The Sonic Hedgehog Signaling System as a Bistable Genetic Switch

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ABSTRACT Sonic hedgehog (Shh) controls critical cellular decisions between distinct fates in many systems, particularly in stem cells. The Shh network functions as a genetic switch, and we have theoretically and computationally analyzed how its structure can endow it with the ability to switch fate choices at a threshold Shh concentration. The network is composed of a positive transcriptional feedback loop embedded within a negative signaling feedback loop. Specifically, positive feedback by the transcription factor Gli, which upregulates its own expression, leads to a switch that can adopt two distinct states as a function of Shh. However, Gli also upregulates the signaling repressor Patched, negative feedback that reins in the strong Gli autoregulatory loop. Mutations that have been associated with cancer are predicted to yield an irreversible switch to a high Gli state. Finally, stochastic simulation reveals the negative Patched feedback loop serves a critical function of dampening Gli fluctuations to reduce spontaneous state switching and preserve the network's robust, switch-like behavior. Tightly linked positive and negative feedback loops are present in many signaling systems, and the Shh system is therefore likely representative of a large set of gene regulation networks that control stem cell fate throughout development and into adulthood.

INTRODUCTION

Throughout development and adulthood, cells are exposed to complex regulatory signals and must correctly interpret these signals to implement necessary functional decisions. Signal transduction and gene regulation cascades are therefore information processing mechanisms that translate extracellular information into intracellular decisions. In many cases, cells are exposed to a variable or graded concentration of an external signal, and at key intermediate levels, they switch between two alternate behaviors or fates in an ''all-or-none'' fashion. Important examples of these alternate decisions include cell survival versus apoptosis, stem cell proliferation versus differentiation (Lillien, 1995; Marshall, 1995; Weissman et al., 2001), chemoattraction versus chemorepulsion (Song and Poo, 2001), and the numerous critical cell fate choices that stem cells face either individually or as part of an integrated tissue patterning process during development (Wagers et al., 2002; Weissman et al., 2001). Analyzing the signal transduction and gene regulation mechanisms that mediate such biological or genetic switches can therefore yield insights into numerous cellular processes.

Drosophila hedgehog and Sonic hedgehog (Shh), one of its three mammalian homologs, are canonical, secreted signaling factors that regulate cell function and fate in numerous systems. Among its many roles during development, Sonic hedgehog patterns spinal cord and limb bud tissue differentiation and controls midbrain and ventral forebrain neuronal differentiation (Ericson et al., 1995; Hynes et al., 1995; