Gastrointestinal defects of the Gas1 mutant involve dysregulated Hedgehog and Ret signaling

Sandrine Biau1,2,*, Shiying Jin1,* and Chen-Ming Fan1,‡

1Department of Embryology, Carnegie Institution of Washington, 3520 San Martin Drive, Baltimore, Maryland 21218, USA
22IE Foundation, International Institute for Water and Environmental Engineering, Rue de la Science, 01 BP 594, Ouagadougou 01, Burkina Faso

*These authors contributed equally to this work
‡Author for correspondence (fan@ciwemb.edu)

Introduction

The mammalian gastrointestinal (GI) tract is a complex digestive system. The entire tract develops from two ventral invaginations that fuse in the midline to form a straight gut tube in the gastrulating embryo. Subsequent morphogenic events divide the tube into distinct organs such as the stomach, intestine and colon. These organs have a similar radial organization: the endoderm-derived internal luminal epithelium, the splanchnic mesoderm-derived mucosa underneath the epithelium and smooth muscle layers (the circular and longitudinal) at the outer edge, and the neural crest-derived enteric neurons embedded in the mucosa and smooth muscles. The enteric neurons establish a mesh-work of innervations (collectively, the enteric nervous system or ENS) (reviewed by de Santa Barbara et al., 2003; Furness, 2006; Heanue innervations (collectively, the enteric nervous system or ENS) (reviewed by de Santa Barbara et al., 2003; Furness, 2006; Heanue

Summary

The gastrointestinal (GI) tract defines the digestive system and is composed of the stomach, intestine and colon. Among the major cell types lining radially along the GI tract are the epithelium, mucosa, smooth muscles and enteric neurons. The Hedgehog (Hh) pathway has been implicated in directing various aspects of the developing GI tract, notably the mucosa and smooth muscle growth, and enteric neuron patterning, while the Ret signaling pathway is selectively required for enteric neuron migration, proliferation, and differentiation. The growth arrest specific gene 1 (Gas1) encodes a GPI-anchored protein known to bind to Sonic Hh (Shh), Indian Hh (Ihh), and Ret. However, its role in the GI tract has not been examined. Here we show that the Gas1 mutant GI tract, compared to the control, is shorter, has thinner smooth muscles, and contains more enteric progenitors that are abnormally distributed. These phenotypes are similar to those of the Shh mutant, supporting that Gas1 mediates most of the Shh activity in the GI tract. Because Gas1 has been shown to inhibit Ret signaling elicited by Glial cell line-derived neurotrophic factor (Gdnf), we explored whether Gas1 mutant enteric neurons displayed any alteration of Ret signaling levels. Indeed, isolated mutant enteric progenitors not only showed increased levels of phospho-Ret and its downstream effectors, phospho-Akt and phospho-Erk, but also displayed altered responses to Gdnf and Shh. We therefore conclude that phenotypes observed in the Gas1 mutant are due to a combination of reduced Hh signaling and increased Ret signaling.

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Key words: Gas1, Gastrointestinal development, Ret, Shh

Introduction

The mammalian gastrointestinal (GI) tract is a complex digestive system. The entire tract develops from two ventral invaginations that fuse in the midline to form a straight gut tube in the gastrulating embryo. Subsequent morphogenic events divide the tube into distinct organs such as the stomach, intestine and colon. These organs have a similar radial organization: the endoderm-derived internal luminal epithelium, the splanchnic mesoderm-derived mucosa underneath the epithelium and smooth muscle layers (the circular and longitudinal) at the outer edge, and the neural crest-derived enteric neurons embedded in the mucosa and smooth muscles. The enteric neurons establish a mesh-work of innervations (collectively, the enteric nervous system or ENS) (reviewed by de Santa Barbara et al., 2003; Furness, 2006; Heanue and Pachnis, 2007) to control gut movement, digestive enzyme secretion, and nutrient absorption.

