GAS1 is required for NOTCH-dependent facilitation of SHH signaling in the ventral forebrain neuroepithelium

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ABSTRACT

Growth arrest-specific 1 (GAS1) acts as a co-receptor to patched 1, promoting sonic hedgehog (SHH) signaling in the developing nervous system. GAS1 mutations in humans and animal models result in forebrain and craniofacial malformations, defects ascribed to a function for GAS1 in SHH signaling during early neurulation. Here, we confirm loss of SHH activity in the forebrain neuroepithelium in GAS1-deficient mice and in induced pluripotent stem cell-derived cell models of human neuroepithelial differentiation. However, our studies document that this defect can be attributed, at least in part, to a novel role for GAS1 in facilitating NOTCH signaling, which is essential to sustain a persistent SHH activity domain in the forebrain neuroepithelium. GAS1 directly binds NOTCH1, enhancing ligand-induced processing of the NOTCH1 intracellular domain, which drives NOTCH pathway activity in the developing forebrain. Our findings identify a unique role for GAS1 in integrating NOTCH and SHH signal reception in neuroepithelial cells, and they suggest that loss of GAS1-dependent NOTCH1 activation contributes to forebrain malformations in individuals carrying GAS1 mutations.

KEY WORDS: Forebrain organizer region, Holoprosencephaly, NOTCH intracellular domain, Neuroepithelial precursor cells, HH co-receptors

INTRODUCTION

The mammalian forebrain develops from a simple neuroepithelial sheet at the anterior end of the neural plate: the anterior neuroectoderm. Several morphogen pathways provide instructive signals during early neurulation, including sonic hedgehog (SHH), which governs patterning processes along the dorso-ventral axis of the developing neural tube (reviewed by Dessaud et al., 2008). In the embryonic forebrain, SHH is initially produced from the prechordal plate (PrCP) at the anterior tip of the embryo. It acts on the overlying rostral diencephalon ventral midline (RDVM) to induce its own production and the expression of ventral forebrain markers. SHH transcriptional targets, such as NK2 homeobox 1 (NKX2.1), specify ventral midline identity and counteract dorsalizing signals by bone morphogenetic protein 4 (BMP4) (Hoch et al., 2009; Sousa and Fishell, 2010). In line with a prominent role for SHH in forebrain development, defects in SHH signaling in humans (Roessler et al., 1996) and in mouse models (Chiang et al., 1996) result in midline formation defects, ultimately causing craniofacial malformation and holoprosencephaly (HPE). HPE is the most frequent forebrain anomaly in humans and may include improper division of the forebrain hemispheres, as well as cyclopia and formation of a proboscis (Muenke and Beachy, 2000). Inheritable mutations in components of the SHH signaling pathway have been associated with human HPE, including mutations in SHH, in its receptor patched 1 (PTCH1), or in downstream transcription factors, such as GLI family zinc finger 2 (GLI2), SIX homeobox 3 (SIX3) and zinc-finger protein of the cerebellum 2 (ZIC2) (Roessler and Muenke, 2010).

Besides the canonical SHH receptor PTCH1, previous studies have identified additional cell surface proteins that facilitate SHH signal reception in the neuroepithelium and that cause midline malformations and HPE when mutated (reviewed by Christ et al., 2016). One of these auxiliary SHH receptors is growth arrest-specific 1 (GAS1), a 45 kDa glycoprotein attached to the plasma membrane via a glycosylphosphatidylinositol (GPI) anchor. Among other cell types, GAS1 is expressed in progenitor cells of the developing central nervous system (Allen et al., 2007; Lee and Fan, 2001). GAS1 facilitates interaction of SHH with PTCH1 under limiting SHH concentrations by forming co-receptor complexes, promoting SHH signaling in the cerebellum (Izzi et al., 2011) and spinal cord (Allen et al., 2011, 2007; Martinelli and Fan, 2007).

Loss of GAS1 in gene-targeted mice (Allen et al., 2007; Khonsari et al., 2013; Martinelli and Fan, 2007; Seppala et al., 2007, 2014) or in individuals with GAS1 missense mutation (Pineda-Alvarez et al., 2012; Ribeiro et al., 2010) result in a range of craniofacial and forebrain malformations, including small eyes, cleft palate, fusion of nasal processes and HPE. These malformations are believed to originate from defects in early development of the rostral forebrain neuroepithelium, as judged from impaired expression of Shh (Seppala et al., 2014) as well as its targets Nkx2.1 (Allen et al., 2007; Echevarria-Andino and Allen, 2020) and Glil (Echevarria-Andino and Allen, 2020; Khonsari et al., 2013; Seppala et al., 2007) in this tissue in Gas1 mutant mice.

Our findings now corroborate loss of SHH activity in the GAS1-deficient forebrain neuroepithelium in vitro and in vivo. Surprisingly, this defect may be attributed, at least in part, to a novel role for GAS1 in promoting NOTCH signaling, required to sustain the SHH activity domain in this forebrain organizer region. Loss of GAS1 impairs NOTCH-mediated facilitation of
SHH signaling in neural progenitors and results in a failure to permanently establish the ventral SHH activity domain in the embryonic forebrain, which is the ultimate cause of forebrain and craniofacial anomalies in individuals lacking GAS1.

