Gli1 were expressed in response to this signaling throughout the frontonasal region of WT embryos (Figure 2, C, D, H, and I, arrows); however, in Gas1+/– mice there was a clear reduction in the extent of this expression. Ptc1 and Gli1 failed to extend throughout the length of the frontonasal process in Gas1+/– mice at both stages (Figure 2, M, N, Q, and R, arrows). This reduction was in the peripheral regions of Ptc1 and Gli1 expression, at a distance from the source of Shh transcription and in regions coincident

<table>
<thead>
<tr>
<th>Deficiency</th>
<th>PO Gas1+/– (n = 10)</th>
<th>E18.5 Gas1+/–Shh+/– (n = 5)</th>
<th>E18.5 Gas1+/–Shh+/– (n = 4)</th>
</tr>
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<tr>
<td>Fused premaxillary incisor</td>
<td>40% (4/10)</td>
<td>40% (2/5)</td>
<td>25% (1/4)</td>
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<tr>
<td>Synostic premaxilla</td>
<td>50% (5/10)</td>
<td>60% (3/5)</td>
<td>100% (4/4)</td>
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<tr>
<td>Complete secondary cleft palate</td>
<td>30% (3/10)</td>
<td>20% (1/5)</td>
<td>100% (4/4)</td>
</tr>
<tr>
<td>Partial secondary cleft palate</td>
<td>30% (3/10)</td>
<td>0% (0/5)</td>
<td>0% (0/4)</td>
</tr>
<tr>
<td>Basisphenoid defect</td>
<td>67% (4/6)</td>
<td>80% (4/5)</td>
<td>100% (4/4)</td>
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<tr>
<td>Premaxillary incisor agenesis</td>
<td>0% (0/10)</td>
<td>0% (0/5)</td>
<td>75% (3/4)</td>
</tr>
<tr>
<td>Synostotic mandible/symphysis</td>
<td>0% (0/10)</td>
<td>0% (0/5)</td>
<td>75% (3/4)</td>
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<tr>
<td>Fused mandibular incisor</td>
<td>0% (0/10)</td>
<td>0% (0/5)</td>
<td>75% (3/4)</td>
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<td>Clefing of the basisphenoid</td>
<td>0% (0/10)</td>
<td>0% (0/5)</td>
<td>75% (3/4)</td>
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<td>Ectopic proximal dentarya</td>
<td>0% (0/10)</td>
<td>0% (0/5)</td>
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*Not considered a midline-centered deficiency.

incus. Moreover, a process extended from either the incal or the malleal portion of the fused elements toward the stapes. This process was often synchondrotically fused with the stapes. While the stapes was less affected, it was not completely normal and was occasionally synchondrotic with the otic capsule. The styloid process was always, although variably, affected, typically being markedly hypoplastic and separated into tympanohyal and stylohyal portions. The otic capsule was dysmorphic, as were the middle ear–associated regimens tympani and retroarticular processes of the squamosals (Figure 1, E–L).

Gas1 and Ptc1 are coexpressed in peripheral regions of hedgehog transduction in the early craniofacial region. Shh signaling is known to be important for normal development of the facial midline (7, 8, 42). The findings that lack of Gas1 function is associated with abnormalities in development of the facial midline led us to examine and compare expression domains of Gas1 and members of the Shh signaling pathway during early development of the facial region.

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with those expressing Gas1. Gas1 transcripts were only detected at background levels in the Gas1−/− mice as a result of the targeting strategy (data not shown and refs. 39, 40). Therefore, a reduction in Shh signaling activity, as assayed by Shh-responsive genes, was identified in the frontonasal region of Gas1−/− embryos, suggesting that in regions of coexpression, Gas1 might act as an agonist of Shh signal transduction.