The gut endoderm expresses Sonic hedgehog (Shh) and Indian hh (Ihh) (Ramalho-Santos et al., 2000). Elimination of Hedgehog (Hh) signaling, by either removing the function of both Shh and Ihh or their obligatory signaling component Smoothed (Smoo), leads to early lethality (Zhang et al., 2001). Shh single mutants develop further with a severely shortened GI tract, and a partial transformation of the stomach to intestine epithelium (Ramalho-Santos et al., 2000). Ihh mutants have defects in hindgut epithelium proliferation. Both Shh and Ihh single mutants also have thinner smooth muscle layers, indicating that they stimulate smooth muscle proliferation. Importantly, conditional inactivation of Shh and Ihh via ShhIhhror revealed their critical role in gut mesoderm expansion (Mao et al., 2010). Mice mutant for Gli2 or Gli3, both downstream mediators of Hh signaling, also display GI tract defects similar to, albeit milder than, that of the Shh mutant (Mo et al., 2001; Kim et al., 2005). Shh mutants have an apparent increase in ectopic localized enteric neurons whereas a fraction of Ihh mutants has virtually no enteric neurons (Ramalho-Santos et al., 2000), suggesting their opposing roles in enteric neuron development. As the primary effect of Hh signal is in the mesenchyme, whether the documented enteric phenotypes result from a direct action of Hh is uncertain. For example, Hh signaling was reported not active in the enteric neurons between the smooth muscle layers, i.e. the myenteric plexus, based on the expression of its downstream reporters, Ptc1-LacZ and Gli1-LacZ (Kolterud et al., 2009). On the other hand, endogenous Ptc1 expression was detected in the myenteric plexus (Fu et al., 2004). In addition, the recombinant N-terminal fragment of the active portion of Shh (Shh-N) can stimulate proliferation of enteric progenitors, which is in agreement with Ihh mutant phenotype but contrast with the apparent Shh mutant phenotype (Fu et al., 2004). Thus, how Hh signaling controls enteric neuron development along the GI tract remains elusive.
The majority of the neurons in the ENS originate from the enteric neural crest (ENC) at the vagal level (reviewed by Burns, 2005; Heanue and Pachnis, 2007; Newgreen and Young, 2002; Taraviras and Pachnis, 1999). They migrate to reach the rostral end of the intestine and enter the mesodermal layer, in which they migrate rostrocaudally and circumferentially along the GI tract, continuing to proliferate while they migrate and to differentiate into a variety of neuronal subtypes (Furness, 2006). The migration and colonization of the ENCs depends on Glial-derived neurotrophic factor (Gdnf) expressed in the gut mesenchyme (Golden et al., 1999; Natarajan et al., 2002). Gdnf appears to act as a chemoattractant to guide ENCs (Asai et al., 2006; Natarajan et al., 2002; Yan et al., 2004; Young et al., 2001). Consistently, the ENCs express Gdnf receptor-α (Gfrα-1) (Chalazonitis et al., 1998; Worley et al., 2000) and the receptor tyrosine kinase Ret (Taraviras et al., 1999): the former binds to Gdnf and couples to the latter for signal transduction. This signaling axis is also critical to enteric neuron progenitor survival, proliferation, and differentiation (Gianino et al., 2003) and involves downstream effectors such as Akt and Erk (Asai et al., 2006; Hayashi et al., 2000; Mograbi et al., 2001; reviewed by Airaksinen and Saarma, 2002). Importantly, mice mutant for Gdnf, Gfrα-1, or Ret, lack enteric neurons in the small and large intestine (Enomoto et al., 1998; Jain et al., 2004; Moore et al., 1996; Pichel et al., 1996; Sánchez et al., 1996; Schuchardt et al., 1994; Tomac et al., 1999). Of clinical relevance, lack of enteric neurons in segments of the intestine causes congenital intestinal obstruction in humans known as the Hirschsprung’s disease (HSCR) (Brooks et al., 2005; Gershon and Ratcliffe, 2004).

Enteric neuron progenitors in the GI tract can be isolated and cultured as enteric neurospheres (Bondurand et al., 2003). Shh-N and Gdnf have been shown to counteract each other’s activity in such a culture system. Shh enhances enteric progenitor proliferation, whereas Gdnf promotes differentiation (Fu et al., 2004). Such observation, coupled with Shh and Ihh expression in the epithelium and Gdnf in the mesenchyme, supports a notion that they coordinately control enteric progenitor and neuron numbers via long-range versus local actions. Recent data revealed that Growth arrest-specific gene 1 (Gas1) is both a Hh and a Ret binding protein (Cabrera et al., 2006; Lee et al., 2001a), suggesting that it can play a role in coordinating these two pathways. Although Gas1 was thought to be an unique membrane protein without homologs (Schneider et al., 1988), advanced computational studies re-assigned it to be a member of the Gfrα family (Cabrera et al., 2006; Schueler-Furman et al., 2006). For the Ihh pathway, Gas1 appears to act as a co-receptor by facilitating Ihh binding to its receptor Ptc1, which then results in canonical pathway activation (Martinelli and Fan, 2007a). For the Ret pathway, over-expressed Gas1 was shown to suppress Gdnf-induced tyrosine phosphorylation of Ret at Y1062 (López-Ramirez et al., 2008), as well as phosphorylation of its downstream effector Akt in cell lines (Cabrera et al., 2006; López-Ramirez et al., 2008). While the involvement of Gas1 in Ihh signaling is well established in *in vitro* and *in vivo*, the role of Gas1 in modulating Ret signaling has not been investigated beyond these initial *in vitro* studies.

To explore the possibility that Gas1 integrates Shh and Ret signaling *in vivo*, we first established that *Gas1* is expressed in the developing GI tract. We found that *Gas1* mutant GI tract has circular smooth muscle defects that can be attributed to defective Hh signaling. The abnormality of enteric progenitor organization and proliferation found in *Gas1* mutants is more likely explained by a combination of reduced Hh signaling and increased Ret signaling. We further utilized the enteric neurosphere cultures derived from *Gas1* control and mutant embryonic GI tracts to delineate the contribution of Shh and Ret in the proliferation and differentiation of enteric progenitors. Our results have implications to other organs and cell types where both Hh and Ret signaling pathways are active.

**Materials and Methods**

**Generation of embryos** *Gas1* mutant (Martinelli and Fan, 2007a) and *Shh* (Chiang et al., 1996) mutant alleles were described previously. Heterozygous mating was used to generate mutant embryos of desired genotypes (determined by PCR reactions) and specific stages described in the text. The vaginal plug date is designated as embryonic day 0.5 (E0.5) following convention. All procedures are approved by Carnegie IACUC.

**Histology, histochemistry, and immunostaining**

For histology, embryos younger than E13.5 or dissected GI tracts (for embryos older than E13.5) were fixed in Methacarn, dehydrated in methanol, embedded in paraffin, sectioned, and stained by hematoxylin and eosin (Surgipath). For X-gal reaction and immunofluorescence, embryos or GI tracts were fixed in 4% paraformaldehyde/PBS for 2 hrs, extensively washed in PBS, transferred through serial sucrose/PBS, and embedded in OCT for cryosectioning. Primary antibodies used were: anti-β-gal (rabbit, Chemicon, 1:1000, or mouse, Promega, 1:1000), anti-smooth muscle actin (SMA, rabbit, Abcam, 1:200), anti-neuronal-specific β-tubulin (Tuj1, mouse, Covance, 1:800), anti-Neurotrophin receptor p75 (Rabbit, Millipore, 1:200), Ret (goat, R&D system, 1:100), and anti-mouse Gas1 (goat, R&D system, 1:200). Alexa fluor 488 and Alexa fluor 568 conjugated secondary antibodies against specific species (goat, mouse, and rabbit) were used for detection (Molecular Probes, all at 1:1000). DAPI was used at 1 μM for counter staining of DNA.

