# Gas1 is a receptor for sonic hedgehog to repel enteric axons

Shiying Jin[a], David C. Martinelli[a,1], Xiaobin Zheng[a], Marc Tessier-Lavigne[b], and Chen-Ming Fan[a,2]

[a]Department of Embryology, Carnegie Institution of Washington, Baltimore, MD 21218; and [b]Rockefeller University, New York, NY 10065

Edited by Kathryn V. Anderson, Sloan-Kettering Institute, New York, NY, and approved November 26, 2014 (received for review September 26, 2014)

**The myenteric plexus of the enteric nervous system controls the movement of smooth muscles in the gastrointestinal system. They extend their axons between two peripheral smooth muscle layers to form a tubular meshwork arborizing the gut wall. How a tubular axonal meshwork becomes established without invading centrally toward the gut epithelium has not been addressed. We provide evidence here that sonic hedgehog (Shh) secreted from the gut epithelium prevents central projections of enteric axons, thereby forcing their peripheral tubular distribution. Exclusion of enteric central projections by Shh requires its binding partner growth arrest specific gene 1 (Gas1) and its signaling component smoothened (Smo) in enteric neurons. Using enteric neurons differentiated from neurospheres in vitro, we show that enteric axon growth is not inhibited by Shh. Rather, when Shh is presented as a point source, enteric axons turn away from it in a Gas1-dependent manner. Of the Gαi proteins that can couple with Smo, G protein α Z (Gnaz) is found in enteric axons. Knockdown and dominant negative inhibition of Gnaz dampen the axon-repulsive response to Shh, and *Gnaz* mutant intestines contain centrally projected enteric axons. Together, our data uncover a previously unsuspected mechanism underlying development of centrifugal tubular organization and identify a previously unidentified effector of Shh in axon guidance.**

enteric neuron | axon guidance | chemorepellent | Hedgehog | Gas1

The enteric nervous system is the largest peripheral nervous subsystem, often likened to a second brain (1). Embedded in the gastrointestinal tract, it controls various aspects of digestive function ranging from movement and secretion to absorption. Despite their final location, enteric neurons are of neural crest origin, and the majority of enteric progenitors are derived from vagal neural crest cells at midgestation stages of the mouse embryo (1, 2). Under the control of glial cell-derived neurotrophic factor (GDNF) and Endothelin 3 (END3) signaling, they migrate into the anterior gut mesenchyme. Once there, they continue to propagate and migrate posteriorly through most of the length of the gut. The first appearing enteric arbor is the myenteric plexus, composed of islands of neurons interconnected by longitudinal and circumferential axons between the inner circular and outer longitudinal smooth muscle layers in the stomach, intestine, and colon to effect contraction. In cross-section, this plexus presents a simple centrifugal ring structure, but when viewed superficially, it is organized as an extensive mesh-like tubular arborization encasing the gut wall. Axonal arborization along the gut smooth muscles is controlled by overlapping and sequential actions of GDNF and Neuturin, a GDNF family member acting mainly postnatally (3, 4). Recently, the planar polarity pathway has also been implicated in enteric connectivity (5). Patients carrying mutations that cause enteric neuron deficiency, including in genes of the GDNF signaling pathway, present with megacolon and constipation associated with poor bowel movement, a condition known as Hirschsbrung's disease (1, 2).

Growth of the gut mucosal mesenchyme and smooth muscle, on the other hand, depends on Hedgehog (Hh) signaling during embryonic development (6–8). Both *Sonic Hh* (*Shh*) and *Indian Hh* (*Ihh*) are expressed in the gut endoderm (6), which is radially surrounded by mucosa mesenchyme, smooth muscles, and enteric neurons (1, 2). Germ-line and endoderm-specific conditional mouse mutants for *Shh* and/or *Ihh* display reduced mesenchyme and smooth muscle mass (6, 8). Shh and Ihh directly act on the gut mesoderm, as inactivation of smoothened (*Smo*), the obligatory Hh signaling component, in gut mesoderm causes the same defect (8). Mice mutant for growth arrest specific gene 1 (*Gas1*), which encodes an Hh surface binding receptor (9, 10), also share this defect (11).

The role of Hh signaling in enteric neuron development is less defined. *Shh* mutant intestines contained more punctuated Tuj1 (an antibody recognizing neuronal βIII-tubulin) staining signals erroneously located near the base of the villi and were interpreted to have a substantial number of mislocalized enteric neurons, whereas *Ihh* mutants had no enteric neurons (6). However, endoderm-specific conditional *Shh;Ihh* mutants were reported to have enteric neurons (8). Using the enteric progenitor/neuron marker P75 to better assess the cell body, we showed that *Gas1* mutant intestines had quantitatively more P75-positive ($P75^+$) enteric progenitors/neurons due to increased proliferation, and a small fraction of them were abnormally located near the base of the villi (11). However, the enteric abnormalities of *Shh* and *Gas1* mutants may arise as a secondary patterning defect (12) due to reduced mesenchyme/smooth muscle mass or reduced levels of BMP4 in the mesenchyme (6), as ectopic expression of BMP4 could negatively influence enteric progenitor positioning and proliferation (12). Conversely, recombinant Shh can inhibit enteric progenitor differentiation and migration in vitro (7). Single nucleotide polymorphisms (SNPs) of the *PTCH1* gene, a conserved Hh pathway inhibitory component, are associated with a high risk of Hirschsbrung's disease (13, 14). Furthermore, *Ptch1* conditional

## Significance

The enteric nervous system is the largest peripheral nervous subsystem. Enteric axons form a tubular meshwork arborizing the smooth muscles of the digestive tract to control gut movement. Deficiencies of enteric neurons cause megacolon and constipation—that is, Hirschsprung's disease. How enteric axons are kept in the smooth muscle layers without projecting toward the gut epithelium is unknown. We provide, to our knowledge, the first insights into how these axons are confined to the periphery and, in the process, define a novel mechanism of sonic hedgehog signaling for axon repulsion involving the guidance receptor growth arrest specific gene 1 and the signaling G protein α z. We believe this to be a conceptual advance in understanding a key topological problem in axonal arborization and to have important health implications.

Author contributions: S.J., M.T.-L., and C.-M.F. designed research; S.J., D.C.M., and C.-M.F. performed research; S.J. and D.C.M. contributed new reagents/analytic tools; S.J. and X.Z. analyzed data; and S.J., M.T.-L., and C.-M.F. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

[1]Present address: School of Medicine, Stanford University, Stanford, CA 94305.

[2]To whom correspondence should be addressed. Email: fan@ciwemb.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1418629112/-/DCSupplemental.

mutant mouse enteric progenitors have a reduced propensity for neurogenesis in vitro (13). Thus, Shh signaling may play a direct role in enteric neuron development.

