

Review

The Role of *Gas1* in Embryonic Development and its Implications for Human Disease

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ABSTRACT

Growth arrest specific gene 1 (Gas1) has long been regarded as a cell cycle inhibitor of the G₀ to S phase transition. How GAS1, a GPI-anchored plasma membrane protein, directs intracellular changes without an extracellular ligand or a transmembrane protein partner has been puzzling. A recent series of biochemical and molecular genetic studies assigned the mammalian Hedgehog (HH) growth factor to be a ligand for GAS1 in vitro and in vivo. HH has enjoyed considerable attention for its profound role in embryonic patterning as a classic morphogen, i.e. inducing various cell types in a concentration-dependent manner. GAS1 appears to help transform the HH concentration gradient into its morphogenic activity gradient by acting cooperatively with the HH receptor, the 12-transmembrane protein Patched 1 (PTC1). These findings provoke intriguing thoughts on how HH and GAS1 may coordinate cell proliferation and differentiation to create biological patterns. The role of HH extends to human genetic diseases, stem cell renewal, and cancer growth, and we consider the possibility of GAS1's involvement in these processes as well.

Precise integration between cell proliferation and cell type specification during embryogenesis directs the formation and function of each tissue. Integration between these two programs in a given cell is largely determined by intercellular signaling molecules. Here, we review the recent 'unlikely marriage' between two extracellular molecules, GAS1 and HH, and the implications on coordinated cell proliferation and cell type specification.

Gas1 THE CELL CYCLE INHIBITOR

In 1988, *Gas1* was identified as one of six genes that were transcriptionally up-regulated in NIH3T3 cells arrested in cell cycle upon serum starvation, a presumed G₀ arrest.¹ Among the six initially identified growth arrest specific (hence *Gas*) genes, only *Gas1* could suppress cell cycle progression when overexpressed in non-transformed and certain oncogene-transformed NIH3T3 cells.²⁻⁴ Because GAS1 overexpressing cells did not incorporate BrdU, it was thought that GAS1 blocked the G₀ to S phase transition. Deletion analysis of GAS1, an outer plasma membrane glycosylphosphatidylinositol (GPI)-linked protein of 37 kDa, concluded that it could induce growth arrest without its GPI anchor, suggesting that it acts through another membrane partner.^{5,6} Interestingly, GAS1-induced growth arrest depended on the tumor suppressor gene p53.^{7,8} Furthermore, c-Myc and v-Src, powerful proliferation promoting factors, repressed *Gas1* expression, suggesting that *Gas1* downregulation is an associated step in the promotion of proliferation.^{9,10} By contrast, senescent fibroblast cells, though cease proliferating, do not express *Gas1*.¹¹ These studies together suggest that GAS1 is a negative cell growth regulator rather than a senescence mediator. However, evidence to demonstrate such a function for *Gas1* in an animal model is lacking.

IS *Gas1* A TUMOR SUPPRESSOR?

More recently, *Gas1* was also shown to regulate apoptosis in a context dependent manner. *Gas1* expression was upregulated after excitotoxic insult in the rat hippocampus and cultured hippocampal neurons, and suggested to be a pro-apoptotic gene in this context.¹² Ectopic expression of *Gas1* in the C6 glioma cell line induced apoptosis via activating the apoptotic protease Caspase 3.¹³ On the other hand, in cultured blood vessel endothelial cells, *Gas1* was suggested to prevent apoptosis.¹⁴ Despite these seemingly

contradictory results in different cell types, *Gas1* overexpression has been used to slow tumor growth. When the C6 glioma cells were injected into nude mice, tumor growth was inhibited if the cells were made to express *Gas1*.¹⁵ Similarly after injection, lung adenocarcinoma cells overexpressing *Gas1* also exhibited reduced growth of the subsequently arising tumors.¹⁶ Although it was not clear if the reduction in tumor growth was due to reduced proliferation or increased apoptosis. Further suggestive that *Gas1* is a tumor suppressor gene is that multiple gene expression profiling studies on cancer cells have noted the downregulation of *GAS1*.^{17,18} Lastly, the human *GAS1* gene is located in a fragile chromosome site that is frequently found to be deleted in cancerous cells.¹⁹⁻²¹ Whether the loss of *Gas1* expression is a direct cause of tumor formation and if *Gas1* is a true endogenous tumor suppressor has not yet been proven. Nonetheless, modulating its expression or function appears to have potential for therapeutic treatment.