Premaxillary hypoplasia and incisor fusion in Gas1−/− mice. At E12.5, a clear restriction in development of the facial midline was identified in Gas1−/− embryos. The frontonasal region was narrower, and the early incisor tooth buds were approximated together (Figure 3, A and B, double arrows). During early tooth development, Shh expression was restricted to these early thickenings of dental epithelium, and in the frontonasal process of WT embryos, these early dental placodes were separated by a region of non–Shh-expressing epithelium (Figure 3, C and E). However, analysis of Shh in the Gas1−/− mice demonstrated a continuous zone of expression, representing fusion of the incisor placodes (Figure 3, D and F). Furthermore, the response of the frontonasal tissue to Shh signaling was reduced in Gas1−/− mice: Ptc1 expression, while normally detectable in the epithelium and mesenchyme of the frontonasal process up to the boundary of the nasal capsule, was not seen in the mutant (Figure 3, G and H). Consistent with these findings, both histological analysis and differential staining of skeletal preparations derived from perinatal Gas1−/− mice demonstrated fusion between the premaxillary incisors (see Figure 1D and Figure 3J). Instead of 2 separate tooth germs situated adjacent to the nasal cavity, the Gas1−/− mouse had a fused incisor crown positioned below the nasal septum in the midline (Figure 3, I and J). Thus, the single premaxillary incisor crown was a direct result of a lack of development in the facial midline of the Gas1−/− embryo.

Reduced Shh signal transduction and cell proliferation in the developing palate of Gas1−/− mice. A complete or partial cleft of the secondary palate was present in 60% of Gas1−/− mice analyzed (Figure 1 and Figure 4, A and B). Standard histological analysis suggested that the initially vertical palatal shelves elevated above the tongue and oriented themselves into a horizontal position; however, in affected mice these shelves were unable to contact each other in the midline (Figure 4, C–F). By E15.5 in WT mice, the shelves began the process of fusion along the anteroposterior axis, while in Gas1−/− mice these shelves failed to meet their counterparts in the midline, resulting in cleft secondary palate.

The Shh signaling pathway is known to play an important role in mediating growth of the palatal shelves (45, 46). At E13.5, Shh was strongly expressed in epithelium of WT palatal processes, and Ptc1 exhibited a gradient of activity within the mesenchyme in response to signaling (Figure 5, A, B, E, and F). In addition, Ptc1 was also expressed within bilateral regions of mesenchyme in the bend of the palatal vault (Figure 5F, arrow). We examined Shh signaling activity in palatal shelves of Gas1−/− mice at E13.5 and found a noticeable reduction in the range of Ptc1 transcriptional activity within the mesenchyme (n = 3 out of 5 mice analyzed); however, Shh was expressed in the palatal epithelium of these mutants (Figure 5, C, D, G, and H). Ptc1 expression was present in close proximity to the palatal shelf epithelium in affected Gas1−/− mice, but the extent of this expression within the mesenchyme was less than that seen in the WT embryos. In addition, Ptc1 expression was absent from palatal bend mesenchyme in Gas1−/− mice (Figure 5H, arrow).
Figure 3

The frontonasal region is reduced in Gas1+/− embryos. (A and B) At E12.5, histological analysis revealed a lack of transverse development in the maxillary incisor region in Gas1+/− compared with WT embryos (double arrows). In addition, the vomerinal organ surrounded by the early palatal plate was detectable in Gas1+/− embryos. (C–F) This reduced development caused fusion of the early incisor placodes and continuous expression of Shh across the frontonasal region of Gas1+/− mice. (C and D) Radioactive section in situ hybridization. (E and F) Whole-mount in situ hybridization. (G and H) Ptc1 expression was reduced in the frontonasal region of Gas1+/− compared with WT embryos. (I and J) In perinatal WT embryos, the bilateral maxillary incisor teeth formed in their normal positions adjacent to the nasal capsule, while in Gas1+/− mice, a single enlarged maxillary incisor crown was present, representing fusion between 2 separate tooth germs. The paraseptal cartilage, which failed to form in Gas1−/− mice, is denoted by an arrow. i, premaxillary incisor. Scale bar: 250 μm (A–D, G and H); 500 μm (E and F); 750 μm (I and J).