Whether the gut epithelium directly acts on the myenteric axons to confine their periphery connectivity within the smooth muscles is not known. During our continuous investigation of *Shh* and *Gas1* mutant gut defects, we found that their intestines contained Tuj1 staining signals deep inside the villi, almost reaching the gut epithelium, suggesting a previously unappreciated axonal projection defect. Given that Shh is an axon guidance molecule in the central nervous system (CNS) (15, 16), we were motivated to investigate whether Shh in the gut epithelium acts to prevent erroneous entry of enteric axons into the villi. Below, we provide evidence for a previously unknown enteric axon repulsion mechanism mediated directly by Shh via its receptor Gas1 and signaling component Smo. We further implicate a unique Gαi protein, the pertussis toxin (PTX)-insensitive Gnaz (17), in mediating Shh-directed chemorepulsion.

## Results

***Shh* Mutants Have Centrally Projecting Enteric Axons.** To assess whether Hh signaling plays a role in enteric axon projection, we began by comparing the patterns of enteric axons in wild-type and *Shh* mutant intestines using the Tuj1 antibody at embryonic day 18.5 (E18.5), before their perinatal death (18). At this time point, wild-type enteric axons are mainly of the myenteric plexus (between circular and longitudinal smooth muscles) and organized in a tightly restricted centrifugal ring by cross-section view (Fig. 1*A*). We confirmed that *Shh* mutant intestines have more Tuj1 signals along the gut wall than wild-type intestines (6). In contrast to the study that interpreted punctuated Tuj1 signals near the base of the villi as mislocalized enteric neurons (6), we found abundant long thin Tuj1 signals extending deep into the villi, indicating an additional phenotype of erroneously projecting axons (Fig. 1*B*). The difference between these studies may lie in sample preparations, staining protocols, or planes of section. Upon quantification, 68 ± 14% of the villi examined contain axons projecting toward the central region where the gut epithelium lies (Fig. 1*B*; quantification in Fig. 1*I*). Erroneously projecting axons were also found in the stomach, but not in the colon (Fig. S1 *A* and *B*). These data encouraged further investigation into whether the Shh signaling pathway plays a direct role in enteric axon guidance.

***Gas1*, but Not *Cdo* or *Boc*, Mutants Show Centrally Projecting Enteric Exons.** We next sought to define which of the three mammalian Hh binding proteins on the plasma membrane—Gas1, Cdo, or Boc (9, 10, 19, 20)—might be the relevant surface component(s). We have previously shown *Gas1-LacZ* (10) knock-in reporter activity present in the enteric system of the stomach and intestine, but low or absent activity in the colon (11). Gas1 proteins are also coexpressed with enteric progenitors and neurons (11). Similarly, we used the activity of the LacZ reporter inserted into the *Cdo* and the *Boc* loci (19) as a proxy for their expression. Both genes were expressed in smooth muscle with temporal differences from E11.5 to E18.5, but displayed little to no expression in the enteric system (Fig. S1 *C* and *D*). We next asked whether mouse mutants for these genes displayed enteric axon

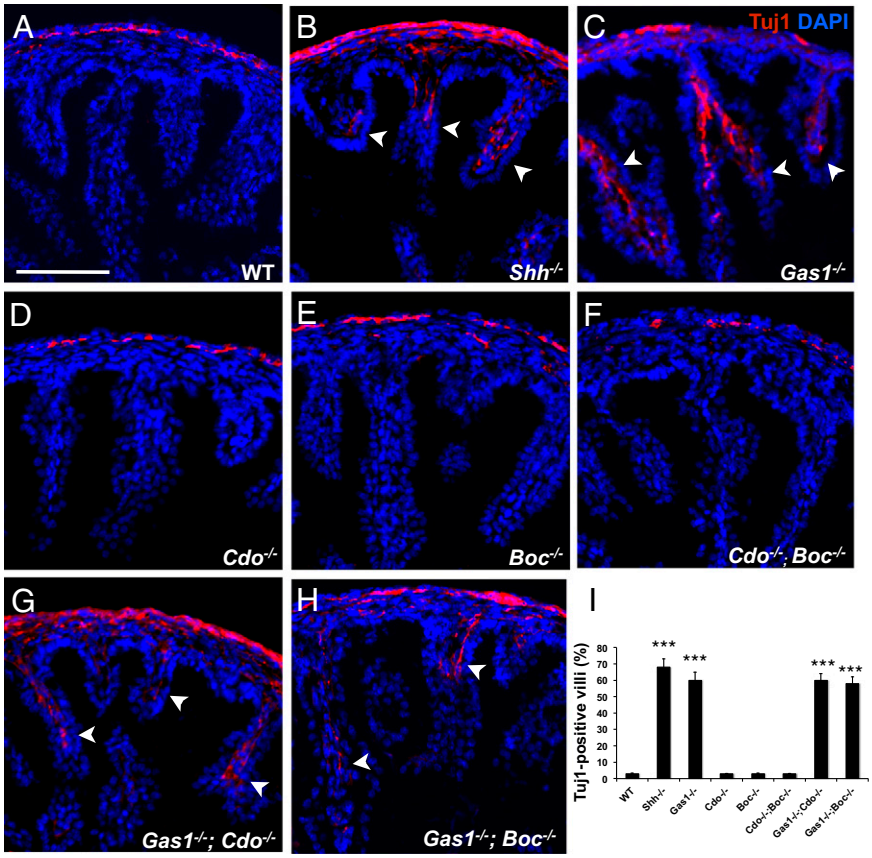


**Fig. 1.** Enteric axons erroneously project into intestinal villi in *Shh* and *Gas1* mutants. (*A–H*) Tuj1 (red)-stained E18.5 small intestine cross-sections (counterstained with DAPI in blue) of (*A*) wild type (WT), (*B*) $Shh^{-/-}$, (*C*) $Gas1^{-/-}$, (*D*) $Cdo^{-/-}$, (*E*) $Boc^{-/-}$, (*F*) $Cdo^{-/-}$;$Boc^{-/-}$, (*G*) $Gas1^{-/-}$;$Cdo^{-/-}$, and (*H*) $Gas1^{-/-}$;$Boc^{-/-}$. Arrowheads point to abnormal axons found underneath the villi. (*I*) Quantification of percentages of villi that contain Tuj1+ axons; ***$P < 0.001$ (compared with wild type) by Student's *t* test; three animals for each genotype, 10 sections (≥70 villi) per intestine sample were counted. Error bars, SEM. (Scale bars, 50 μm.)

projection defects, as seen in the *Shh* mutant. Only the *Gas1* mutant intestine shared this defect, with 60 ± 13% of villi containing axons at E18.5 (Fig. 1 *C* and *I*), whereas *Cdo*, *Boc*, and *Cdo;Boc* mutants did not (Fig. 1 *D–F* and *I*). *Gas1;Cdo* and *Gas1;Boc* mutants did not show more severe defects than *Gas1* mutants (Fig. 1 *G–I*). *Gas1* mutants also displayed defects in the stomach, but not in the colon (Fig. S1 *A* and *B*), similar to the *Shh* mutant. The differential defects among the stomach, intestine, and colon in *Gas1* and *Shh* mutants likely reflect regional differences. In addition, *Shh* and *Gas1* mutants have more axons in the intestine, consistent with them having more enteric neurons (6, 11). Thus, Gas1 appears to be the primary surface mediator of Shh signaling to control enteric axon projections.