**In situ hybridization (ISH)**

Freshly dissected guts were fixed in Methancarn, dehydrated in methanol, embedded in paraffin, and sectioned. ISH was performed using a standard protocol (Schaefer-Wimmers and Gerfin-Moser, 1993) with digoxigenin-labeled antisense RNA probes. The Shh probe was a gift from Dr A. McMahon (Harvard University), the Ptc1 probe, a gift from Dr M. Scott (Stanford University).

**RT-PCR**

For each sample, 1 μg of total RNA was used for standard reverse transcription (RT) using random primers and MMLV reverse transcriptase (Invitrogen) in a 15 μl reaction. One μl of the RT reaction was then used in a 20 μl Polymerase Chain Reaction (PCR) for 30–35 cycles. The oligonucleotide primers used for each are as follows. *Gdnf*: GAAGTATGGAGATGTCCGAC and CTTGACCAAAATCCAGACTG; *Shh*: GAAGATCACAAGAACTCCGAG and TGGATTCAATGACCCCACTGGA; *Gas1*: ATCCACCGTGGAGATGCTGATT and GGGCTGAAATGACCTCCGACAC; *Act*: TGGAAGATGCTCAGAAGG- ACT and GGCTCATCCTTTTCAGGTTGAC. Products were resolved on 2% agarose gels and presented as qualitative assessments.

**Neurosphere culture**

Neurosphere-like bodies were generated with E11.5 whole guts of specified genotypes (determined by PCR reactions) and specific stages described in the text. The vaginal plug date is designated as embryonic day 0.5 (E0.5) following convention. All procedures are approved by Carnegie IACUC.

**Proliferation assay**

For *in vivo* labeling, EdU (5 μg/gram of body weight) in PBS was injected into the pregnant mice perinatally, and the embryos were harvested 1 hr later. For *in vitro* labeling, EdU (0.5 μg/ml) was added 1 hr before the termination of culture. EdU detection was performed using the Click-iT kit (Invitrogen) after immunostaining procedure.

**Western blots**

Control and mutant neurospheres were incubated in serum-free medium for 4 h and then mock-stimulated or stimulated with 10 nM of Shh-N or 50 nM of Gdnf in serum free medium for 10 min. Approximately 20–30 neurospheres were used per condition. They were lysed in ice-cold RIPA buffer supplemented with protease inhibitor, phosphatase inhibitors and 1 mM PMSF (Promega). Nuclei
were removed by centrifugation. 4× SDS-PAGE sample buffer was added, and samples were resolved by 7.5% SDS-PAGE. Western blots were performed using rabbit antibodies against Akt, phospho-Akt (P-Akt), Erk1/2 (collectively, Erk), phospho-Erk1/2 (collectively, P-Erk), Ret, and phospho-Ret-Y1062 (P-Ret; Cell Signaling) followed by HRP-conjugated secondary antibody and ECL detection (Amersham). Band intensities were quantified using densitometry, followed by Image J program. At least 3 independent experiments were performed for each condition for statistical evaluation.

**Quantitation and statistical analyses**
Circular smooth muscle thickness was measured based on anti-SMA staining. P75+ and EdU+ cells were counted on digital images of ≥5 sections of each of ≥3 embryos of each genotype. Bar graphs represent mean ± standard deviations. All statistical data considered significant were with P values <0.05 as assessed by the Student’s t-test; t-test was performed for paired samples, and ANOVA with Tukey post hoc corrections performed for paired and multiple comparisons. They are presented in text or legends as appropriate.

**Results**

**Gas1** is expressed in the developing mouse GI tract

As the first step towards investigating *Gas1* function in the developing GI tract, we examined its expression. Using a *LacZ* knock-in allele of *Gas1* (*Gas1*LacZ) (Martinelli and Fan, 2007a), we monitored β-gal activity (by X-gal histochemical reaction) as a reporter for *Gas1* expression in *Gas1*+/LacZ embryos. At E8.5, β-gal positive cells were found at the dorsal gut endoderm and the splanchnic mesoderm (Fig. 1A). At the E9.5 midgut level, the dorsal endoderm staining was preferentially localized to the left side and the splanchnic mesoderm staining becomes more intensified (Fig. 1B). The significance for such asymmetric expression is currently unknown. At E11.5, X-gal staining was detected in the gut mesenchyme (Fig. 1C), but not longer in the endodermal epithelium of the stomach and midgut. From E13.5 to E15.5 and E18.5 (Fig. 1D–L), staining in the mesenchyme between the epithelium and smooth muscles becomes progressively reduced, and eventually staining is only seen in the two smooth muscle layers. Similar changes were found in the stomach (Fig. 1D,G,J), intestine (Fig. 1E,H,K) and colon (Fig. 1F,I,L). At E13.5 and E15.5, we noted some cells lightly stained with variable intensities scattered in between the smooth muscle layers, i.e. the myenteric plexus. The latter suggests that enteric progenitors or neurons express *Gas1*. At E18.5, X-gal staining was detected intensely throughout the two muscle layers as well as cells between them in the stomach and small intestine, while much weaker or no X-gal signal was found in cells in the myenteric plexus of the colon. *Gas1* expression in the smooth muscles suggests a role in mediating Hh signaling for the growth of these muscles, while expression in myenteric plexus suggests a role in mediating Hh and/or Ret signaling for enteric progenitor/neuron development.