We further addressed the cleft phenotype of Gas1−/− mice by analyzing cell proliferation in epithelium and mesenchyme of the palatal shelves at E12.5 and E13.5 (Figure 6, A–F). No significant difference in epithelial-like and mesenchymal proliferation within the anterior palate between WT and Gas1−/− embryos, and while some reduction was seen in epithelium of the apex in the middle and posterior regions, this was not identifiable in the bend region. However, there was a significant reduction in the percentage of mesenchymal cells proliferating within the middle and posterior regions of the palatal shelves at the apex (42% for both; P < 0.001) and bend (40% and 32%, respectively; P < 0.001) in Gas1−/− compared with WT embryos (Figure 6, G and H). Therefore, in the absence of Gas1 function, a reduction in Ptc1 transcription in mesenchyme of both the palatal apex and bend was associated with a statistically significant reduction in cell proliferation within these regions.

Gas1 and Shh genetically interact. These data suggest that Gas1 is able to positively regulate Shh signal transduction and that this activity is relevant for signaling at a distance from the source of transcription. It is clear that the absence of Gas1 function had important consequences for normal midline development of the craniofacial region. In order to determine whether Gas1 and Shh interact genetically, we analyzed the craniofacial phenotype of mice lacking a single Shh allele in a Gas1+/− background.

Individually, both Gas1+/− and Shh+/− mice were phenotypically normal in the craniofacial region; however, the loss of a Shh allele in a Gas1+/− background caused a progressively more severe HPE phenotype. Gas1+/− Shh+/− embryos survived to birth but only had a single external nostril, while Gas1−/− Shh+/− mice had severe craniofacial defects and only survived to around E9.5 (data not shown). Indicative of a significant genetic interaction, Gas1−/− Shh+/− perinatal skulls exhibited exacerbated neurocranial, splanchnocranial, and dermocranial defects relative to the Gas1−/− single mutants, as well as significant and novel defects of patterning and development (Figure 7).

Skull size was further reduced in compound Gas1−/− Shh+/− perinatal mice, and patent sutures between the frontal and parietal ossifications were not evident. Development of the neurocranial base was severely disrupted: the trabecular basal plate, which runs from the rostral basisphenoid, presphenoid, and ethmoid through the nasal septum, was discontinuous and cleft. The nasal capsules and associated dermal ossifications were drastically reduced, without proper midline manifestations. The maxillaries were hypoplastic, synostotic, and lacking teeth. Lateral extensions from the neurocranial base were all disrupted, the ala temporalis were hypoplastic, the lamina obliterans failed to properly invest the cartilage of the ala, and ectopic preotic cartilaginous pillars extended toward the crista parotica of the otic capsule (Figure 7, A–D).
Meckel’s cartilage was truncated and lacked a malleal end. Instead, the proximal end bifurcated slightly, apparently being shared by the dysmorphic proximal dentary and an ectopic ossification that appeared to be a mirror-image duplication of the proximal dentary, which included a secondary cartilage-containing condylar process and a molar alveolus. The distal dentary lacked a symphysis, as it was synostotic across the midline, and contained a single lower incisor (Figure 7, C and E). Middle ear–associated splanchnocranial elements either failed to develop (malleus and incus) or were represented by a cartilaginous remnant (stapes). The associated dermatocranial elements, the ectotympanic and gonial bones, also failed to form. The squamosal bones were repatterned and separated into distinct retrotympanic and squamosal portions (Figure 7F). Together, these data demonstrate a marked worsening of the midline craniofacial phenotype with the loss of a single Shh allele in a Gas1–/– background.

Discussion

HPE, cleft palate, and congenital deafness are 3 of the most common craniofacial anomalies that affect human populations. Both cleft palate and congenital deafness have a birth prevalence of around 1 in 1,000 (47, 48), while HPE is seen in 1 in 10,000–20,000 live births and in 1 in 250 during embryogenesis, making it the most common cause of brain abnormality in humans (1, 49). In many cases the etiology of these conditions remains unknown; however, the 9q21.3–q22 locus has previously been associated with both neurosensory deafness (34, 35) and nonsyndromic cleft palate (36), while mosaic trisomy 9 has been implicated in HPE (37) and isolated defects of the facial midline (38). Given that the human GAS1 gene resides at this locus, we examined the consequences of a loss in Gas1 function using a mouse model.