***Gas1* and *Smo* Are Required in Enteric Neurons to Prevent Central Projection.** To address whether *Gas1* acts autonomously in enteric neurons, we examined *Wnt1:Cre;Gas1$^{f/f}$* embryos in which the floxed *Gas1* (*Gas1$^{f}$*; Fig. S2*A*) alleles were conditionally inactivated in the dorsal neural tube and neural crest cells (the cells of origin of enteric neurons) by the *Wnt1:Cre* driver (21); the recombined *Gas1$^{f}$* allele is a null (Fig. S2*A*). Because the small intestine showed robust defects in *Shh* and *Gas1* mutants, we focused our analyses on this gut segment. The *Wnt1:Cre* control intestine villi contained minimal enteric axons (Fig. 2 *A* and *F*), whereas *Wnt1:Cre;Gas1$^{+/f}$* intestines had 17 ± 8% villi containing scattered thin axons (Fig. 2 *B* and *F*). Examination of *Gas1$^{+/-}$* intestines confirmed haploid insufficiency (Fig. S2*B*). Importantly, *Wnt1:Cre;Gas1$^{f/f}$* intestines had a similar degree of defects (62 ± 7% villi with axons; Fig. 2 *C* and *F*) as the *Gas1* germ-line mutant (Fig. 1*I*).

We next asked whether the obligatory Hh signaling component Smo is also required in enteric neurons to prevent central projection. Although *Wnt1:Cre;Smo$^{f/-}$* embryos were described to have normal sensory projections (22, 23), we found that *Wnt1:Cre;Smo$^{f/f}$* intestines had centrally projecting enteric axons in 60 ± 5% of villi examined (Fig. 2 *E* and *F*). Twenty-four percent of villi in the *Wnt1:Cre;Smo$^{+/f}$* also contained thin and scattered axons (Fig. 2 *D* and *F*), indicating haploid insufficiency.

Like *Shh* and *Gas1* germ-line mutants, *Gas1* and *Smo* conditional mutants have more enteric axons along the intestinal wall, indicative of increased enteric neurons. We have previously used the enteric neuron/progenitor marker P75, which is more concentrated at the cell body, to determine mislocalized P75$^{+}$ cells near the base of, but not inside, the villi (11). By contrast, *Smo* and *Gas1* conditional mutants do not show mislocalized P75$^{+}$ cells (Fig. S2*C*). Similar results were obtained using anti-HuC/D to label enteric neuron cell bodies (24, 25) (Fig. S2*C*). Thus, enteric progenitor/neuron positioning is not directly governed by Hh signaling. Furthermore, because central enteric projections were observed in conditional *Gas1* and *Smo* heterozygotes in which overabundance of enteric axons was not found, central projection is unlikely to be a simple consequence of excessive axons. Taken together, these data support the view that Gas1 in enteric neurons mediates Shh signaling via Smo to prevent them from projecting out of the myenteric plexus into the villus mesenchyme.

**Chemorepulsion by Shh-N Depends on Gas1.** The most parsimonious possibility for Shh to control enteric axon projections is for it to act on the axon terminals. Indeed, endogenous Gas1 and Smo-GFP (expressed via lentiviral transduction) were readily detectable at the axon terminals of cultured enteric neurons dissociated from the intestine (Fig. 3*A*).

Mechanistically, Shh may act through Gas1 to prevent centrally projecting enteric axons by pruning, growth inhibition, or repulsion. Upon examining wild-type enteric axons during development (Fig. S3*A*), we found virtually no centrally projected axons, arguing against pruning as a contributing mechanism. By contrast, in the *Gas1* mutant, as soon as intestinal villi started to form, erroneous axons were present (Fig. S3*A*).

To determine whether Shh inhibits enteric axon growth, we developed an in vitro system. Primary gut explants behaved poorly in an axon outgrowth assay. Serendipitously, we discovered that enteric neurospheres derived from E11.5 guts (7, 11, 26) could spontaneously differentiate and send out axons after being placed into 3D collagen gels (27). These enteric neurospheres sent out fasciculated axons radially as determined by Tuj1 staining

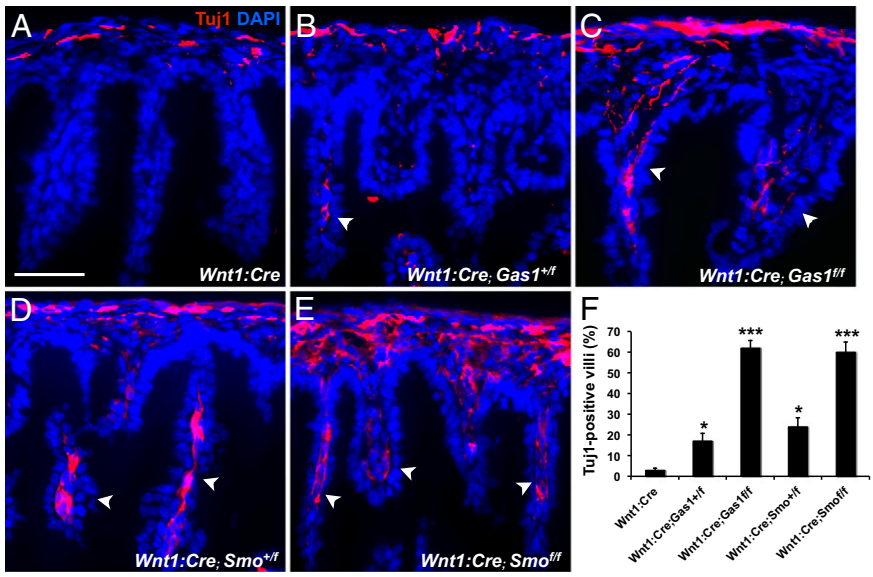


**Fig. 2.** Gas1 and Smo are autonomously required to prevent enteric axons entering intestinal villi. (*A–E*) Tuj1 (red)-stained E18.5 small intestine sections (counterstained with DAPI in blue) of (*A*) *Wnt1:Cre*, (*B*) *Wnt1:Cre;Gas1$^{+/f}$*, (*C*) *Wnt1:Cre;Gas1$^{f/f}$*, (*D*) *Wnt1:Cre;Smo$^{+/f}$*, and (*E*) *Wnt1:Cre;Smo$^{f/f}$*. Arrowheads point to abnormal axons found underneath the villi. (*F*) Quantification of percentages of villi that contain Tuj1+ axons; *$P < 0.05$ and ***$P < 0.001$ (compared with *Wnt1:Cre*) by Student's *t* test; three animals for each genotype and 10 sections (≥70 villi) per intestine sample were counted. Error bars, SEM. (Scale bars, 50 μm.)