RESULTS

Reduced SHH and NOTCH pathway activities in the rostral ventral neuroepithelium of Gas1 mutant mouse embryos

To dissect the contribution of GAS1 to SHH signaling during early neuroepithelial differentiation, we compared expression of Shh and its target Gli1 (Lee et al., 1997) in Gas1−/− mouse embryos and their littermate controls. No differences were detected in Shh transcript and SHH protein in the PrCP and overlying RDVM, a major forebrain organizer region, at E8.5 (8-9 and 10-11 somites; Fig. 1A,B and Fig. S1A). By contrast, Shh and Gli1 transcripts in the rostral ventral neuroepithelium of Gas1−/− embryos were decreased by E9.5 (Fig. 1C) and SHH protein was completely lost by E10.5, when compared with controls (Fig. 1D). These findings extended earlier observations of reduced expression of Shh (Seppala et al., 2014) at E12.5 or Nkx2.1 (Allen et al., 2007; Echevarria-Andino and Allen, 2020) and Gli1 (Echevarria-Andino and Allen, 2020; Khonsari et al., 2013; Seppala et al., 2007) at E9.5-10.5 in the ventral forebrain neuroepithelium of Gas1 mutant mice. Importantly, our findings revealed that GAS1 is not essential for SHH signaling in this tissue. Rather, it promotes persistence of this SHH activity domain, initially established in the absence of this receptor.

At E8.5, expression of Shh in the PrCP provides the major source of the morphogen to pattern the overlying RDVM (Dule et al., 1997). To exclude a primary defect in this source as the reason for loss of SHH activity in Gas1−/− embryos at later embryonic stages, we quantified the size of the mutant PrCP based on Shh fluorescence in situ hybridization (FISH) on E8.5 coronal forebrain sections. These studies failed to detect any difference in the size of the Shh expression domain in the PrCP comparing control and Gas1−/− embryos (Fig. S1B,C).

To elucidate the reasons for loss of SHH activity in Gas1−/− embryos at later stages of development, we performed comparative bulk RNAseq of the microdissected rostral ventral neuroepithelium from E10.0 Gas1+/+ and Gas1−/− embryos (Fig. 2A). Global changes in the transcriptomes were determined by principal component analysis (Fig. 2B) and by hierarchical clustering of 324 identified differentially expressed genes (DEGs; Fig. 2C and Table S3). Gene ontology (GO) term enrichment analysis identified the expected changes in gene expression related to nervous system development, neurulation and Hedgehog signaling (Fig. 2D). Changes in DEGs included decreased levels of transcripts for Shh, Ptch1 and Nkx2.2 (Fig. 2E and Table S1). In addition, manual query of the RNAseq data for established downstream targets of the SHH signaling pathway identified statistically significant decreases...
Fig. 2. See next page for legend.
Fig. 2. Global transcriptomics indicate defects in NOTCH signaling in the GAS1-deficient rostral ventral forebrain. (A) The SHH expression domain (as highlighted by immunodetection of SHH) in the rostral ventral midline of murine E10.0 forebrain sections was isolated by laser capture microdissection and subjected to bulk RNA-sequencing as detailed in the supplementary Materials and Methods. (B,C) Principal component analysis (PCA; B) and column-based hierarchical clustering heatmap (C) for all 324 identified differentially expressed genes (DEGs) of replicate pools (five embryos per pool) of control (n=5) and Gas1−/− (n=4) tissue samples are shown. (D) Gene ontology (GO) term enrichment analysis, including the categories ‘enriched pathway sets’ and ‘associated phenotypes’. The five top hits for each category are shown in decreasing order of evidence based on GO term enrichment test q-value. Numbers indicate the quantity of DEGs related to the respective term. (E,F) Heatmap of DEGs associated with the GO terms ‘biological process’, ‘enriched pathway sets’ and ‘associated phenotypes’. The five top hits for each category are shown in decreasing order of evidence based on GO term enrichment test q-value. Numbers indicate the quantity of DEGs related to the respective term. (E) Heatmap of DEGs associated with the GO terms ‘enriched pathway sets’ (E) and ‘NOTCH signaling pathway’ (F).

In addition to the anticipated change in SHH target gene expression, global transcriptomics identified unexpected alterations in NOTCH signaling in the ventral forebrain midline of Gas1 mutants (Fig. 2D). NOTCH pathway components have been shown to be expressed in the rostral ventral neuroepithelium of the mouse embryo from E8.5 onwards (Ware et al., 2014), but no function for GAS1 in modulating NOTCH signaling in this domain had been described so far. In detail, our RNAseq data revealed decreased transcript levels for the DEGs NOTCH receptor 1 (Notch1), Delta-like proteins 1 (Dll1) and 3 (Dll3), Hes family bHLH transcription factor 5 (Hes5), NOTCH-regulated ankyrin repeat protein (Nrarp), and maniac fringe (Mfng) (Fig. 2F and Table S2). Alterations in NOTCH activity in Gas1−/− embryos were further supported by expression analyses of genes affected by NOTCH deficiency in other models (Ratié et al., 2013; Ware et al., 2016). Genes also downregulated in the rostral forebrain neuroepithelium of Gas1 mutant mice included the transcription factor Hey1, which is a NOTCH target in the murine forebrain neuroepithelium (Ware et al., 2014) (Fig. S3). Of note, some targets, upregulated upon NOTCH pathway disruption in mouse and chick models (Ratié et al., 2013; Ware et al., 2016), were downregulated in the Gas1−/− ventral midline, including Ascl1, Stmm2 and Slit1 (Fig. S3).