Gas1 was originally identified as a gene highly expressed in a fibroblast cell line maintained under conditions of growth arrest (50); it encodes a membrane glycoprotein that in vitro has been implicated as an inhibitor of cell cycle progression (51, 52), a participant in excitotoxic neuronal cell death (53), and a negative regulator of Shh (31, 43). However, Gas1–/– mice do not demonstrate generalized growth excess or tumor progression: while Gas1 does negatively regulate retinal proliferation (39), it is actually required for normal postnatal proliferation in regions of the cerebellum (40). Similarly, the phenotype of these animals is not indicative of excess Shh signal transduction. The limbs develop with small auto-

Figure 4
Palatogenesis in WT and Gas1–/– mice. (A and B) Scanning electron microscopy demonstrating that palate development was complete in WT mice at P0 (A), while there was a full-penetrance cleft of the secondary palate in affected Gas1–/– mice at the same time point (B). (C and D) Histological analysis showed comparable morphology of the palatal shelves at E13.5 in WT and Gas1–/– embryos. (E and F) At E15.5, while the shelves rose above the tongue and fused in the midline in the WT embryos, fusion failed to occur in affected Gas1–/– embryos. Scale bar: 1.25 mm (A and B); 500 μm (C–F).

Figure 5
Shh pathway gene expression in the palatal shelves of WT and Gas1–/– mice. (A–D) At E13.5, Shh was expressed in the epithelium of WT and Gas1–/– palatal shelves. (E and F) Ptc1 was expressed throughout the epithelium and mesenchyme of WT shelves, in addition to isolated regions in the palatal bend mesenchyme (F, arrow). (G and H) In contrast, Gas1–/– palatal shelves had reduced Ptc1 expression, which failed to extend along their length and was lost in the palatal bend mesenchyme (H, arrow). Scale bar: 250 μm.
pods, missing phalanges, and anterior digit syndactyly, but this is secondary to defective proliferation in the apical ectodermal ridge (54). Gas1 also exhibits structural homology to the GFR-α receptor for the GDNF family of ligands (GFL), capable of independently binding the receptor tyrosine kinase ret and interfering with signaling (55). Therefore, Gas1 would appear to have multiple functions, influencing the cell cycle in a cell context–dependent manner and potentially interacting with several signaling pathways.

Our findings suggest that Gas1 can also act cooperatively during Shh signaling in both the early facial processes and developing palate. Gas1 demonstrates coexpression with Ptc1 in peripheral domains of Shh-mediated signal activity; it is within these regions that signaling is reduced in Gas1−/− mice. This is consistent with previous reports that the active signaling form of Shh can bind Gas1 (31). This interaction is known to occur through a common interface with that for Ptc1, inviting speculation that Gas1 might act as a coreceptor, presenting Shh to Ptc protein in regions of coexpression (56). However, our present data are inconsistent with previous reports that Gas1 activity can attenuate the mitogenic response to Shh signaling in the somite and downregulate Ptc1 induction in isolated diastema mesenchyme of the jaw (31, 43). However, these contrary observations might be explained within the context of endogenous Ptc1 and Gas1 protein levels, which would be expected to strongly influence signal output. From our analysis, it is clear that Gas1 positively influences hedgehog transcription at the margins of signal activity, where membrane quantities of Gas1 and Ptc1 are at minimal levels. In regions where either Gas1 or Ptc1 are in gross excess, these effects would appear to be negated.