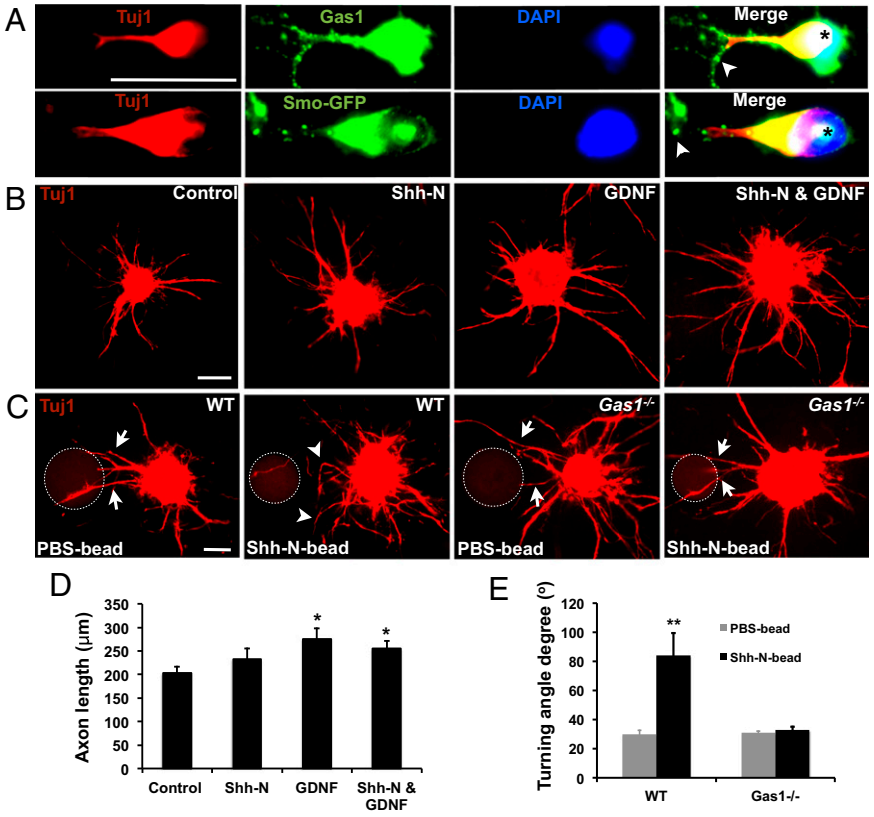


**Fig. 3.** Shh-N repels enteric axons in vitro. (*A*) Immunostaining of endogenous Gas1 (*Top*) and lentiviral-transduced Smo-GFP (*Bottom*) is present in axon terminals (arrowheads) of dissociated enteric neurons cultured from E11.5 gut; Tuj1 in red, DAPI in blue, and asterisks indicate nuclei. (*B*) Dosage effect of recombinant Shh-N and GDNF on enteric neurosphere-derived axons growing in the collagen gels for 48 h. The lengths of axons based on Tuj1 (red) staining were measured (tabulated in *D*); $*P < 0.05$, by Student's *t* test; >150 axons from 20 neurospheres ($n \geq 3$ animals) were counted for each treatment. (*C*) Heparin beads (outlined by white dashed circles) soaked with PBS or 2 μg/mL of Shh-N (in PBS) were placed next to wild-type or *Gas1*$^{-/-}$ enteric neurospheres in collagen gels for 48 h. Tuj1 staining was used to assess axon turning. Control beads have little effect on enteric axons (arrow), whereas Shh-N beads turned away (arrowhead) wild-type axons. Turning angle degree was tabulated in *E*; $**P < 0.01$ by Student's *t* test; >70 axons from 20 neurospheres ($n \geq 3$ animals) were counted for each experiment. Error bars, SEM. (Scale bars, 50 μm.)

(Fig. 3*B*). By titrating the dosage of recombinant Shh-N (the active N-terminal fragment of Shh), we found that even at 2–4 μg/mL, a concentration that saturated the transcriptional response of these spheres (Fig. S3*B*), the axon length emanating from these spheres was not significantly shorter than those from mock-treated control spheres (Fig. S3*B*). GDNF family members expressed in the gut are known trophic factors for enteric neurites (3, 28). As expected, GDNF at and above 50 ng/mL (Fig. 3 *B* and *D* and Fig. S3*B*) stimulated longer axons. We next tested whether Shh-N could counteract the activity of GDNF. When both GDNF and Shh-N were added, Shh-N did not exhibit a detectable inhibitory effect (Fig. 3 *B* and *D*). Thus, Shh signaling prevents enteric central projections in vivo, but Shh-N does not inhibit their growth when applied uniformly in vitro.

We next tested whether Shh acts as a chemorepellant to change the direction of enteric axon projections. To present Shh-N as a point source, we initially used cell aggregates prepared from COS cells transiently transfected with an empty (control) or Shh-N–expressing vector (Fig. S3*C*). These aggregates were placed next to enteric neurospheres. Shh-N–expressing aggregates caused axons to turn away from the source with significantly larger turning angles than control aggregates (Fig. S3*C*). By contrast, axons derived from *Gas1* mutant enteric neurospheres did not turn away from control and Shh-N sources (Fig. S3*C*), consistent with our in vivo data that Gas1 is a necessary component for this repulsive response. To rigorously prove Shh-N is a repulsive cue without contributing factors from COS cells, we used heparin beads soaked with purified recombinant Shh-N as the point source of repellent (Fig. 3*C*). Consistent with the results using cell aggregates, wild-type enteric axons turned away from and avoided Shh-N–treated beads but not control PBS-treated beads, whereas *Gas1* mutant axons did not avoid either type of bead (Fig. 3 *C* and *E*). These data together indicate that Shh acts as a chemorepellant to steer away enteric axons in a Gas1-dependent manner, thereby providing an explanation for the lack of centrally projected enteric axons in vivo.

**Shh-N–Directed Enteric Axon Repulsion Requires Gnaz.** Given that Gα proteins of the inhibitory class (i.e., Gαi) mediate Smo signaling to effect Hh-induced transcription (29, 30), we next investigated whether they played a role in Shh/Gas1/Smo-directed enteric axon turning. Comparison of RNA-seq data between undifferentiated and differentiated (induced by 50 ng/mL GDNF for 48 h; Fig. 4*A*) enteric neurospheres identified many *Gα* genes in the *Gαs*, *Gαi*, and other *Gα* classes that were up-regulated in the differentiated samples (Fig. S4*A*). Among *Gαi* family members, *Gnaz* showed the largest fold increase after differentiation. We further confirmed its up-regulation in differentiated enteric neurospheres by quantitative reverse transcription (qRT)-PCR (Fig. 4*A*) and found that its protein was distributed throughout the axons of cultured enteric neurons (Fig. 4*B*). Coincidentally, Smo stimulates the highest levels of GTPγ's binding of Gnaz among Gαi members (29), yet Gnaz has not to date been linked to Shh/Smo-mediated biological processes.