Impaired NOTCH pathway activity was further validated by expression analyses in Gas1−/− embryos. In detail, Hes5 expression in the rostral neuroepithelium was reduced as early as E8.5 (10-11 somites; Fig. 3B), a time point coinciding with Gas1 expression in this tissue (Fig. S4A). Transcripts for Hes5, and also for Notch1 and
Dll1 in the rostral ventral neuroepithelium of mutants, were further reduced at E9.5 (Fig. 3C) and E10.5 (Fig. 3D). Notably, a reduction in Hes5 expression at E8.5 preceded defects seen in the SHH pathway in Gas1 mutants at E9.5 (Fig. 1C); and they were specific to the forebrain neuroepithelium as no changes in expression of Notch1, Dll1 or Hes5 were detected in the spinal cord of mutant when compared with control embryos (Fig. S4B).

**GAS1 promotes activation of NOTCH1 to facilitate SHH-dependent ventral neuroepithelial cell fate specification**

To further dissect the molecular mechanism underlying GAS1 function in the rostral neuroepithelium, we established isogenic human induced pluripotent stem cell (iPSC) lines, either wild type or genetically deficient for GAS1 (GAS1 KO; Fig. S5A,B). Loss of GAS1 did not impact pluripotency of iPSCs, as shown by normal expression of pluripotency markers (Fig. SSC-F) and by their ability to generate all three germ layers (Fig. S5G-J).

Next, wild-type and GAS1 KO iPSCs were subjected to differentiation into neural progenitor cells (NPCs) of dorsal or ventral cell identity using established protocols (Fig. 4A) (Chambers et al., 2009; Flemming et al., 2020). When treated with noggin, dorsomorphin and small molecule SB431542 to block BMP and TGFβ signaling, both wild-type and GAS1 KO iPSCs downregulated the pluripotency marker OCT4 and induced the neuroectodermal marker Pax6 (Fig. 4B,C; Fig. S6A,B). Consistent with adopting a dorsal neural progenitor fate, both genotypes induced GLI3, an inhibitor of the SHH pathway (Fig. 4D). In addition, wild-type, but not KO, cells induced expression of GAS1 (Fig. 4E,F), a negative SHH transcription target (Allen et al., 2007). By contrast, when iPSCs were treated with SHH, instead of noggin, GLI1 transcription in wild-type NPCs to a much greater extent than in GAS1 KO NPCs (SHH-Np; Fig. 4G-K). Loss of SHH-dependent repression of Pax6 or induction of Nkx2.1 in mutant NPCs was confirmed by immunocytochemistry (Fig. S6B,C). These data documented the inability of GAS1-deficient NPCs to adopt a ventral cell fate, likely due to their impaired response to ventralizing signals provided by SHH.

To substantiate the inability of GAS1 KO cells to respond to SHH, we tested GLI1 transcript levels in NPCs treated with conditioned medium from HEK293 cells secreting SHH-Np (Christ et al., 2012). SHH-Np induced GLI1 transcription in wild-type NPCs to a much greater extent than in GAS1 KO NPCs (SHH-Np; Fig. 4L), a response blocked by the hedgehog inhibitor cycloamine-KAAD in both cell types (SHH-Np+KAAD; Fig. 4L). Treatment with a smoothened (SMO) agonist (SAG) resulted in a similar induction of GLI1 in both genotypes, indicating pathway integrity in GAS1 KO cells downstream of SMO (SAG; Fig. 4L). Taken together, our findings substantiated iPSC-derived NPCs as a faithful model for studying neural progenitor fate decisions, and the importance of GAS1 for interpreting ventralizing signals provided by SHH to this cell type.

In line with our gene expression data from Gas1 mutant embryos, GAS1 KO NPCs also failed to activate the NOTCH pathway during neuroectodermal differentiation. Thus, despite normal expression of NOTCH1 and DLL1 transcripts and proteins (Fig. 5A,B,D), induction of HES5 transcript and protein levels was much lower in GAS1 KO when compared with wild-type NPCs (Fig. 5C,E). This defect was seen for dorsal and ventral cell fates alike, documenting a SHH-independent role for GAS1 in NOTCH signaling. GAS1 deficiency impacted Notch1 and Dll1 expression in E9.5-10.5 embryos (Fig. 3C,D) but not in NPCs (Fig. 5A,B). This distinction likely reflected the fact that iPSC-derived NPCs recapitulate an early stage of neuroepithelial differentiation. Importantly, enhancing or abrogating SHH activity by SHH-Np and SAG or by cycloamine, respectively, did not impact HES5 transcript levels in wild-type NPCs (Fig. S7). These findings confirmed that SHH does not control expression of NOTCH pathway components in neural progenitors and that loss of SHH activity was not the primary cause of NOTCH pathway deficiency in GAS1 KO NPCs.