Gas1 FUNCTION IN EMBRYOGENESIS

The above studies of *Gas1* suggest a potential role for regulating the G₀ phase or apoptosis in an animal. For example, *Gas1* may be expressed in terminally differentiated cells which are arrested in G₀, in stem cells which stay mostly in G₀ unless activated, or in cells that are destined to die for tissue remodeling. These potential roles for *Gas1* during embryonic development were first probed a decade after its original discovery. However, a survey of *Gas1* expression in mouse and chick embryos did not completely match G₀-arrested cells at various developmental stages and tissues examined.^{22,23} In fact, many highly proliferative regions also displayed high levels of *Gas1* expression. Although inactivation of *Gas1* in the mouse does result in multiple growth and patterning defects, including the eye, cerebellum, and limb,²⁴⁻²⁶ its *in vivo* role appears to be more in line with positive rather than negative regulation of cell proliferation and survival. Furthermore, *Gas1* mutant pups are 3/4 the size of control litter mates and do not display gross disproportional/abnormal overgrowth of any tissue (unpublished). These data, at face value, indicate that the role of *Gas1* during embryogenesis differs from originally assigned cellular role.

DISCOVERY OF A GAS1 LIGAND

What follows was an interesting clue for closing in on GAS1 function during embryogenesis: biochemical evidence that it can physically associate with two related Hedgehog (HH) proteins, Sonic Hedgehog (SHH) and Indian Hedgehog (IHH), based on an unbiased expression screen for genes encoding SHH binding proteins.²⁷ SHH and IHH are lipid-modified, secreted proteins that can signal to adjacent or distant cell targets to elicit different responses in a concentration dependent manner—i.e., they act as morphogens.²⁸ They are essential for the development of numerous tissues in the mouse, including those affected in the *Gas1* mutant (*Gas1*^{-/-}).²⁹ A further link between *Gas1* and HH came when it was noted that the *Gas1* expression pattern correlates with regions distant from HH sources of secretion; that is, it exists in a counter gradient to that of the HH signaling gradient.³⁰ Additionally, *Gas1* expression is negatively regulated by HH signaling.^{30,31} Lastly, overexpression of *Gas1* showed that it could inhibit SHH signaling in two embryonic explant culture systems.^{27,32} It was therefore proposed that GAS1

acts as a sink to sequester excessive SHH and prevent its action at an unwanted distance. Again lacking, however, was *in vivo* evidence to demonstrate such a role.

Gas1^{-/-} embryos exhibit gross phenotypes that are too ambiguous to be viewed as a simple consequence of excessive HH signaling. To address this issue, compound mutants between *Gas1* and *Shh* were generated to test for a potential genetic interaction, and we discovered that *Gas1* is a bona fide genetic modifier of *Shh* in the multiple developmental systems examined, including the craniofacial structure, heart, spinal cord, somite and limb.^{30,33} Using a battery of cell type-specific molecular markers as readouts for HH signaling in these compound mutants,^{30,31} it was found that from the genotypes of *Gas1*^{-/-}, *Gas1*^{-/-};*Shh*^{+/-}, *Shh*^{-/-}, to *Gas1*^{-/-};*Shh*^{-/-}, there is a progressive reduction in HH signaling (relative to wild type and *Gas1*^{+/-};*Shh*^{+/-} controls). These data indicate that, contrary to the original expectations, *Gas1* acts positively rather than negatively in HH signaling.

Based on GAS1's ability to bind SHH or IHH, one can envision two simple scenarios for GAS1 action: it either facilitates HH diffusion or HH activity. Immunohistochemistry to detect SHH in the developing limb bud revealed no reduction in its range of diffusion in either *Gas1*^{-/-} or *Gas1*^{-/-};*Shh*^{+/-} genotypes—even though there was clearly reduced domains of SHH downstream gene expression in both genotypes.³⁰ To address a potential role for GAS1 in enhancing HH activity, RNAi and overexpression approaches were used. When GAS1 was reduced by siRNA in a HH-responsive NIH3T3 cell line, the cells became less responsive to exogenously supplied SHH.³⁰ When *Gas1* was over-expressed in the chick neural tube, it extended the range of HH signaling by activating HH downstream genes ectopically at farther distances.^{30,31} Clonal analysis of these ectopically activated cells revealed that GAS1 acts cell-autonomously to enhance HH signaling. Taken together, there is strong evidence that GAS1 functions in the receiving cell to enhance HH responsiveness.