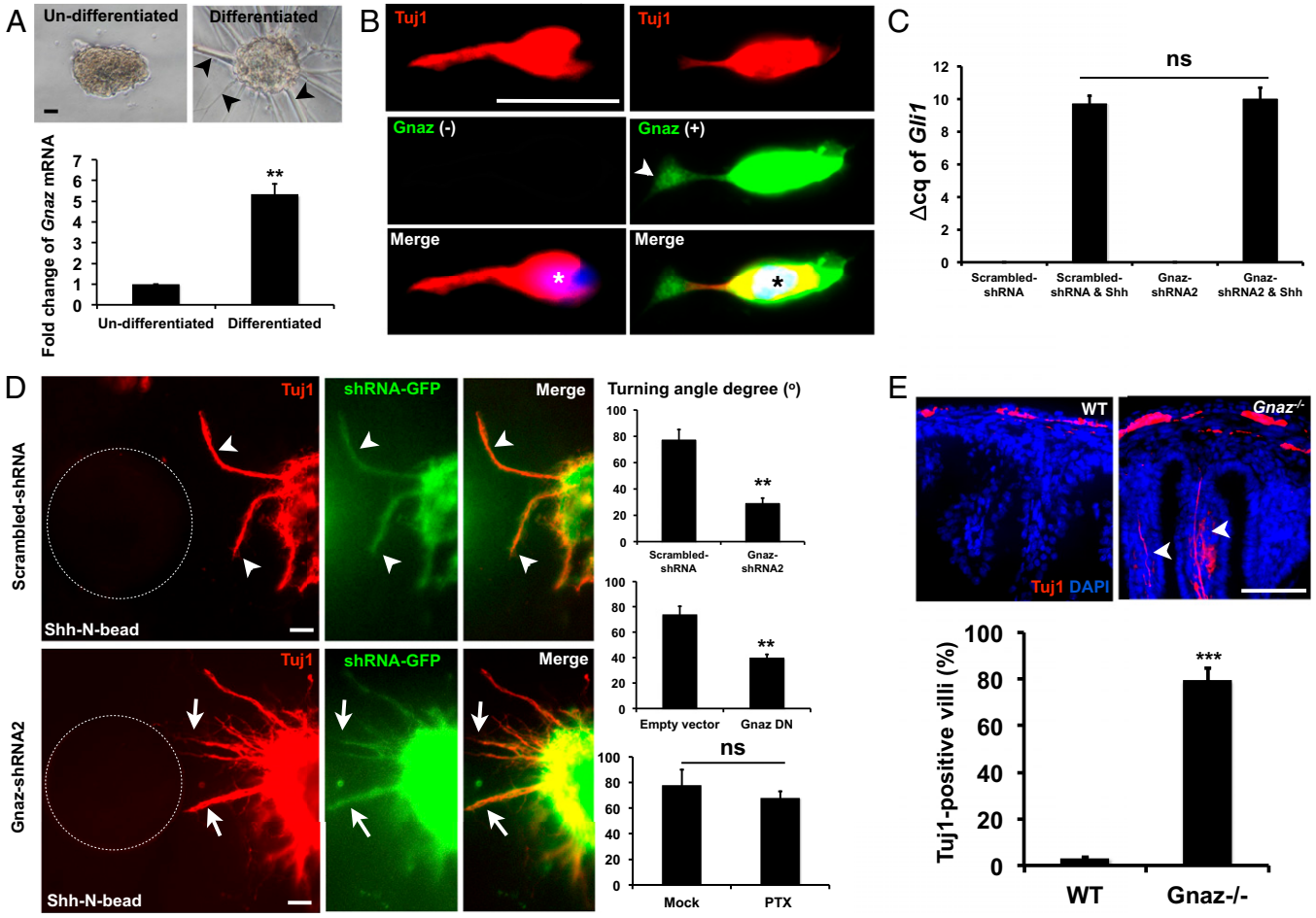


**Fig. 4.** Gnaz participates in Shh-N–mediated enteric axon repulsion. (*A*, *Top*) Images of undifferentiated and differentiated enteric neurospheres with axons (pointed by arrowheads). (*Bottom*) qRT-PCR confirms *Gnaz* up-regulation in differentiated enteric spheres. (*B*) Endogenous Gnaz is present in the enteric neuron axon terminal (arrowhead) cultured from E11.5 gut: *Left* panels, without primary antibodies [Gnaz(–)]; *Right* panels, with anti-Gnaz antibodies [Gnaz(+)]. Gnaz (green) is costained with Tuj1 (red) and DAPI (blue). Superimposed images are at the bottom, and asterisks indicate nuclei. (*C*) Scrambled– and *Gnaz*–shRNA2-transduced enteric neurospheres treated with Shh-N were assayed for *Gli1* expression by qRT-PCR. *Gli1* levels are shown as Δcq values in the *y* axis; no significant difference was found. Without Shh-N, there was no detectable *Gli1*. (*D*) Enteric neurospheres transduced by lentiviruses expressing scrambled–shRNA or Gnaz–shRNA2 were placed next to Shh-N beads (outlined by white dashed circles) in collagen gels for 48 h. Samples are stained by Tuj1 (red) and anti-GFP (green); GFP monitors lentiviral transduction. Arrowheads point to scrambled–shRNA virus-infected axons that have made a decision on turning, and arrows point to Gnaz–shRNA2 virus-infected axons that projected toward the bead. Shown are turning angle degrees tabulated for enteric axons exposed to Shh-N beads under treatment with shRNA expression, empty vector and Gnaz-DN overexpression, and mock (without PTX) and PTX (75 ng/mL). More than 70 axons from 20 neurospheres ($n \geq 3$ animals) were counted for each experiment. Error bars, SEM. (Scale bars, 50 μm.) (*E*) Wild-type and *Gnaz* mutant intestines stained by Tuj1 (*Top*), and quantification of percentages of villi containing Tuj1-stained axons (*Bottom*). $^{***}P < 0.01$, $^{**}P < 0.01$, and ns (not significant) by Student's *t* test. Error bars, SEM. (Scale bars, 50 μm.)

To test whether *Gnaz* participates in Shh-directed enteric axon repulsion, we initially used a lentiviral shRNA delivery system to knock down *Gnaz*. Of two commercial lenti-shRNAs against *Gnaz*, one of them (Gnaz–shRNA2) efficiently knocked down *Gnaz* mRNA to 36 ± 5% of that by scrambled–shRNA (Fig. S4*B*). The transcriptional response to Shh-N was unchanged by *Gnaz* knockdown, as assessed by induction of its direct downstream target gene *Gli1* (Fig. 4*C*). High-efficiency lentiviral infection of enteric neurospheres was obtained, as assessed by the coexpressed GFP reporter (Fig. 4*D*). Enteric neurospheres expressing scrambled–shRNA retained an axonal turning response to Shh-N beads, whereas those expressing Gnaz–shRNA2 displayed a muted response (Fig. 4*D*). Thus, Shh-induced transcriptional response in these neurospheres is not sufficient to cause axon turning, and Gnaz is selectively required in the latter function. We next used the lentivirus to overexpress a dominant negative form of Gnaz (31) (Gnaz-DN) in neurospheres and found it also reduced the enteric axon turning response to Shh-N beads (quantification in Fig. 4*D*). Consistent with the fact that Gnaz is a Gαi member uniquely insensitive to inhibition by PTX (17), we found that the enteric axon turning response to Shh-N beads was insensitive to PTX (quantification in Fig. 4*D*). To further demonstrate the role of Gnaz, we analyzed the Tuj1 staining pattern in the intestine of *Gnaz* mutants (32). Although not as pronounced as in other *Shh* pathway mutants, *Gnaz* mutants had increased Tuj1 signals at the intestinal wall. Importantly, they contained centrally projected axons in the villi (Fig. 4*E*). The percentage of villi containing axons in *Gnaz* mutants was similar to those in *Shh* pathway mutants described above, but *Gnaz* mutants' intravillus enteric axons appeared fewer and/or thinner. Such a difference implies a partial involvement of another Gα protein(s) in vivo. Together, these data argue for differential signaling of Smo mediated in part by Gnaz in response to the Shh gradient at the enteric growth cone as being critical for repulsion to prevent central projection.