The impact of GAS1 on NOTCH signaling manifested at the level of ligand-induced processing of the receptor polypeptide, as shown by quantification of the NOTCH1 intracellular domain (NICD) produced in response to treatment with its ligand DLL1. In wild-type NPCs, treatment with recombinant DLL1 increased NICD levels when compared with the control condition (Fig. 5F,G). NICD formation in wild-type NPCs was blocked by addition of the γ-secretase inhibitor DAPT (Fig. 5F,G). DLL1-induced NICD production correlated with increased levels of HES5 transcripts in wild-type cells, faithfully recapitulating established NICD actions in this cell type (Fig. 5H). By contrast, DLL1-induced NICD generation and downstream signal transduction was completely lost in GAS1 KO NPCs as the levels of NICD (Fig. 5F,G) as well as HES5 transcripts (Fig. 5H) did not increase above the levels seen in cells in the absence of the ligand. These findings documented a complete loss of ligand-induced NOTCH activity in NPCs lacking GAS1.

To investigate the molecular mechanism of GAS1 action in NOTCH signaling, we performed proximity ligation assays, demonstrating the close proximity of GAS1 and NOTCH1 in wild-type NPCs (Fig. 6A). Immunoprecipitation (IP) assays further showed that GAS1 co-immunoprecipitates with PTCH1 (a known interaction) but also with NOTCH1 (a known interaction) but also with NOTCH1 (a known interaction) but also with NOTCH1 (a known interaction) but also with NOTCH1 (a known interaction). To investigate a similar role for GAS1 in the rostral neuroepithelium, and the relevance of GAS1 to the forebrain neuroepithelium as no changes in expression of Notch1, Dll1 or Hes5 were detected in the spinal cord of mutant when compared with control embryos (Fig. S4B).

**Ectopic NOTCH signaling rescues loss of SHH activity in the GAS1-deficient forebrain neuroepithelium**

NOTCH has been shown to facilitate SHH signaling in the retina and spinal cord of mouse, chick and zebrafish models (Huang et al., 2012; Jacobs and Huang, 2019; Kong et al., 2015; Ringuelette et al., 2016; Stasiulewicz et al., 2015). To investigate a similar role for NOTCH in the rostral neuroepithelium, and the relevance of GAS1 in this process, we studied the interdependency of SHH and NOTCH pathways in NPCs. Levels of HES5 were always lower in GAS1 KO when compared with wild-type NPCs, irrespective of the presence or absence of SHH-Np (Fig. 7A), consistent with the notion that GAS1 activation of NOTCH is SHH independent. By contrast, induction of GLI1 and Nkx2.1 expression in wild-type NPCs by addition of SHH-Np was reduced (GLI1) or even...
completely lost (NKX2.1) by blockade of NOTCH signaling using DAPT (Fig. 7B,C). In fact, GLI1 and NKX2.1 transcript levels in wild-type cells treated with SHH-Np and DAPT were comparable with levels in GAS1 KO cells treated with SHH-Np in the absence of DAPT. These findings further argued that GAS1-dependent activation of NOTCH1 is a major contributor to SHH signal strength in neural progenitors.

To further substantiate the relevance of GAS1-dependent activation of NOTCH1 for SHH signaling, we tested SHH activity in GAS1 mutant cells following ectopic induction of NOTCH1 activity. In these experiments, lentiviral overexpression of NICD rescued the defect in HES5 induction in GAS1 KO NPCs independently of SHH as levels of this transcript were comparable in NICD-treated wild-type and KO NPCs, both in the presence or
absence of SHH-Np (Fig. 7D). Importantly, NICD overexpression partially rescued SHH-dependent GLI1 and NKX2.1 induction in GAS1 KO NPCs treated with SHH-Np and NICD when compared with treatment with SHH-Np only (Fig. 7E,F).

So far, our findings corroborated a role for GAS1 in NOTCH-dependent facilitation of SHH signaling in cultured NPCs. To confirm the relevance of this activity for SHH action in the developing forebrain, we tested the ability of NOTCH signaling to rescue loss of SHH activity in the Gas1−/− rostral ventral neuroepithelium. To do so, we used cephalic explants, a utilitarian model to study neuroepithelial differentiation ex vivo (Christ et al., 2012; Echevarria et al., 2001). In the protocol used here, cephalic explants were isolated from E9.5 wild-type and Gas1−/− embryos, and cultured for 48 h, followed by gene expression analyses using ISH (Fig. 8A). In Gas1 mutant explants, correct, albeit slightly reduced, expression of Shh was seen at E9.5 in the rostral ventral neuroepithelium (t=0 h; Fig. 8B). This expression domain was completely lost after 2 days in culture.
By contrast, Shh expression sustained in the rostral ventral neuroepithelium of wild-type explants (Fig. 8B). These findings recapitulated our in vivo data that GAS1 was required to sustain the Shh expression domain, initially established normally around E8.5 in the Gas1 mutant forebrain. Thus, cephalic explants represented a faithful model to recapitulate Shh defects observed in the GAS1-deficient rostral neuroepithelium in vivo.