The core components for transducing the HH signal on the cell surface include Patched1 (PTC1) and Smoothed (SMO) (Fig. 1).³⁴ PTC1 is a 12-transmembrane domain protein that can also bind HH and has been considered “the HH receptor”. SMO is a seven-transmembrane domain protein whose activity is normally inhibited by PTC1. Upon binding to PTC1, HH relieves PTC1's inhibitory effect on SMO, allowing SMO to translocate to the primary cilia and trigger downstream signaling events.³⁵ One such event appears to be generating activator forms of GLI Zn-finger transcription factors to turn on downstream genes.³⁴⁻³⁸ In NIH3T3 cells, a constitutively active form of SMO activates the HH pathway regardless of *Gas1* over-expression or *Gas1* RNAi.³⁰ Conversely, a constitutively active form of PTC1 that cannot bind HH represses HH pathway activation but such repression can be overcome by *Gas1* overexpression plus SHH application, presumably by acting through the endogenous wild type PTC1 and titrating away the effects of the mutant PTC1. These data suggest that GAS1 acts at a level above SMO and at that of PTC1. Furthermore, cells coexpressing GAS1 and PTC1 display a SHH binding capacity that is greater than the combined binding capacity provided by cells expressing each protein individually. Thus, the HH enhancing activity of GAS1 may rest on its ability to facilitate loading of HH onto PTC1 thereby enhancing HH repression of PTC1 activity.

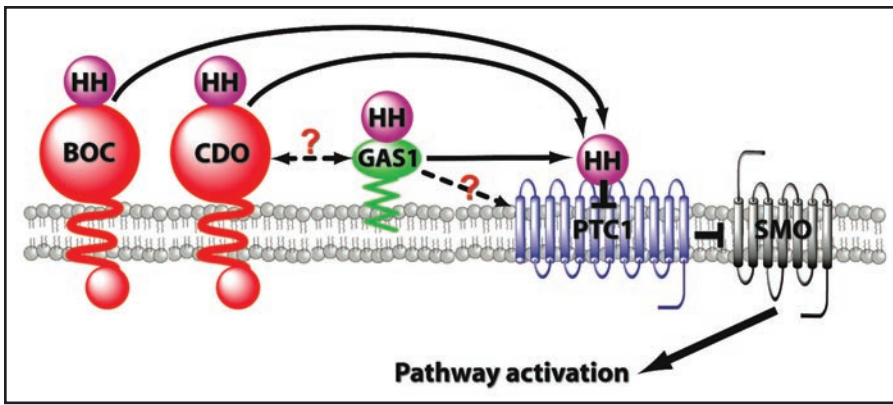


Figure 1. Diagram of cell surface HH signal transduction. GAS1, CDO, and BOC are all HH binding proteins. They appear to facilitate the binding of HH to PTC1, which in turn releases the inhibition of SMO and causes pathway activation. Whether GAS1 has any direct interaction with PTC1, CDO, or BOC is not yet known.

WHAT IS THE RELATIONSHIP BETWEEN GAS1, CDO AND BOC?

The activities described above for GAS1 are similar to those reported for the protein Interference Hedgehog (iHog), a transmembrane protein containing four Immunoglobulin (Ig) and two Fibronectin III (FNIII) repeats, and has been shown to be a co-receptor for the *Drosophila* Hh protein.³⁹ *ihog* mutant flies have general defects in Hh signaling, indicating its importance in Hh pathway activation. There is an iHog-related molecule called Brother of iHog (Boi) in the fly and their respective homologs in mammals are CDO and BOC (Fig. 1). Overexpression or RNAi of *Cdo* or *Boc* yielded similar results as to those obtained by manipulating *Gas1* levels,^{39,40} but mouse single mutants for *Cdo* and *Boc* showed specialized rather than general HH-related defects as found in the *Gas1* mutant. The *Cdo* mutant has HH-related defects in the CNS midline,⁴⁰⁻⁴² while the *Boc* mutant has a defect in SHH-directed commissural axonal guidance, but is otherwise normal.⁴³ Importantly, the CNS midline structure expresses *Cdo* but not *Boc*, while the commissural neurons express *Boc* but not *Cdo*. In other embryonic regions, *Cdo* and *Boc* expression are highly similar, suggesting compensatory roles.