## Discussion

We have explored how developing axons within epithelial structures are kept in peripheral domains of the epithelia and away from their central domains. Using the gastrointestinal tract as a model for this topological problem, we identify a previously unidentified mechanism of Shh signaling for enteric axon repulsion involving Gas1 as a surface receptor and Gnaz as an intracellular effector. This repulsive force confines enteric terminals to the smooth muscle layers where they are needed for gut movement. Our results thus provide a framework for how axons in a tubular structure can be organized by a diffusible signal emanating from the center of the tube.

**Shh Is a Context-Dependent Axon Guidance Molecule.** We report here that Shh uses Gas1 for axonal repulsion of enteric neurons, which express neither *Cdo* nor *Boc*. The unique function of Gas1 in this context may be due to its lone expression, but we note that Gas1 is structurally unrelated to Boc and Cdo, which might contribute to this unique function. In fact, Gas1 is related to the GDNF receptor family (GFRαs) (33), and whereas GFRα1 binds GDNF to recruit Ret association, Gas1 can associate with Ret without binding to GDNF (33). Although this property of Gas1 implies a potential to regulate both Shh and Ret signaling pathways, the *Gas1* mutant phenotype in the gastrointestinal tract can be largely explained by defective Shh signaling (11). Because we observe consistent axonal defects among multiple Shh pathway mutants and enteric axons turning away from the Shh-N source, we propose that the axon repulsion effects described here are primarily due to Shh signaling. Whether Cdo or Boc can substitute Gas1 for the Shh-directed repulsion of these particular axons is an open question.

Among the best characterized axon guidance roles for Shh is in the developing spinal cord. Shh secreted from the floorplate acts in a *Boc*- and *Smo*-dependent manner to attract commissural axons arriving from the dorsal spinal cord (15, 19). *Gas1* mutants share the commissural axon trajectory defect (34) with *Boc* (19) and *Wnt1:Cre;Smo*$^{f/-}$ (15) mutants, whereas *Cdo* is not expressed in commissural neurons (19). Because *Gas1* mutants have a compromised floorplate, which may indirectly affect the commissural trajectory, its role in commissural axon attraction requires clarification. On the other hand, Shh at the optic chiasm acts through Boc to induce retraction of ventro-temporal retinal axons (35, 36). *Cdo* is expressed in retinal neurons but has no apparent roles (36), whereas *Gas1* is required earlier to pattern the neural retina (37). Furthermore, Gas1, Boc, and Cdo act cooperatively in several Hh-mediated embryonic patterning events, but they also show unique prevalence for each specific compound mutant phenotype depending on dosage and expression (38, 39). Thus, Shh employs Gas1, Cdo, and/or Boc in a context-dependent manner.

**Gas1–Smo–Gnaz Defines a Signaling Pathway for Shh-Directed Enteric Axon Repulsion.** Endogenous Gas1 and Gnaz, and transduced Smo-GFP, are localized to enteric axonal terminals, consistent with their function at the growth cone for Shh-directed repulsion. In the CNS, Shh also acts as a chemorepulsive cue for commissural axons after midline crossing (40, 41), although in that context the receptor(s) and Smo-coupled G protein(s) have yet to be identified. Our findings thus generalize the role of Shh in chemo-repulsion and extend the signaling mechanism by defining components upstream and downstream of Smo in axonal repulsion.

Although we have excluded a role for Cdo and Boc, we have not been able to evaluate the involvement of Ptch1, as *Wnt1*$^{Cre}$*; Ptch1*$^{f/f}$ embryos die before E12.5, days before villus formation (13). Given that Ptch1 has a conserved role in suppressing Smo activity, it seems likely to be involved. Curiously, Gorlin Syndrome caused by *PTCH1* haploid insufficiency has no noted symptoms related to Hirschsbrung's disease, although a patient with gastrointestinal mesenchymal growth and small bowel adenoma was reported (42). Because the *PTCH1* SNPs associated with Hirschbrung's disease risk appear ethnic group-dependent, contribution from additional genetic variations may be needed for increased risk (13, 14). It will be interesting to test whether *PTCH1* SNPs associated with high risk of Hirschsbrung's disease are neomorphic variants that disrupt enteric axon projections in defined genetic backgrounds.

Shh-mediated Smo-dependent signaling has been implicated in MEF chemotaxis (43) and scratch healing (44), but the surface receptors are also not defined. In both cases, PTX-sensitive Gαi proteins (Gαi 1–3) have been implicated. Identification of PTX-insensitive Gnaz for chemorepulsion provides the first hint that Smo may distinguish attraction versus repulsion by selective coupling to distinct Gαis. The enteric defect of *Gnaz* mutants appears qualitatively different from that of other Shh pathway mutants (i.e., fewer axons at the gut wall and thinner/fewer centrally projected axons), suggesting partial compensation by another Gα protein(s) in vivo. *Gnaz* mutants may thus exhibit partial defects in other Shh-directed guidance processes. A recent study shows that removing Gαs function leads to increased Shh signaling for cell fate determination, implying the importance of balanced Gα signaling (45). By analogy, we posit that the combined activity of Gα proteins at the growth cone, regardless of coupling to Smo, contributes to Shh-induced enteric axon repulsion. Changes in any number of these Gα proteins may therefore alter the sense of Shh-directed guidance. Given the multiplicity of Gα proteins, unraveling their complex network and defining each one's contribution in modulating Shh-induced events in vivo will be challenging.

**Shh Acts Directly on Enteric Axons.** It was proposed that Shh induces BMP4 in the mesenchyme, which in turn reduces enteric progenitor/neuron density and positions them away from the central epithelium (12). Our *Gas1* and *Smo* conditional mutant data delineate these two processes: Hh signaling plays a direct and negative role in enteric neuron/axon abundance but an indirect role in their positioning. We also show that Gnaz knockdown diminishes Shh-directed enteric axon repulsion without affecting Shh-induced transcription, supporting the idea that transcriptional response is not sufficient to mediate repulsion. This alone, however, cannot definitively exclude a contribution from Shh-induced transcription. Importantly, it has been shown that the inner circular muscle, but not the myenteric plexus and outer longitudinal muscle, displays an Hh transcriptional response, as assessed using a *Gli1*$^{LacZ}$ reporter mouse (46). We therefore propose that as the gut mesenchyme and smooth muscles expand, the maturing myenteric plexus becomes organized just outside the limit (i.e., circular muscle) of sufficient Hh levels for *Gli1* activation. Instead, enteric axons erroneously traversing into the circular muscle layer encounter an Hh gradient and turn back by repulsion, and this guidance response is mainly mediated via Gnaz signaling at the axonal terminal without a retrograde transcriptional response.