Next, we treated cephalic explants with lentiviral constructs encoding NICD to ectopically induce NOTCH signaling. In line with this strategy, expression of Hes5, which is absent from GAS1-deficient explants treated with control virus, was rescued in mutants by ectopic expression of NICD (Fig. 8C). Importantly, overexpression of NICD also increased Shh transcript levels in the rostral ventral neuroepithelium in approximately half of the Gas1−/− explants to levels similar to that in wild-type tissue (Fig. 8D). Similarly, NICD expression also partially rescued levels of Nkx2.1 in the rostral ventral neuroepithelium of mutant explants (Fig. 8E).

In conclusion, our findings identified a novel role for GAS1 in integrating SHH and NOTCH signaling pathways, a function specific to neural progenitors in the rostral forebrain neuroepithelium destined to adopt a ventral cell fate (Fig. 8F).
According to our model, GAS1 interacts with PTCH1 to promote SHH-dependent gene expression, including induction of Shh, Nkx2.1 or Gli1. However, GAS1 also interacts with NOTCH1 to facilitate ligand-induced production of NICD. GAS1-dependent NICD production induces prototypic NOTCH targets, such as Hes5 and Gli1, but it also acts on the SHH pathway to increase strength and persistence of SHH signal reception. The latter mode of action is unclear at present but, based on work by others, may act at the level of several HH pathway components, including PTCH1, SMO or GLI2.

**DISCUSSION**

Previous studies have identified multiple developmental abnormalities in mouse models and patients carrying mutations in *Gas1*. These defects have been phenotypically characterized in great detail in *Gas1*−/− mice and include microforms of HPE, but also a range of craniofacial anomalies, such as midfacial hypoplasia, premaxillary incisor fusion, cleft palate or malformation of the anterior pituitary (Allen et al., 2007; Echevarria-Andino and Allen, 2020; Khonsari et al., 2013; Martinelli and Fan, 2007; Seppala et al., 2007). Malformations phenocopy aspects of SHH deficiency, including hypothalamic defects and pituitary hypoplasia (Allen et al., 2011, 2007; Martinelli and Fan, 2007), they also serve a role for GAS1 in patterning of the caudal neural tube and limbs (Carreno et al., 2017; Zhao et al., 2012). Phenotypes increase in severity with haploinsufficiency for *Shh* (Allen et al., 2007; Khonsari et al., 2013; Martinelli and Fan, 2007; Seppala et al., 2007), documenting interaction of *Gas1* and *Shh* in formation of the midline and structures derived thereof. Increasing or decreasing GAS1 activity in the neural tube of chick or mouse positively correlates with SHH pathway activity, corroborating an agonistic role for GAS1 in the graded response of neuroepithelial cells to ventralizing signals by this morphogen (Allen et al., 2007; Khonsari et al., 2013; Martinelli and Fan, 2007; Seppala et al., 2007).

Although studies on the cellular response to SHH mainly concern a role for GAS1 in patterning of the caudal neural tube and limbs (Allen et al., 2011, 2007; Martinelli and Fan, 2007), they also serve as an explanatory model for GAS1 action in SHH signaling in the rostral neuroepithelium. This assumption is supported by loss of expression of *Shh* and downstream targets in this tissue around E9.5-12.5 (Allen et al., 2007; Echevarria-Andino and Allen, 2020; Khonsari et al., 2013; Seppala et al., 2014).

Our studies aimed at corroborating a role for GAS1 in the cellular response of neural progenitors of the forebrain neuroepithelium to SHH signals. To do so, we applied unbiased as well as targeted approaches of molecular phenotyping of the forebrain neuroepithelium during early neurulation (E8.5-10.5), and we queried our findings by modeling morphogen actions in iPSC-derived NPCs. NPCs faithfully recapitulate the in vivo response of neuroepithelial cells to morphogen signals and the consequential dorsal versus ventral cell fate choices (Fig. 4B-K). In addition, this cell model faithfully recapitulates defects in the immediate cellular response to SHH signals observed in the GAS1-deficient forebrain neuroepithelium in vivo and ex vivo. Importantly, this cell model enables quantitative assessment of the cellular response to morphogen signals using agonists and antagonists, an experimental strategy difficult to apply to forebrain patterning in vivo.

Concerning the presumed role for GAS1 in the cellular response of the forebrain neuroepithelium to SHH, our studies extended previous work by confirming loss of morphogen expression and activity around E9.5 (Fig. 1C). These defects are consistent with SHH deficiency at early neurulation, as seen in *Shh* mutant mouse embryos (Carreno et al., 2017; Corman et al., 2018; Crane-Smith et al., 2021; Szabo et al., 2009). However, contrary to prior analysis that focused on developmental stages after E9.5, our data uncovered that GAS1 is not required for initial establishment of the SHH...
domain in the RDVM at E8.5, but is necessary to sustain its activity at later stages of neurulation. The same effect is also seen in cephalic explants (Fig. 8B). This mode of action distinguishes GAS1 from other SHH-binding proteins, such as LRP2, which is required for initial establishment of the SHH activity domain in the RDVM at E8.5 (Christ et al., 2012). A facilitatory role for GAS1 in SHH signal reception in neural progenitors is substantiated by quantitative assessment of their response to pathway stimulation. In these experiments, the response of GAS1 KO cells to SHH-Np is lower than in wild-type cells, yet significant when compared with non-