If CDO and BOC function in a similar way as GAS1, why is then the *Cdo* and *Boc* mutant phenotype more limited than the *Gas1* mutant phenotype? The answer may be that the presence of the *Gas1* gene minimizes the *Cdo* and *Boc* single mutant phenotype. Not only do they have similar activities, but they also have a strikingly similar expression pattern during embryogenesis.^{23,44,45} Even more correlative is that all three genes are negatively regulated by HH signaling. Indeed, *Gas1;Cdo* compound mutant analysis revealed that they act in a dosage-dependent manner to mediate HH signaling in some (e.g., neural tube) but not in all tissues (e.g., limb).³¹ It was particularly interesting to learn that the double mutant's neural tube has no detectable HH signaling response, suggesting that *Gas1* and *Cdo*, though potentially redundant, are together indispensable for HH pathway activation in this context. For tissues that *Gas1* and *Cdo* do not show a genetic interaction, *Boc* may compensate. Examining phenotypes in various HH target tissues of *Boc;Cdo* and *Boc;Gas1* double mutants and eventually their triple mutant will be needed to resolve their contributions in each tissue context.

THE BIOCHEMICAL MECHANISM OF GAS1 ACTION

While we know that GAS1 helps cells expressing PTC1 to have a higher capacity for SHH binding, any further insight into the biochemical mechanism of GAS1 is not known (Fig. 1). There are several possible mechanisms for the nature of its action. GAS1 may bind to PTC1, thus forming a higher affinity receptor complex for HH binding. Alternatively, GAS1 may increase PTC1 surface presentation or alter PTC1 conformation, thereby allowing more surface binding of HH. A simpler possibility is that GAS1 acts as a net to capture HH from the extracellular space, thereby increasing the local concentration of HH and eventually transferring HH to PTC1. This model would be particularly applicable to cells that are located distant from

the HH source and thus have low levels of PTC1 and high levels of GAS1. Given the importance of primary cilia in HH signaling, it remains possible that GAS1 has a role in cilia form or function. Investigation of these different possibilities is currently underway in our laboratory.

FINE-TUNING THE HH MORPHOGENIC GRADIENT

HH is a classic morphogen that acts in a concentration-dependent manner to induce an array of cell types in a defined biological pattern of a given tissue. A particular range of HH concentrations will activate a specific HH target gene, but this range does not have infinitely precise boundaries. For cells located in these boundary regions, activation of the target gene may be stochastic because two similar HH concentrations may not be consistently interpreted as distinct (Fig. 2). Thus, the HH activity gradient must be steep enough so that these boundary regions represent a narrow portion of the patterning field, and the resulting border(s) of a given target gene expression domain is 'sharp'.

Current data support a model in which HH signaling down-regulates *Gas1* expression and creates a gradient of GAS1 that is countered to that of the HH signaling gradient.³⁰ This creates a situation where at any given point in the HH morphogenic field, *Gas1* is correspondingly down-regulated to a level appropriate for the required magnitude of HH signaling amplification. In the *Gas1* mutant, HH signaling suffers in range and amplitude as exemplified by the ventral shifts of the expression domains of NKX6.1 and DBX1, both HH target genes in the spinal cord. Re-examining the *Gas1* mutant spinal cord data,³⁰ we noticed a defect not previously stressed: the NKX6.1 and DBX1 domains have 'jagged' boundary regions (Fig. 2). This can be explained if the absence of GAS1 function causes the HH activity gradient to be more shallow or flattened when compared to the wild type. Such a shallow gradient causes an increase in the number of cells exposed to an ambiguous level of HH signaling in the boundary regions of the NKX6.1 and DBX1 domains, resulting in the jagged appearance (Fig. 2). Stated alternatively, by transforming the HH concentration gradient into graded activity thresholds, GAS1 helps two adjacent cells exposed to almost identical HH concentrations to each interpret the activity of HH input as distinct. The countered