**A Double Assurance Program Provides a Solution to a Topological Problem.** To assure proper targeting and connectivity, a combination of chemoattractive and chemorepulsive cues along the path of an axon is often used (16). Enteric axonal arborization represents a unique topological challenge, as enteric neuron cell bodies and axons have to be kept in the gut wall along the whole length of the tract. We propose that, in this scenario, trophic GDNF and chemorepulsive Shh operate together as a double-assurance mechanism to ensure enteric axons stay and terminate at the smooth muscle layers and form a radial meshwork in the stomach and intestine, with the repulsive guidance emanating from the center and the permissive trophic cue at the periphery.

The tubular topology of the gut naturally constrains the longitudinally and circumferentially traversing enteric axons and their growth cones to be perpendicular to the endoderm and sense the largest gradient differences along the radial axis. Given the extensive morphological changes of the endodermal epithelium and mesenchyme during villi morphogenesis, an accompanying developmental strategy may have evolved to exclude myenteric axons from the endoderm and re-enforce their local innervation in the smooth muscle layers. The contiguously folded epithelium of the villi further strengthens the repulsive signal, both at the bottom of the epithelial crypt near the smooth muscles and by surrounding each villus mesenchyme radially.

## Materials and Methods

For more details, see *SI Materials and Methods*.

**Animals.** *Gas1*$^{-}$ (10), *Shh*$^{-}$ (18), *Cdo*$^{-}$, *Boc*$^{-}$ (19), *Smo*$^{f}$ (22), *Gnaz*$^{-}$ (32), and transgenic *Wnt1:Cre* (21) alleles were described. The vaginal plug date is designated as E0.5. The *Gas1*$^{f}$ allele generated for this work is detailed in Fig. S2. All procedures are approved by the Carnegie Institutional Animal Care and Use Committee.

**Histochemistry and Immunostaining.** For X-gal reactions and immunofluorescence, samples were fixed in 4% (wt/vol) paraformaldyhyde/PBS and embedded in O.C.T. (Tissue Tek) for sectioning at 10 μm thickness using LEICA CM3050S cryostat. X-gal reactions were counterstained with Nuclear Fast Red (Sigma). For immunostaining, the following primary antibodies were used: anti–β-gal (rabbit, Chemicon, 1:1,000, or mouse, Promega, 1:1,000), anti-Gnaz (rabbit, Santa Cruz Biotechnology, 1:100), anti–neural-specific β-tubulin (Tuj1, mouse, Millipore, 1:800), anti-Neurotrophin receptor P75 (Rabbit, Millipore, 1:200), anti-GFP (rabbit, Invitrogen, 1:100), anti-mouse Gas1 (goat, R&D Systems, 1:200), and anti-HuC/D (mouse, Molecular Probes, 1:100). Alexa Fluor 488- and Alexa Fluor 568-conjugated secondary antibodies against specific species (goat, mouse, and rabbit) were from Molecular Probes (all at 1:1,000). DAPI (Sigma) was used at 1 μg/mL for DNA staining. They were imaged by a Nikon Eclipse TE200 scope.

**Neurosphere Culture and Axon Outgrowth and Turning Assays.** Neurospheres were generated using E11.5 guts (7, 11, 26). For axon outgrowth assay, they were embedded in collagen matrices (BD Biosciences) and cultured in different concentrations of Shh-N or GDNF proteins for 48 h (15). For turning assay, COS cell aggregates transfected with a control or Shh-expression-plasmid or heparin beads (Sigma) soaked in PBS or PBS with Shh-N (2 μg/mL, R&D Systems) were embedded next to neurospheres and cultured for 48 h. PTX (Calbiochem) at 75 ng/mL was used. Based on Tuj1 staining, axon length was measured with MetaMorph software, and axon turning angle degrees were measured with ImageJ software.

**RNA-Seq and qRT-PCR.** Undifferentiated or differentiated (induced by 50 ng/mL of GDNF for 48 h) neurospheres were collected for RNA-seq using a HiSeq2000 (Illumina). RNA-seq reads were mapped using TopHat to the mouse genome (mm9) and RefSeq annotations. Differentially expressed *Gα* genes were analyzed using edgeR. qRT-PCR condition and quantification for *Gnaz* and *Gli1* are detailed in *SI Materials and Methods*.

**Lentiviral shRNA Transduction.** Lentiviral shRNA vectors of the GIPZ system were from Thermo Scientific: Gnaz shRNAs (1, V2LMM-78357; 2, V2LMM-80266) and nonspecific shRNA (nonshRNA, RHS4346). The pLEX vector (Thermo Scientific) was used to express Smo-GFP and the DN form of Gnaz (31).

**Quantitation and Statistical Analyses.** Villi containing Tuj1$^{+}$-stained axons were counted from 10 sections of each of three embryos of each genotype. For the in vitro neurosphere assay, 20 neurospheres prepared from at least three embryos were used for each experiment. Bar graphs represent mean ± SEs. All statistical data considered significant were with $P$ values $<0.05$, $<0.01$, or $<0.001$, as assessed by Student's $t$ test.

**ACKNOWLEDGMENTS.** We thank C.-M.F. laboratory members for manuscript reading, Ms. S. Satchell and Ms. E. Dikovsky for animal genotyping and husbandry, and Ms. A. Pinder for RNA-Seq. This work is supported by National Institutes of Health Grant R01 DK084963 and the Carnegie Institution of Washington.