**Gas1** mutant mice have multiple morphological GI tract defects

To assess the function of *Gas1* in GI development, we examined the phenotype of *Gas1* mutants. Although *Gas1*-LacZ expression in the GI system is detected early, we did not find appreciable defects in the gut tube at E10.5 and E11.5. However, at postnatal day 0, the mutant GI tract was ~60% the length of the control GI tract, while the mutant embryo weighed at 75–80% of the control sibling. However, the mutant stomach was ~1/4 the size of the control stomach, indicating a disproportional deficiency in the growth of this organ. The mutant gut also displayed slight malrotation (Fig. 2A).

Because most *Gas1* mutants died immediately after birth and were cannibalized, we focused our analysis at E18.5, one day prior to birth. The *Gas1* mutant not only had a much smaller stomach, but also displayed an overgrown stomach epithelium by histological analysis (compare Fig. 2B and Fig. 2C), a phenotype previously reported for the *Shh* mutant (Fig. 2D) (Ramalho-Santos et al., 2000). Although *Shh* mutant has a partial stomach-to-midgut transformation, as its epithelium is positive for Alkaline Phosphatase activity (specific for midgut) (Ramalho-Santos et al., 2000). Although *Shh* mutant has a partial stomach-to-midgut transformation, as its epithelium is positive for Alkaline Phosphatase activity (specific for midgut) (Ramalho-Santos et al., 2000). Although *Shh* mutant has a partial stomach-to-midgut transformation, as its epithelium is positive for Alkaline Phosphatase activity (specific for midgut) (Ramalho-Santos et al., 2000). Although *Shh* mutant has a partial stomach-to-midgut transformation, as its epithelium is positive for Alkaline Phosphatase activity (specific for midgut) (Ramalho-Santos et al., 2000). Although *Shh* mutant has a partial stomach-to-midgut transformation, as its epithelium is positive for Alkaline Phosphatase activity (specific for midgut) (Ramalho-Santos et al., 2000). Although *Shh* mutant has a partial stomach-to-midgut transformation, as its epithelium is positive for Alkaline Phosphatase activity (specific for midgut) (Ramalho-Santos et al., 2000). Although *Shh* mutant has a partial stomach-to-midgut transformation, as its epithelium is positive for Alkaline Phosphatase activity (specific for midgut) (Ramalho-Santos et al., 2000). Although *Shh* mutant has a partial stomach-to-midgut transformation, as its epithelium is positive for Alkaline Phosphatase activity (specific for midgut) (Ramalho-Santos et al., 2000). Although *Shh* mutant has a partial stomach-to-midgut transformation, as its epithelium is positive for Alkaline Phosphatase activity (specific for midgut) (Ramalho-Santos et al., 2000). Although *Shh* mutant has a partial stomach-to-midgut transformation, as its epithelium is positive for Alkaline Phosphatase activity (specific for midgut) (Ramalho-Santos et al., 2000). Although *Shh* mutant has a partial stomach-to-midgut transformation, as its epithelium is positive for Alkaline Phosphatase activity (specific for midgut) (Ramalho-Santos et al., 2000). Although *Shh* mutant has a partial stomach-to-midgut transformation, as its epithelium is posi...
Fan, 2007a), the Gas1 mutant displays milder defects than those found in the Shh mutant, including the GI tract.

**Gas1 mutants display circular smooth muscle defects**

Both Shh and Ihh single mutants were reported to have reduced circular smooth muscle thickness. To determine whether endogenous Gas1 is expressed in the circular smooth muscles to mediate their function, we performed double immunofluorescence with anti-Gas1 and anti-SMA antibodies. We found that their expression overlapped in both circular and longitudinal smooth muscle layers (Fig. 3A–C), consistent with the X-gal histochemical data.

While we confirmed the circular muscle defects in the Shh mutant (Fig. 3F), we did not find such a defect in the Ihh mutant.
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quantified the circular muscle thickness of small intestine, compared to the control (Fig. 3D–F). We further as
find reduced expression of Hh signal transcriptional targets, such Gas1 mutants have reduced Hh signaling
like Gas1+/LacZ in the stomach, small intestine, and colon. In comparison to normal in the mutant epithelium (Kolterud et al., 2009; Ramalho-Santos et al., 2000), where Gas1 helps to enhance the signaling output.

Gas1 mutants have reduced Hh signaling
If Gas1 indeed mediates Hh signaling in the gut, we expect to find reduced expression of Hh signal transcriptional targets, such as Ptc1, in the Gas1 mutant. At E11.5, Shh was expressed normally in the mutant epithelium (compare Fig. 4A and Fig. 4B), whereas occasional patches of the mutant mesenchyme showed reduced Ptc1 (compare Fig. 4C and Fig. 4D), suggesting not an overt reduction of Hh signaling at this point. At E15.5, reduced Ptc1 expression in the intestine (Fig. 4G,H) and colon (Fig. 4K,L) was evident: only the mesenchyme immediately adjacent to the epithelium expressed elevated levels of Ptc1 in the mutant, while the control showed the up-regulated Ptc1 expression domain extending further into the surrounding mesenchyme and apparently in the circular muscle layer. Although Shh expression in the mutant small intestine appeared normal, its expression in the mutant colon was visibly reduced (Fig. 4E,F,I,J). On the other hand, Ihh expression levels did not appear qualitative different between control and mutant small intestines and colons (supplementary material Fig. S1). As Gas1 is not expressed in the epithelium after E11.5, these data indicate a feedback regulation from defective smooth muscles (or other cell types) in the Gas1 mutant to down-regulate Shh expression in the colon epithelium. The reduced range and level of Ptc1 expression likely reflect the lack of Gas1 to extend Shh’s range of action in the small intestine. In the colon, however, Ptc1 reduction may be attributed to the reduction of Shh and loss of Gas1. Nevertheless, the complementary expression pattern of Shh in the secretion site and Gas1 in the receiving site supports them as a ligand-receptor pair. We note that the endocrine secreting CCK+ cells in the mutant duodenum