Fig. 8. Ectopic expression of NICD rescues loss of the SHH activity in GAS1-deficient rostral ventral forebrain explants. (A) Preparation of cephalic explants as detailed elsewhere (Christ et al., 2012; Echevarria et al., 2001). Scale bars: 500 µm. (B) Cephalic explants of E9.5 control and Gas1−/− embryos were fixed 2 h (t=0 h) or 48 h (t=48 h) after dissection and subjected to in situ hybridization (ISH) for Shh. The expression domain for Shh in the rostral ventral neuroepithelium (marked by dotted circles) is seen in Gas1−/− embryos at t=0 h, albeit slightly reduced when compared with wild type. However, this expression domain is completely lost in Gas1−/− embryos at t=48 h. (C-E) Cephalic explants of E9.5 control or Gas1−/− embryos were treated with lentiviral constructs encoding EF.PGK.GFP (GFP control) or NICD-pcw107-V5 (NICD), and subjected to ISH for Hes5 (C), Shh (D) or Nkx2.1 (E) 48 h later. Expression domains for Hes5, Shh and Nkx2.1 in the rostral ventral neuroepithelium (circled by dotted lines) are absent from EF.PGK.GFP-treated Gas1−/− explants, but are partially rescued in Gas1−/− explants by NICD-pcw107-V5. The number of explants with robust signal for Hes5, Shh or Nkx2.1 in the rostral ventral neuroepithelium, out of all explants analyzed, are given for each condition and genotype. Scale bars: 500 µm. (F) Model for GAS1 integrating SHH and NOTCH signaling pathways in neural progenitor cells.
treated cells, and comparable with the extent of pathway stimulation seen with SAG in either genotype. Still, baseline stimulation of the SHH pathway in the absence of GAS1 is clearly insufficient to trigger a ventral cell fate decision (Fig. 4B-K), providing a molecular correlate for ventralizing defects seen in the Gas1 mutant neural tube in vivo (Allen et al., 2007; Khonsari et al., 2013; Martinelli and Fan, 2007; Seppala et al., 2007).

Although a role for GAS1 in the response of the forebrain neuroepithelium to SHH may have been anticipated based on previous work, a role for this receptor in NOTCH signaling in this cell type is novel and surprising. In NPCs, loss of GAS1 completely abrogates the ability to induce the NOTCH target HES5, an effect independent of dorsal or ventral cell fate decisions (Fig. 5C,E). In addition, GAS1-deficient NPCs fail to respond to the NOTCH ligand DLL1 with induction of NICD production and HES5 transcription (Fig. 5F-H). Deficiency in NOTCH signaling is confirmed in GAS1-deficient embryos as early as E8.5 (Fig. 3B). Phenotypes include features observed in mice with targeted disruption of the NOTCH pathway component RBPJ, such as loss of Hes5 in the rostral ventral diencephalon (Ware et al., 2016). Some targets, such as Ascl1, which is upregulated upon NOTCH pathway disruption in the RBPJ KO mouse model (Rattié et al., 2013; Ware et al., 2016), were downregulated in the Gas1−/− ventral midline (Fig. S3). These distinctions are likely due to the fact that Gas1−/− mice still retain the activity of RBPJ, which acts as a transcriptional repressor in the absence of NICD (Castel et al., 2013). NOTCH signaling promotes progenitor cell maintenance in the developing CNS and controls neuronal/glial cell fate decisions (reviewed by Gaiano and Fishell, 2002). Specifically, recent work identified the importance of NOTCH signaling for maintenance and differentiation of prosenecphalic structures, including hypothalamic neurons and pituitary gland. Consequently, NOTCH signaling defects in the RBPJ KO mouse model causes malformation of the pituitary gland (Ajula et al., 2015, 2013), a defect shared by GAS1-deficient mice (Khonsari et al., 2013). Although not explored in this study in detail, loss of NOTCH activity in the rostral neuroepithelium of Gas1−/− embryos may be expected to cause additional phenotypes related to NOTCH deficiency.

Concerning the molecular mechanism whereby GAS1 promotes NOTCH activation, this action likely involves direct interaction with NOTCH1, a co-receptor concept also operable for GAS1 action on PTCH1 (Izzi et al., 2011). At present, we can only speculate about the mode of GAS1 action in this context. Because the ability of GAS1 to promote NOTCH1 activation is lost when the GPI anchor is deleted, one may argue that GPI-anchored GAS1 targets NOTCH1 to specialized lipid raft compartments where secretases or ligands reside. Such a NOTCH-sorting function has been shown for GPI-anchored Cripto-1 (Watanabe et al., 2009). Whatever the mode of action, it is operable in the rostral but not the caudal neural tube, supporting a unique role for GAS1 in NOTCH signaling during early forebrain patterning.