Gas1−/− mice demonstrated a number of features associated with HPE, each with varying levels of penetrance. The evidence of reduced hedgehog target transcriptional activity in the craniofacial region of these mice is consistent with Gas1 mediating its effects through the Shh pathway. HPE is a common congenital anomaly, which in the most severe form is characterized by a complete failure of forebrain division and cyclopia (57). However, the phenotypic spectrum associated with this condition is extensive, and a gradation of severity exists in the malformations that can affect both the brain and the face (58). The basis of this clinical heterogeneity is not fully understood, but it has been suggested that HPE represents a multifactorial condition (even in cases demonstrating autosomal-dominant inheritance patterns), as penetrance of the phenotype reflects the varying contributions of multiple genetic and environmental influences within the individual (3). Indeed, the association between hedgehog signaling and HPE provides clear evidence of this complexity. The early craniofacial region is sensitive to perturbations in Shh signaling and this changes with time; disruption of the pathway during discrete periods of embryonic development can recapitulate the phenotypic spectrum of HPE, inducing hypotelorism, midfacial hypoplasia, facial clefting, or frank cyclopia, depending on the timing and magnitude of signal loss (42). In addition, modifying loci have also been identified that act in concert with SHH in the induction of HPE in human populations; these include TGIF and ZIC2 (12). Here, we provide evidence to suggest that GAS1 might represent a further locus for HPE in humans.

We also analyzed the palatal phenotype of Gas1 mutants and provided further evidence that Shh signaling plays an important role in mediating secondary palatogenesis, particularly in mesenchymal proliferation within the palatal shelves (46). Gas1−/− mice demonstrated a reduction in the range of Shh signal response across mesenchyme of the palatal shelf. In these mutants, Ptc1 expression remained localized to superficial regions of palatal mesenchyme adjacent to the epithelium and failed to form a normal gradient of activity across the shelves. Given that the cleft phenotype was not completely penetrant, this implies that mesenchymal proliferation must reach a certain threshold to ensure that the shelves elevate above the tongue and meet their counterparts in the midline. The incomplete penetrance associated with the palatal phenotype, in
addition to the finding that some Gas1−/− mice demonstrating microform HPE were unaffected by cleft palate, indicates that this phenotype can occur independently of HPE and shows the importance of modifying factors in the phenotypic manifestation of this condition. Shh signaling also plays an important role during development of the inner ear: morphogenesis of this region was disrupted in Shh−/− mice, causing a lack of organization into distinct vestibular and cochlear portions and ventral otic derivatives, including the failure of the cochlear duct and cochleovestibular ganglia to develop (59, 60). Gas1−/− mice exhibited severe disruption in both the middle and inner ear, and it is possible that this is secondary to defective hedgehog signaling.

Importantly, the loss of a single Shh allele in a Gas1−/− background significantly worsened the midline craniofacial phenotype and auditory defects of these animals. In particular, the neurocranial base became severely disrupted with a cleft and discontinuous trabecular basal plate, while the premaxillary region lacked incisor teeth. Notably, Gas1−/−Shh+/− perinates exhibited what appeared to be a duplicated proximal dentary that included a condylar process (green arrow). White arrowheads highlight truncation and proximal bifurcation of Meckel’s cartilage. Original magnification, ×4. (D) Calvaria of Gas1−/−Shh+/−, Gas1−/−Shh++, and Gas1−/−Shh−/− perinates. Black arrowheads highlight the loss of a patent coronal suture in the calvarium of the Gas1−/−Shh−/− skull. (E) Histological sections of the developing ectopic molar (red arrow) and dentary. MC, Meckel’s cartilage; oc, otic capsule. (F) Comparison of WT and Gas1−/−Shh−/− middle ears. Outlined in the WT image are styloid process (green), stapes (orange), incus (yellow), and malleus (purple). Yellow arrows highlight the vestigial stapes; green arrows indicate the ectopic preotic pillar running from the neurocranial base to the otic capsule; red arrows indicate persistent cartilage of the ala temporalis; blue arrows indicate changes at the squamosal; black arrows indicate the ectopic condyles; dotted yellow line denotes axis of symmetry. Original magnification, ×2.