Fig. 3. Gas1 mutants have a thinner circular smooth muscle layer similar to that of Shh mutants. (A–C) in E18.5 wild type small intestine, endogenous Gas1 (C) and SMA (B) expression overlaps (A, with DAPI) in both layers of smooth muscles; asterisks in (A) indicate the overlap; cm, circular smooth muscle; Im, longitudinal muscle. (D–F) Circular smooth muscle defects are shown for small intestines of Gas1+/− (E) and Shh−/− (F) at E18.5, compared to the Gas1+/+ control (D); longitudinal muscle (Im) layers are slightly disorganized in both mutants. The thickness of the circular smooth muscle layers in Gas1+/−, Gas1−/− and Shh−/− is quantified in μm (mean; error bars = standard deviation) for stomach (G), small intestine (H), and colon (I). For Gas1−/− stomach, 18.5±1.9; small intestine, 10.85±1.1; and colon, 15.9±1.5. For Gas1+/− stomach, 13.5±1.3; small intestine, 4.51±0.76; and colon, 9.5±1.09. For Shh−/− stomach, 12.4±1.4; small intestine, 7.23±0.65; and colon, 5.9±0.56. In stomach, small intestine and colon, Gas1+/− has 27.3%, 58.4% and 40.3% reduction, and Shh−/− has 33.2%, 30.6% and 62.5% reduction, respectively, compared to Gas1+/+. Scale bars: 25 μm in A (also applies to B,C) and in D (also applies to E,F).
epithelium are present, implying not an overt change in the Gas1 mutant epithelium patterning (supplementary material Fig. S2).

Gas1 mutants have altered enteric progenitor number and distribution

Shh mutants were described to have substantial enteric neurons, suggesting that it inhibits enteric progenitor proliferation (Ramalho-Santos et al., 2000). We therefore examined whether Gas1 mutants have a similar change in enteric neurons to that found in Shh mutants.

At E18.5, the control myenteric neurons formed coalesced clusters between the two muscle layers (stained by Tuj1) (supplementary material Fig. S2). The Gas1 mutant had abundant enteric neurons that were scattered and disorganized, and some were mis-localized to near the base of the epithelium. This patterning defect was also documented for the Shh mutant (Ramalho-Santos et al., 2000). As Tuj1 and GFAP positive cells were found abundantly in the mutant (supplementary material Fig. S2), there appeared no major defects in the terminal differentiation of neurons and glia per se. Because Shh is implicated in enteric progenitor proliferation (Fu et al., 2004), we next examined whether endogenous Gas1 is expressed in the enteric progenitors using P75 as a marker. Indeed, we observed P75+ enteric progenitors in the small intestine stained positively for endogenous Gas1 antigen (Fig. 5A–C). Importantly, in the small intestine, there were approximately 1.6-fold more P75+ progenitors per cross section at E18.5 in the Gas1 mutant than in the control (Fig. 5D,E, and quantification in Fig. 5F). Furthermore, the mutant P75+ cells were less organized and some of them were found ectopically located near the epithelium (Fig. 5E). To determine whether the increase in enteric progenitors in the mutant is due to increased proliferation, we monitored their proliferation rate at E13.5, an earlier time-point when the enteric progenitors actively expand. At this time-point, the mutant P75+ progenitors appeared normally distributed (Fig. 5H).

In vivo EdU incorporation assay revealed that among the P75+ cells, P75+EdU+ cells were found at a 2-fold higher rate in the Gas1 mutant than in the control (Fig. 5G,H, and quantification in Fig. 5I). Thus, the Gas1 mutant displays similar enteric neural defects to that described for the Shh mutant.

**Fig. 4.** Hh signaling is altered in the Gas1 mutant GI tract. At E11.5, the Gas1−/− (B,D) does not have an altered expression of Shh in the endoderm epithelium (ep), but a slightly reduced level of Ptc1 in patches of the mesenchyme (me) at stomach level compared to the control (A,C). At E15.5 small intestine level, the Gas1−/− (F) has a similar level of Shh expression in the epithelium as the control (E). Ptc1 expression is reduced in the submucosa and nearly absent at the circular muscle layer (H). In the colon, Shh expression level is reduced in the Gas1 mutant (J) compare to the control (I), and Ptc1 expression is restricted to the mesenchyme immediately below the epithelium in the Gas1 mutant (L), compared to the control (K). Arrows point to signals in the mesenchyme, the mucosa immediately underneath the epithelium, and the outer layer, presumably the circular smooth muscle; arrowheads in epithelium. Scale bars: 100 μm in A (also applies to B–D), and in E (also applies to F–L).
The Gas1 mutant small intestine has altered levels of phosphorylated Akt and phosphorylated Erk

Because Gas1 was suggested to inhibit Ret signaling by in vitro cell line assays (Cabrera et al., 2006; López-Ramírez et al., 2008), we wanted to determine whether the defect observed in Gas1 mutant enteric system is associated with elevated Ret signaling. Despite multiple attempts, we failed to reliably detect phosphorylated Ret using anti-phospho-Ret-Y1062 (P-Ret), a phosphorylation event critical for Ret signaling (Jain et al., 2006; Jijiwa et al., 2004; Wong et al., 2005), on small intestine sections or tissue extracts by Western blot. However, we were able to detect phosphorylation of Ret downstream effectors, Akt (P-Akt) and Erk (P-Erk), in extracts. After normalization to total Akt and Erk, both P-Akt and P-Erk levels were consistently found to be slightly increased in the Gas1 mutant compared to the control (supplementary material Fig. S3) intestine. Although this change is consistent with increased Ret signaling, a plethora of signaling pathways converging to Akt and intestine. Although this change is consistent with increased Ret signaling, a plethora of signaling pathways converging to Akt and Erk of size ranges similar to those of control cells. In order to have sufficient neurospheres of similar sizes for assay, subsequent experiments used neurospheres after secondary expansion and derived from multiple independent control and mutant embryos.