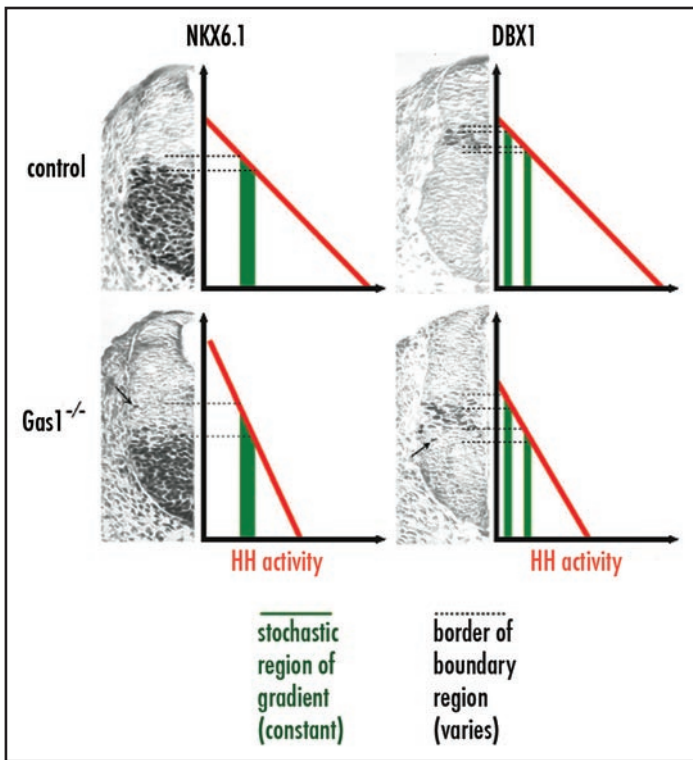


Figure 2. Inactivation of *Gas1* causes a flattened HH activity gradient, resulting in enlarged boundary regions for various HH target gene expression domains. Immunohistochemistry of E9.5 mouse spinal cords; genotypes labeled at left, and target genes analyzed at top. Dashed lines demark the borders at which the decision to activate the HH target gene appears definitive. Green area indicates the HH concentrations at which target gene activation appears stochastic, i.e., the “boundary regions”. Red lines are hypothetical HH activity gradients based on the locations of the borders for each gene. Pictures modified from previous publication (ref. 30).

gradient of GAS1 levels to that of HH diffusion may also help to sequester HH at the diffusion front where HH is low and GAS1 is high—a way to create an even sharper HH diffusion border.

How is *Gas1* repressed by HH? One possible candidate is Myc. There are lines of evidence supporting that HH induces N-Myc to promote cell proliferation.^{46–48} It happens that Myc can repress *Gas1*, and N-Myc and Myc are essentially interchangeable in their function.⁴⁹ This proposal places *Gas1* repression as a secondary event of HH signaling, contrasting to *Ptc1* up-regulation as a direct event. The timing delay between their regulation likely presents a window to fine-tune the HH response. Conversely, GAS1 is up-regulated by WNT proteins, also lipid-modified, secreted factors implicated in many developmental events and tumor types.^{27,28} The countering effects of WNT and HH signaling are well documented.^{50,51} It is tempting to speculate that GAS1 may be utilized by both pathways as a mediator to balance their influences.

HUMAN DISEASE

Decreased SHH signaling has been associated with holoprosencephaly in human and in mouse.⁵² *Gas1* or *Cdo* single mutant mice have microform holoprosencephaly, a phenotype that is further exacerbated in the *Shh*^{+/-} background.^{30,31,33,40–42} Are *Gas1* and *Cdo* modifier loci that contribute to the large spectrum of

holoprosencephaly in humans? Conversely, increased SHH signaling, either by reducing PTC1 activity or increasing SHH, SMO, or GLI activities, leads to several types of tumors such as basal cell carcinoma, medullablastoma and prostate cancer.^{34,53} Due to their signal enhancing function, increased GAS1, CDO, and possibly also BOC activity may also aid HH-stimulated tumorigenesis. However, it is possible that for cells that do not receive HH, they can act as a tumor suppressor independent of their role in the HH pathway. This would be in line with the originally ascribed function of *Gas1*, described above.