Figure 7 Demonstration of a genetic interaction between Gas1 and Shh. (A–D) Comparison of Gas1−/−Shh++, Gas1−/−Shh−/−, and Gas1−/−Shh−/− perinatal skulls. The left 3 mice were littermates. (A) Norma basalis. The black line aligns the skulls, which were photographed at the same magnification. (B) Norma lateralis. (C) Dentaries of Gas1−/−Shh−/−, Gas1−/−Shh++, and Gas1−/−Shh−/− perinates. Notably, the Gas1−/−Shh−/− perinates, in addition to midline fusion and a single incisor, exhibited what appears to be a duplicated proximal dentary that includes a secondary cartilage-containing condylar process (black arrows) and an alveolus containing an ectopic molar (green arrow). White arrowheads indicate truncation and proximal bifurcation of Meckel’s cartilage. Original magnification, ×4. (D) Calvaria of Gas1−/−Shh++, Gas1−/−Shh−/−, and Gas1−/−Shh−/− skulls. Black arrowheads highlight the loss of a patent coronal suture in the calvarium of the Gas1−/−Shh−/− skull. (E) Histological sections of the developing ectopic molar (red arrow) and dentary. MC, Meckel’s cartilage; oc, otic capsule. (F) Comparison of WT and Gas1−/−Shh−/− middle ears. Outlined in the WT image are styloid process (green), stapes (orange), incus (yellow), and malleus (purple). Yellow arrows highlight the vestigial stapes; green arrows indicate the ectopic preotic pillar running from the neurocranial base to the otic capsule; red arrows indicate persistent cartilage of the ala temporalis; blue arrows indicate changes at the squamosal; black arrows indicate the ectopic condyles; dotted yellow line denotes axis of symmetry. Original magnification, ×2.
Methods

Generation of Gas1 and Shh mutant mice. Gas1<sup>+/–</sup> and Shh<sup>+/–</sup> mice were generated and genotyped as previously described (11, 39) and maintained in a 129Sv-C57BL/6 mixed background. Timed matings were set up such that no litter was allowed to develop. All animal procedures were reviewed, approved, and carried out under license issued by the Animals and Scientific Procedures Division of the Home Office, Her Majesty’s Government, London, United Kingdom.

Histological and skeletal analysis. For histological analysis, embryos were fixed in 4% paraformaldehyde at 4°C, dehydrated through a graded ethanol series, embedded in paraffin wax, sectioned at 7 μm, and stained with H&E. For differential staining of bone and cartilage, E18.5 and newborn (i.e., P0) mice were fixed overnight in 95% ethanol, stained, and embedded with H&E. For scanning electron microscopy, whole mounts were labeled with BrdU via intraperitoneal injection into pregnant females (5 mg/100 g body wt) 2 hours prior to sacrifice. Embryos were fixed in 4% paraformaldehyde at 4°C, dehydrated in ethanol, and sectioned in paraffin wax, and sectioned at 7 μm. The percentage of BrdU-positive cells was calculated after counting 1 researcher blinded to experimental group on 2 separate occasions 1 week apart, and the mean value was calculated. A total of 4 WT and 4 Gas1<sup>+/–</sup> mice were analyzed for each embryonic stage, which included a total of 45 WT and 55 Gas1<sup>+/–</sup> sections. BrdU-positive cells were counted in the epithelium and mesenchyme of the anterior and posterior palate, using an ocular scale oriented at the apex of the palatal projection and in the bend region of the palatal shelf (46). Specifically, at E12.5 this covered an area of mesenchyme that was 0.02 mm<sup>2</sup> at the palatal apex and 0.012 mm<sup>2</sup> at the bend; these regions were bounded by epithelium of lengths of 132 μm and 88 μm, respectively. At E13.5, the areas of mesenchyme counted were 0.03 mm<sup>2</sup> and 0.015 mm<sup>2</sup> and bounded by 165-μm lengths of epithelium at the palatal apex and bend, respectively.

Scanning electron microscopy. For scanning electron microscopy, tissues were fixed and stored in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, rinsed in 0.1 M cacodylate buffer, and postfixed in 1% osmium tetroxide in water for 90 minutes, followed by dehydration through a graded series of acetone in water, critical point drying in liquid CO₂, and sputter coating with gold. Tissues were examined and recorded in a Phillips SEM501B scanning electron microscope fitted with a Deben Pixie digital scan generator and recorder.

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