Double immunofluorescence for β-gal and Ret of Gas1+/LacZ enteric neurospheres showed that neurospheres contained many Gas1+ cells (Fig. 6A,B). X-gal reactions on these spheres revealed that the Gas1 promoter remained active during the culturing condition for both control and mutant. After secondary expansion, the mutant cells gave rise to neurospheres in efficiency and of size ranges similar to those of control cells. In order to have sufficient neurospheres of similar sizes for assay, subsequent experiments used neurospheres after secondary expansion and derived from multiple independent control and mutant embryos.

We noted that primary Gas1−/− neurospheres initially formed more abundantly but smaller than Gas1+/− control neurospheres in a reproducible manner (Fig. 6A,B). X-gal reactions on these spheres revealed that the Gas1 promoter remained active during the culturing condition for both control and mutant. After secondary expansion, the mutant cells gave rise to neurospheres in efficiency and of size ranges similar to those of control cells. In order to have sufficient neurospheres of similar sizes for assay, subsequent experiments used neurospheres after secondary expansion and derived from multiple independent control and mutant embryos.

Double immunofluorescence for β-gal and Ret of Gas1+/LacZ enteric neurospheres showed that neurospheres contained many β-gal+ Ret+ cells (Fig. 6C–E), legitimizing the use of this system to assess the role of Gas1 in modulating Ret signaling. Control and mutant neurospheres established by this method expressed minimal levels of Gdnf or Shh transcripts by RT-PCR compared to E11.5 gut tubes of corresponding genotypes (Fig. 6F), making them suitable for determining the consequences of exogenously applied factors. Consistently, neither control nor mutant neurospheres expressed detectable levels of the Hh downstream gene Gli1 without exogenously applied Shh-N. To confirm that these neurospheres were responsive to Shh signaling, we applied recombinant Shh-N at 10 nM (previously determined to induce sub-maximal response) (Martinelli and Fan, 2007a) for 24 hrs and assayed for the induction of Gli1. We found that mutant neurospheres were qualitatively less responsive than control neurospheres (Fig. 6G), suggesting the positive role of Gas1 in facilitating Hh signaling in this system, as in other tissues reported previously (Martinelli and Fan, 2007a).
Gas1 modulates Ret and its downstream effectors

We next wanted to test whether there was an alteration of Ret signaling in the Gas1 mutant neurosphere, because Gas1 was suggested to impact Gdnf-induced Ret signaling (Cabrera et al., 2006; López-Ramírez et al., 2008). We also monitored two effectors that have been implicated to mediate Ret-regulated cell proliferation and differentiation, i.e. Akt and Erk (Airaksinen and Saarma, 2002; Asai et al., 2006; Hayashi et al., 2000; Mograbi et al., 2001). For signaling levels, Western analysis was performed using antibodies to Y1062 phosphorylated Ret (P-Ret), P-Erk and P-Akt. Total Ret, Erk and Akt levels were also monitored to determine relative ratios of their respective phosphorylated forms. For optimization of monitoring above phosphorylation events, we varied the dosages of Shh-N (10–40 nM) and Gdnf (0.75–100 ng/ml), and conducted time-course studies (5–20 min, at 5 min intervals). Consistent with previous reports, we found that 10 nM Shh-N and 50 ng/ml of Gdnf are at sub-maximal for response (Fu et al., 2004; Martinelli and Fan, 2007a), and that 10 min after Gdnf and Shh-N application resulted in the most robust effect. Below, we describe data from these conditions.

We were first surprised to find that after switching to the basal medium without addition of Gdnf or Shh-N, Gas1 mutant neurospheres reproducibly displayed an increased level of P-Ret (Fig. 7) than that in the control neurospheres (1.56 fold, $P=0.014$). Similarly, P-Erk (2.02 fold, $P=0.012$) and P-Akt (1.98 fold, $P=0.003$) levels were also increased in the mutant neurospheres. After acute application of Gdnf for 10 min, both control and mutant neurospheres were stimulated to display significantly increased levels of P-Ret as well as P-Akt and P-Erk, compared to untreated control ($0.0007 \leq P \leq 0.016$ for all).

Gas1 mutant neurospheres treated with Gdnf showed further increased levels of P-Ret ($P=0.079$), P-Erk ($P=0.018$), and P-Akt ($P=0.047$) compared to the mutant untreated sample.

Unexpectedly, we found that acutely applied Shh-N (10 min) also stimulated P-Erk (2.89 fold, $P=0.023$) and P-Akt (2.17 fold, $P=0.004$) in control neurospheres (compared to untreated), without significantly inducing P-Ret as with Gdnf. These data indicate that Shh-N pulse elicits Erk and Akt pathway activation in enteric neurospheres. Although Shh-N appeared to increase P-Erk and P-Akt levels in the mutant neurosphere relative to mutant untreated sample, but the increases were not significant (for P-Erk, 2.24 vs 2.02 fold, $P=0.15$; for P-Akt, 1.98 vs 1.72 fold, $P=0.13$). These results together indicate that removal of Gas1 function in enteric neurospheres makes them spuriously activate Ret signaling, and become less responsive to Shh-N-induced P-Erk and P-Akt activation, as well as Gli1 expression (Fig. 6G).