1. Furness JB (2006) *The Enteric Nervous System* (Blackwell Publishing, Malden, MA).
2. Heanue TA, Pachnis V (2007) Enteric nervous system development and Hirschsprung's disease: Advances in genetic and stem cell studies. *Nat Rev Neurosci* 8(6):466–479.
3. Heuckeroth RO, Lampe PA, Johnson EM, Milbrandt J (1998) Neurturin and GDNF promote proliferation and survival of enteric neuron and glial progenitors in vitro. *Dev Biol* 200(1):116–129.
4. Heuckeroth RO, et al. (1999) Gene targeting reveals a critical role for neurturin in the development and maintenance of enteric, sensory, and parasympathetic neurons. *Neuron* 22(2):253–263.
5. Sasselli V, et al. (2013) Planar cell polarity genes control the connectivity of enteric neurons. *J Clin Invest* 123(4):1763–1772.
6. Ramalho-Santos M, Melton DA, McMahon AP (2000) Hedgehog signals regulate multiple aspects of gastrointestinal development. *Development* 127(12):2763–2772.
7. Fu M, Lui VC, Sham MH, Pachnis V, Tam PK (2004) Sonic hedgehog regulates the proliferation, differentiation, and migration of enteric neural crest cells in gut. *J Cell Biol* 166(5):673–684.
8. Mao J, Kim BM, Rajurkar M, Shivdasani RA, McMahon AP (2010) Hedgehog signaling controls mesenchymal growth in the developing mammalian digestive tract. *Development* 137(10):1721–1729.
9. Lee CS, Buttitta L, Fan CM (2001) Evidence that the WNT-inducible growth arrest-specific gene 1 encodes an antagonist of sonic hedgehog signaling in the somite. *Proc Natl Acad Sci USA* 98(20):11347–11352.
10. Martinelli DC, Fan CM (2007) Gas1 extends the range of Hedgehog action by facilitating its signaling. *Genes Dev* 21(10):1231–1243.
11. Biau S, Jin S, Fan CM (2013) Gastrointestinal defects of the Gas1 mutant involve dysregulated Hedgehog and Ret signaling. *Biol Open* 2(2):144–155.
12. Sukegawa A, et al. (2000) The concentric structure of the developing gut is regulated by Sonic hedgehog derived from endodermal epithelium. *Development* 127(9):1971–1980.
13. Ngan ES, et al. (2011) Hedgehog/Notch-induced premature gliogenesis represents a new disease mechanism for Hirschsprung disease in mice and humans. *J Clin Invest* 121(9):3467–3478.
14. Wang Y, et al. (2013) Common genetic variations in Patched1 (PTCH1) gene and risk of hirschsprung disease in the Han Chinese population. *PLoS ONE* 8(9):e75407.
15. Charron F, Stein E, Jeong J, McMahon AP, Tessier-Lavigne M (2003) The morphogen sonic hedgehog is an axonal chemoattractant that collaborates with netrin-1 in midline axon guidance. *Cell* 113(1):11–23.
16. Kolodkin AL, Tessier-Lavigne M (2011) Mechanisms and molecules of neuronal wiring: A primer. *Cold Spring Harbor Perspect Biol* 3(6):a:001727.
17. Casey PJ, Fong HK, Simon MI, Gilman AG (1990) Gz, a guanine nucleotide-binding protein with unique biochemical properties. *J Biol Chem* 265(4):2383–2390.
18. Chiang C, et al. (1996) Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature* 383(6599):407–413.
19. Okada A, et al. (2006) Boc is a receptor for sonic hedgehog in the guidance of commissural axons. *Nature* 444(7117):369–373.
20. Tenzen T, et al. (2006) The cell surface membrane proteins Cdo and Boc are components and targets of the Hedgehog signaling pathway and feedback network in mice. *Dev Cell* 10(5):647–656.
21. Danielian PS, Muccino D, Rowitch DH, Michael SK, McMahon AP (1998) Modification of gene activity in mouse embryos in utero by a tamoxifen-inducible form of Cre recombinase. *Curr Biol* 8(24):1323–1326.
22. Long F, Zhang XM, Karp S, Yang Y, McMahon AP (2001) Genetic manipulation of hedgehog signaling in the endochondral skeleton reveals a direct role in the regulation of chondrocyte proliferation. *Development* 128(24):5099–5108.
23. Jeong J, Mao J, Tenzen T, Kottmann AH, McMahon AP (2004) Hedgehog signaling in the neural crest cells regulates the patterning and growth of facial primordia. *Genes Dev* 18(8):937–951.
24. Lin Z, et al. (2002) Immunoreactivity of Hu proteins facilitates identification of myenteric neurones in guinea-pig small intestine. *Neurogastroenterol Motil* 14(2):197–204.
25. Hao MM, Young HM (2009) Development of enteric neuron diversity. *J Cell Mol Med* 13(7):1193–1210.
26. Bondurand N, Natarajan D, Thapar N, Atkins C, Pachnis V (2003) Neuron and glia generating progenitors of the mammalian enteric nervous system isolated from foetal and postnatal gut cultures. *Development* 130(25):6387–6400.
27. Tessier-Lavigne M, Placzek M, Lumsden AG, Dodd J, Jessell TM (1988) Chemotropic guidance of developing axons in the mammalian central nervous system. *Nature* 336(6201):775–778.
28. Worley DS, et al. (2000) Developmental regulation of GDNF response and receptor expression in the enteric nervous system. *Development* 127(20):4383–4393.
29. Riobo NA, Saucy B, Dilizio C, Manning DR (2006) Activation of heterotrimeric G proteins by Smoothened. *Proc Natl Acad Sci USA* 103(33):12607–12612.
30. Ogden SK, et al. (2008) G protein Galphai functions immediately downstream of Smoothened in Hedgehog signalling. *Nature* 456(7224):967–970.
31. Nagahama M, et al. (2002) Inactivation of Galpha(z) causes disassembly of the Golgi apparatus. *J Cell Sci* 115(Pt 23):4483–4493.
32. Yang J, et al. (2000) Loss of signaling through the G protein, Gz, results in abnormal platelet activation and altered responses to psychoactive drugs. *Proc Natl Acad Sci USA* 97(18):9984–9989.

33. Cabrera JR, et al. (2006) Gas1 is related to the glial cell-derived neurotrophic factor family receptors alpha and regulates Ret signaling. *J Biol Chem* 281(20):14330–14339.
34. Allen BL, Tenzen T, McMahon AP (2007) The Hedgehog-binding proteins Gas1 and Cdo cooperate to positively regulate Shh signaling during mouse development. *Genes Dev* 21(10):1244–1257.
35. Trousse F, Martí E, Gruss P, Torres M, Bovolenta P (2001) Control of retinal ganglion cell axon growth: A new role for Sonic hedgehog. *Development* 128(20):3927–3936.
36. Fabre PJ, Shimogori T, Charron F (2010) Segregation of ipsilateral retinal ganglion cell axons at the optic chiasm requires the Shh receptor Boc. *J Neurosci* 30(1):266–275.
37. Lee CS, May NR, Fan CM (2001) Transdifferentiation of the ventral retinal pigmented epithelium to neural retina in the growth arrest specific gene 1 mutant. *Dev Biol* 236(1):17–29.
38. Allen BL, et al. (2011) Overlapping roles and collective requirement for the coreceptors GAS1, CDO, and BOC in SHH pathway function. *Dev Cell* 20(6):775–787.
39. Izzi L, et al. (2011) Boc and Gas1 each form distinct Shh receptor complexes with Ptch1 and are required for Shh-mediated cell proliferation. *Dev Cell* 20(6):788–801.
40. Bourikas D, et al. (2005) Sonic hedgehog guides commissural axons along the longitudinal axis of the spinal cord. *Nat Neurosci* 8(3):297–304.
41. Yam PT, et al. (2012) 14-3-3 proteins regulate a cell-intrinsic switch from sonic hedgehog-mediated commissural axon attraction to repulsion after midline crossing. *Neuron* 76(4):735–749.
42. Prodinger PM, et al. (2010) Gorlin syndrome associated with small bowel carcinoma and mesenchymal proliferation of the gastrointestinal tract: Case report and review of literature. *BMC Cancer* 10:360.
43. Bijlsma MF, Damhofer H, Roelink H (2012) Hedgehog-stimulated chemotaxis is mediated by smoothened located outside the primary cilium. *Sci Signal* 5(238): ra60.
44. Polizio AH, et al. (2011) Heterotrimeric Gi proteins link Hedgehog signaling to activation of Rho small GTPases to promote fibroblast migration. *J Biol Chem* 286(22): 19589–19596.
45. Regard JB, et al. (2013) Activation of Hedgehog signaling by loss of *GNAS* causes heterotopic ossification. *Nat Med* 19(11):1505–1512.
46. Kolterud A, et al. (2009) Paracrine Hedgehog signaling in stomach and intestine: New roles for hedgehog in gastrointestinal patterning. *Gastroenterology* 137(2): 618–628.