Conceptually, GAS1 deficiency phenotypes may represent a combination of NOTCH and SHH defects, originating from independent functions of GAS1 in activation of PTCH1 and NOTCH1. More exciting is the hypothesis that both functions for GAS1 converge on its ability to promote SHH signal strength and persistence in the forebrain neuroepithelium (see schematic in Fig. 8F). NOTCH is known to exert some of its actions by facilitating SHH signal reception and maintaining SHH responsiveness in target cells. These actions may work through different mechanisms. On the one hand, NOTCH signaling has been shown to regulate the availability and stability of GLI proteins in mouse retinal progenitor cells (Ringuette et al., 2016) as well as in neural progenitor cells of the zebrafish spinal cord (Jacobs and Huang, 2019). On the other hand, NOTCH signals prime neural progenitor cells of the mouse and chick neural tube for response to SHH by regulating trafficking of PTCH1 and SMO (Huang et al., 2012; Kong et al., 2015; Stasiulewicz et al., 2015). Haploinsufficiency for NOTCH pathway components in some patients with HPE further suggests that NOTCH-dependent facilitation of SHH signaling is required for forebrain formation (Dupe et al., 2011). This conclusion is supported by loss of NOTCH-dependent SHH activity in the embryonic mouse and chick forebrains following pharmacological or genetic perturbation of NOTCH activity, defects that include disruption of the hypothalamo-pituitary axis (Hamdi-Rozé et al., 2020).

Although dissection of NOTCH-dependent versus NOTCH-independent effects of GAS1 on SHH signaling will be challenging in vivo, iPSC-based modelling of neural progenitor differentiation enables quantitative assessment of the contribution of both pathways to SHH signal strength. In wild-type NPCs, SHH-Np-induced gene expression is largely reduced (GLI1) or even completely abolished (NKX2.1) by blockade of NOTCH using DAPT (Fig. 7B,C). In support of a prominent role for NOTCH in SHH signal strength, SHH signaling defects in Gas1 mutant NPCs can be partially rescued in vitro (Fig. 7E,F) and in cephalic explants ex vivo (Fig. 8D,E) by NICD. Although this experimental approach does not formally rule out a GAS1-independent role for NOTCH in SHH signal transduction, SHH-Np-induced expression of GLI1 is comparable in wild-type and Gas1 KO cells in the presence of DAPT, arguing that a major contribution to SHH signal strength in wild-type cells stems from the action of GAS1 on NOTCH (Fig. 7B).

In conclusion, our findings suggest a new concept concerning the role of SHH co-receptors in control of morphogen signaling in forebrain neuroepithelial cells. Specifically, they document that GAS1 acts as a co-receptor for both PTCH1 and NOTCH1 to integrate instructive signals by SHH and NOTCH ligands in this cell type; and they argue that loss of GAS1-dependent NOTCH activation may contribute to forebrain malformations in individuals carrying GAS1 mutations.

MATERIALS AND METHODS

Mouse models

Mice carrying a targeted disruption of Gas1 (Martinelli and Fan, 2007) have been described. The Gas1−/− mutant line was kept by breeding Gas1−/− animals on a C57BL/6N genetic background. As no phenotypic differences in forebrain formation were observed between Gas1−/− and Gas1+/− embryos in this study, both genotypes were used as matched littermate controls for Gas1−/− embryos. All animal experimentation was performed following approval by authorities of the State of Berlin (X9007/17). In situ hybridization (ISH) and immunohistology on mouse tissues were performed according to published protocols (Christ et al., 2012) and as detailed in the supplementary Materials and Methods.

A GAS1-deficient human iPSC model

Human induced pluripotent stem cell line HPSI1131i-wetu_2 was kindly provided by the Wellcome Trust (Sanger Institute, UK) and used as wild-type control cell line (WT). iPSCs were cultured on Matrigel (354277, Corning)−coated culture plates in Essential 8 (E8) or E8 Flex medium ( Gibco). Culture medium was changed daily. Cells were passaged every 3-4 days at a density of 70-80% using StemPro Accutase (Gibco) and 10 µM of Rock inhibitor Y27632 (SEL-S1049, Selleck Chemicals). A GAS1-deficient subclone of HPSI1131i-wetu_2 (GAS1 KO) was generated by targeting the GAS1 gene using the CRISPR/Cas9 system. Single guide RNA
Neural progenitor cells were differentiated from iPSCs using standard protocols based on inhibition of BMP and TGF signaling, as well as induction of SHH signaling (ventral fate). For more details, see supplementary Materials and Methods.

**Microdissection and bulk RNAseq**

Tissues were dissected from cryosections of the embryonic forebrain region by manually guided laser capture microdissection on a Zeiss Axio Observer Z1. Total RNA was isolated from the dissected tissue samples (RNA extraction kit, Qiagen) and used for cDNA library preparation (SMARTer Stranded Total RNA-Seq Pico Kit; Takara). Libraries were sequenced in a 2×75 bp paired end run on an Illumina HiSeq 4000 system with 20 million reads per sample. For more details, see supplementary Materials and Methods.

**Generation of neural progenitor cells**

Neural progenitor cells were differentiated from iPSCs using standard protocols based on inhibition of BMP and TGF signaling, as well as induction of SHH signaling (ventral fate). For more details, see supplementary Materials and Methods.

**Immunofluorescence staining and immunohistology of iPSCs/ NPCs**

Immunodetection of marker proteins was performed on monolayers of iPSCs or NPCs fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. Proteins were visualized by overnight incubation with primary antibodies (at 4°C), followed by incubation with the respective secondary antibodies conjugated with Alexa Fluor fluorophores (1-2 h at room temperature). For more details, see supplementary Materials and Methods.