GAS1, CDO AND HH IN CELL CYCLE REGULATION

SHH acts on at least two points of the cell cycle by (1) upregulation of cyclin D1 and D2 (G_1 cyclins) and by (2) release of cyclin B1 from PTC1 to allow M progression.^{54–56} Do GAS1 and CDO modulate HH-induced cell cycle regulation or vice versa? Clues for GAS1's and CDO's parallel cell cycle function may be drawn from their regulated expression: *Cdo* was found as a gene to be down-regulated by oncogene expression in rat embryonic fibroblast,⁵⁷ while *Gas1*, upregulated by serum starvation of NIH3T3 cells.¹ *Gas1* is also downregulated in NIH3T3 cells transformed by the v-Src oncogene.⁹ Both of their expression is upregulated by cell confluency and down-regulated by serum application (unpublished). For NIH3T3 cells to respond to SHH, they need to be confluent,⁵⁸ a ‘quasi’- G_0 state that happens to also have high levels of GAS1 and CDO expression. Is this a simple coincidence? Or at G_0 , do GAS1 and CDO sensitize cells to be more responsive to HH as a way to jump start G_1/S phase re-entry? Conversely, may SHH's down-regulation of GAS1 and CDO be a prerequisite for the receiving cell to enter the S phase? Would their interplay influence a cell's decision to proliferate versus to differentiate? A direct study in the embryo to correlate GAS1 and CDO expression with the phases of the cell cycle on a single cell basis is incomplete. There is also no stringent study for a particular phase(s) of cell cycle that is permissive for cells to be HH-responsive. Investigation in these directions is likely to enrich our understanding of their action at the cellular level.

IS GAS1 A TRUE GFR α ?

As the GAS1-HH connection is entering center stage, another theoretical platform of GAS1 function is preparing to take off. Recently, bioinformatic investigation has predicted GAS1 to contain two cysteine-knot structural repeats similar to members of the glial cell-derived neurotrophic factor (GDNF) family receptor α (GFR α).^{59–62} There are four GFR α s, GFR α 1–4. They are co-receptors for the GDNF family of ligands to engage and activate a common signaling component, the membrane receptor tyrosine kinase RET. Based on the genomic structures of the GFR α s and *Gas1*, and *Gas1*'s lack on introns, the *Gas1* gene was likely created by retroposition.⁶² The existence of *Gas1*s only in vertebrate genomes perhaps helps to date this event.³⁰ Furthermore, over-expressed GAS1 can bind to and inhibit RET activation, suggesting its antagonistic role to all GFR α s and all GDNF-related ligands.⁶⁰ Puzzling is that none of the conserved amino acids among GFR α s proposed to contact RET are found in GAS1.^{62,63} The driving force for GAS1 to evolve as a RET and HH binding protein is ponderous, and whether GAS1 modulates these two signaling pathways simultaneously, sequentially, or independently is worthy of exploration.

STEM CELL BIOLOGY

In the postnatal brain, *Gas1* is expressed in the dentate gyrus and rostral migratory stream (RMS), where brain stem cells can be found.²³ Perhaps *Gas1* regulates their cell cycle exit or entry there. This proposal has strong potential as SHH signaling has been shown to operate in these self-renewing neuronal stem cells and their transient amplifying progeny.⁶⁴ Compared to fast dividing embryonic cells, brain stem cells may proliferate slow enough to allow investigation of the in vivo role of GAS1 in cell cycle control. Other adult tissues may also utilize GAS1-HH circuitry to regulate stem cell renewal, perhaps even tumor stem cells. There is tantalizing evidence that GDNF acts as a chemoattractant for the RMS.⁶⁵ Does GAS1 juggle between SHH and GDNF signaling there to coordinate the proliferation vs. migration of newly generated neurons? It is certainly possible that GAS1 utilizes a different pathway here beyond what we can predict from current data.

FINAL WORDS

The finding of GAS1's direct connection to the HH pathway promotes GAS1 from a cell cycle regulator to an intercellular signaling modulator. Its potential role in regulating GDNF signaling broadens the scope of its action in this regard. With an available animal model, new gene family assignment, and a variety of new assays and tools, the stage is set for investigation of the leads linking *Gas1* as an integrator of growth factor(s), controlled cell cycle progression, cell type specification, and even stem cell biology.

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