Thus, we have uncovered Akt and Erk as potential Gas1-modulated nodal points of crosstalk between Hh and Ret signaling.

Gas1 mutant neurospheres have altered response to Shh-N and Gdnf

The above data prompted us to investigate whether the biochemical alterations observed in the Gas1 mutant neurosphere had any significance in functional alteration. It was established that the enteric neurosphere system allowed detection of Shh-N-induced proliferation and Gdnf-induced neuronal differentiation (Fu et al., 2004) – despite Gdnf-Ret signaling is also known to stimulate enteric progenitor proliferation (Gianino et al., 2003; Hearn et al., 1998;
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Heuckeroth et al., 1998). To study their effects, we cultured the control and mutant neurospheres in the absence or presence of recombinant Shh-N or Gdnf. We used EdU incorporation to monitor proliferation (Fig. 8A–F) and TuJ1 immunostaining to monitor neuronal differentiation (Fig. 8G–L).

Consistent with previous report (Fu et al., 2004), we found that exogenous Shh-N increased the proliferation rate of the control neurosphere relative to that of the untreated sample (Fig. 8A,B, and quantification in Fig. 8M). Gas1 mutant neuroospheres also responded to applied Shh-N, but to a lesser extent (Fig. 8D,E,M). By contrast, when Gdnf was used to stimulate proliferation, EdU incorporation rate was increased but no significant differences between control and mutant neuroospheres were obtained (Fig. 8C,F,M). When TuJ1 was used for assessing neuronal differentiation, we noted that Shh-N did not enhance enteric neuron differentiation in control and mutant, compared to the untreated condition (Fig. 8G,H,J,K, and quantification in Fig. 8N). Gdnf, on the hand, effectively enhanced neuronal differentiation of the control neurospheres, and this effect was further increased in the Gas1 mutant (Fig. 8L, and quantification in Fig. 8N). Thus, removal of Gas1 leads to blunted Shh-N response in proliferation and elevated Gdnf response in differentiation. Below we discuss the ramifications of our findings.

Discussion

The role of Hh signaling in gastrointestinal development has been firmly established (Kim et al., 2005; Litingtung et al., 1998; Mao et al., 2010; Ramalho-Santos et al., 2000). Such a role has been shown in the zebrafish GI tract (Reichenbach et al., 2008). Here we extend this observation by describing the role of the Hh binding protein Gas1. We further provide evidence that Gas1 mutant enteric neurospheres display elevated levels of Ret signaling as well as its downstream effectors. It appears that Gas1 has diverged from the core Gfrz family to acquire Hh binding and signaling capacity, while gaining constitutive Ret binding property in an inhibitory manner, to effect enteric neuron development.

Gas1 and Hh signaling

We have previously shown that Gas1 mutants display many phenotypes related to the Shh mutant, albeit to a lesser degree (Martinelli and Fan, 2007a; Martinelli and Fan, 2007b). In the Ihh signaling cascade Gas1 is placed parallel to the Ihh receptor Ptc1. Gas1 and Ptc1 together display a greater Shh-N binding activity than either one alone, but the precise biophysical nature for this synergy is not known. Here, we show that inactivation of the Gas1 gene alone is sufficient to cause GI defects related to reduced Hh signaling, including reduced GI tract length, malrotation of the gut, reduced circular muscle thickness, overgrowth of the villi, and hyper abundance of enteric neurons. Aspects of Gas1 mutant GI defects are more severe than those of the Shh mutant, while other aspects milder. The more severe phenotypes, e.g. circular smooth muscle thickness at the midgut, may be due to Gas1 also mediating Ihh signaling. However, the Ihh allele in our mouse colony did not render GI defects reported for the Ihh mutant (Ramalho-Santos et al., 2000) – likely due to different genetic backgrounds used. We note that the circular smooth muscles of Shh+/−Gas1−/− and Ihh+/−Gas1−/− midgut were not statistically thinner than that of Gas1+/−midgut (not shown), suggesting that Gas1 mutation is dominant to produce the phenotype and precludes observable genetic interaction with Shh and Ihh in their heterozygous backgrounds. This perhaps explains the more severe phenotype in the Gas1 mutant than in the Shh mutant in this context. The milder phenotypes, e.g. the GI tract length, may be explained by the compensation by Cdo and/or Boc, two additional Hh binding proteins. Recent studies have shown that Cdo and Boc play a redundant role with Gas1 to mediate Hh signaling at various tissues examined (Allen et al., 2011; Allen et al., 2007). Notably, the Gas1;Cdo;Boc triple mutant is similar to the Smo mutant (i.e. a complete loss of Hh signaling) (Allen et al., 2011; Zhang et al., 2001). The precise contribution of each to the GI tract needs extensive future studies. It is also important to keep in mind that Gas1, Cdo, and Boc have functions seemingly unrelated to Hh signaling (Lee et al., 2001b; Liu et al., 2002; Lu and Krauss, 2010; Wegorzewska et al., 2003).

Because Gas1 is expressed in the circular smooth muscle, we propose that it directly mediates the reception of Hh secreted from the epithelium for growth. Indeed, expression of Hh signaling reporters indicates that Hh signaling is active in the gut mesenchyme and circular muscle (Kolterud et al., 2009). Furthermore, Shh-Cre driven conditional Ihh;Shh mutant has a...
drastic reduction of gut mesenchyme and smooth muscles, and expression of an activated Smo (Smo-M2) drastically increases mesenchymal mass (Mao et al., 2010). Mice mutant for the transcriptional mediators of Hh signaling, Gli2 or Gli3, also display GI defects similar to that of the Shh mutant (Mo et al., 2001; Kim et al., 2005). Lastly, transgenic expression of Ihh via a Villin promoter causes overt villus smooth muscle differentiation (Kolterud et al., 2009). All these results support the role of Hh signaling in the expansion of embryonic gut mesoderm.