**Proximity ligation assay**

Spatial proximity of target proteins was tested on fixed and permeabilized NPCs using primary antibodies directly conjugated with oligonucleotides (Duolink In Situ Probes from Spotlight Biosciences), followed by rolling circle amplification using real-time PCR (Duolink In Situ Detection Reagent Kit Orange). Experiments were performed according to the manufacturer’s instructions (Sigma-Aldrich). For more details, see supplementary Materials and Methods.

**Co-immunoprecipitation**

Co-immunoprecipitation was performed in total protein lysates from NPCs using 10 µg/ml goat anti-GAS1 antibody (AF2636; R&D Systems) or a non-immune goat IgG control (01-6202, Invitrogen) and the Pierce Crosslink Magnetic IP/Co-IP Kit (Thermo Fisher Scientific), according to the manufacturer’s instructions. For more details, see supplementary Materials and Methods.

**Lentiviruses production**

For lentivirus production, HEK293T cells were transfected with lentivirus envelope and packaging plasmids pMD2.G, pMDLg/PRRE and pRSV-Rev (Addgene plasmids #12259, #12251 and #12253, respectively), and the control plasmid EF.PKG.GFP (Addgene plasmid #17618) or the NICD-expressing plasmid NOTCH1 intracellular domain-pcwis107-VS (Addgene plasmid #64622). Cells were further cultivated for 2-3 days and virus particles purified from collected cell media overnight on ice using Lenti-X Concentrator (Clontech Laboratories). For more details, see supplementary Materials and Methods.

**SHH signaling in NPCs**

NPCs of differentiation day 7-9 were incubated with 5-10% conditioned medium from control or SHH-Np-secreting HEK293 cells (Christ et al., 2012), 200 nM SAG (SML1314, Sigma-Aldrich), 50 nM cyclopamine-KAAD (239804, Calbiochem) or 25 µM DAPT (565770, Sigma-Aldrich) diluted in N2B27 medium overnight or for 3 days (for studies including DAPT). The medium was changed daily with freshly added compounds. For rescue experiments, NPCs from differentiation day 5 were dissociated using Accutase and seeded onto Matrigel-coated 24-well-plates at a density of 400,000 cells/well. Cells were transduced with 20 µl/well NICD or GFP control expressing lentivirus solutions and 8 µg/ml polybrene overnight. 48 h after transduction, cells were incubated overnight with 5-10% conditioned medium from control or SHH-Np secreting HEK293 cells. Cells were then subjected to gene expression analysis by quantitative real-time PCR (for more details, see the supplementary Materials and Methods).

**Analysis of NOTCH1 signaling in NPCs and HEK293**

NPCs at differentiation day 7-9 were treated with recombinant DLL1-Fc (10184-DL, R&D Systems) or Fc control (110-HG, R&D Systems) coupled to Pierce Protein G Magnetic Beads (Thermo Fisher Scientific) and, where applicable, additionally with 25 µM DAPT overnight. Human GAS1 constructs (HA-hGAS1, HA-hGAS1ΔGPI, HA-hGAS1ΔN and HA-hGAS1ΔDC) were generated by PCR-based cloning into the pCIG vector (Megason and McMahon, 2002) and confirmed by sanger sequencing. HEK293T cells were transfected with the various GAS1 constructs using Lipofectamine2000. 48 h after transfection, cells were incubated with DLL1-Fc or Fc control magnetic beads overnight. Cells were washed with PBS to remove beads and subjected to protein analysis by western blotting. For western blotting, cell lysates were subjected to standard SDS-polyacrylamide gel electrophoresis, followed by transfer onto nitrocellulose membranes. Bound proteins were detected using primary and horseradish peroxidase-conjugated secondary antibodies and the SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) (for more details, see supplementary Materials and Methods). For each reaction, 20 µl protein G magnetic beads were first equilibrated in PBS and then incubated with 500 ng DLL1-Fc or Fc control overnight at 4°C on a rotator. The next day, the beads were washed and stored in PBS at 4°C until use.

**Rescue of SHH signaling in explants**

Cephalic explants were prepared as described elsewhere (Christ et al., 2012; Echevarria et al., 2001) with minor modifications. Briefly, E9.5 embryos were dissected in DMEM without phenol red supplemented with 1×GlutaMAX and 1×penicillin-streptomycin (Gibco). Embryonic heads were opened along the dorsal midline and the floor plate was cut at the level of the cephalic flexure. Explants were placed on polycarbonate membrane filters of 0.8-1 µm pore size (Millipore) with the ventricular side facing up and transferred to 24-well-plates containing DMEM supplemented with 10% FCS, 1×GlutaMAX and 1×penicillin-streptomycin (Gibco). Explants were recovered for 2-3 h at 37°C with 5% CO2 and 95% humidity. 20 µl lentivirus solution containing NICD or GFP-encoding lentivirus particles was applied on top and explants were further cultivated for 48 h. After gently washing with culture medium and subsequently with PBS, the explants were fixed in 4% PFA at 4°C overnight before subjecting to whole-mount ISH for Shh, Nkx2.1 or Hes5.

**Statistical analysis**

Data are represented as mean±s.d. All statistical analyses were performed using GraphPad Prism 7.0. The applied statistical tests are indicated in the respective figure legends.

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