Transient expression of Gas1 in the dorsal endoderm is similarly to that of Ptc1-LacZ (Kolterud et al., 2009). Their early expression suggests autocrine signaling in the endoderm and may help explain the GI malrotation phenotype shared by Shh and Gas1 mutants. Since Gas1 is not detected in the villi, villi overgrowth in the mutant likely reflects a secondary consequence. Whether myenteric progenitors and neurons receive Hh signaling in vivo remain unresolved by different downstream reporter studies (Fu et al., 2004; Kolterud et al., 2009). Functional studies also generated puzzling results. In the zebrafish, Hh signaling appears essential for enteric progenitor migration/proliferation via mutational and pharmacological assays (Reichenbach et al., 2008). In the mouse, however, inactivating both Shh and Ihh in the endoderm did not lead to a deficiency of enteric neurons (Mao et al., 2010), whereas overexpression of GLI1 (presumably activating Hh signaling) causes patchy absence of enteric neurons along the GI tract.

Fig. 8. Shh-N and Gdnf exert differential effects on Gas1 mutant neurospheres. Gas1+/− (A–C,G–I) and Gas1−/− (D–F,J–L) neurospheres were mock-treated (control; A,D,G,J), treated with 10 nM Shh-N (B,E,H,K) or treated with 50 ng/ml Gdnf (C,F,I,L). EdU was added one hour before harvesting. Controls were performed in side-by-side experiments (A,D,G,J). Proliferation was detected by EdU (red) incorporation (A–F), while neuronal differentiation was detected by Tuj1 (green) immunostaining (G–L). All neurospheres were counterstained with DAPI (blue). Scale bar: 0.1 mm in A–L. (M) Quantification of proliferation: compared to the mock-treated, Gas1−/− neurospheres treated with Shh-N displayed higher rate of proliferation (11±2% vs 24±3%, P<0.01). Gas1−/− neurospheres were less responsive to Shh-N than Gas1+/− neurospheres (17±2% vs 24±3%, P<0.05). No significant differences were found for Gdnf treatment. (N) Quantification of differentiation: for both Gas1+/− and Gas1−/− neurospheres, Gdnf stimulated significant differentiation related to control mock-treated (P<0.01 for both). Gas1−/− neurospheres were more responsive to Gdnf than Gas1+/− neurospheres (51±3% vs 41±3%, P<0.05).
Here we show that Gas1 impinges on Hh and Ret signaling levels. Not only is Gas1 needed to suppress P-Ret, P-Erk and P-Akt levels, it is also needed for maximal P-Akt and P-Erk induction by Shh-N. We suggest that the proliferative effect of Shh-N is mediated by its canonical pathway, while the immediate Erk and Akt activation reflects its cooperation with other signaling pathways. In myoblast cultures Shh-N has also been shown to activate Akt and Erk and cooperate with IGF-1, which signals through a receptor tyrosine kinase (Elia et al., 2007; Madhala-Levy et al., 2012). Cellular levels of activated Akt and Erk conversely impact canonical Hh signaling (Riobo et al., 2006a; Riobo et al., 2006b). In enteric progenitors and myoblasts, we presume P-Erk and P-Akt activation by Hh potentiates receptor tyrosine kinase signaling. We imagine that the integration between Hh and Ret via Gas1 is extended to other contexts as a general regulatory theme.

Further defining the physical interfaces of Gas1-Shh and Gas1-Ret binding should allow the design of function distinguishing mutations of Gas1. For example, a missense mutation in GAS1 associated with holoprosencephaly was recently characterized as deficient for Shh-N binding (Pineda-Alvarez et al., 2012). It is possible that this mutated GAS1 retains Ret binding property. Conversely, based on the modeled interactions between Ret and Gfrαs (Cabrera et al., 2006; Schueler-Furman et al., 2006), a mutation in Gas1 may be engineered to selectively disrupt Ret but not Hh binding. Exclusive binding mutations of Gas1 for Shh and Ret will be the key future tools to resolve Gas1’s contribution to each pathway.

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Competing Interests
The authors have no competing interests to declare.

References
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Supplementary Material
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Fig. S1. ISH of Ihh at the levels of small intestine (A,B) and colon (C,D) at E15.5. There are no dramatic qualitative differences of signal intensities between Gas1+/− (A,C) and Gas1−/− (B,D) samples. Arrows point to ISH signals in the epithelium. Scale bar: 100 μm in A–D.

Fig. S2. CCK+ (arrows) endocrine cells were found in E18.5 duodenums of Gas1+/− (A) and Gas1−/− (B) embryos. Epifluorescent images are overlays between CCK (green) and Dapi (blue) staining. Tuj1+ (white arrows) neurons were found in E18.5 small intestines of Gas1+/− (C) and Gas1−/− (D) embryos. Confocal images are overlays between Tuj1 (red) and Dapi (blue) staining. GFAP+ (arrows, green) glial cells were found in E18.5 small intestines of Gas1+/− (E) and Gas1−/− (F) embryos. Scale bar: 25 μm in A–F.

Fig. S3. Tissue extracts of E15.5 Gas1+/− and Gas1−/− small intestines were subjected to Western analysis using antibodies labeled on the left. Equal amounts of total proteins were loaded in each lane. Total Akt and Erk were probed for loading control. The mutant small intestine contained slightly higher relative levels of P-Akt and P-Erk than the control.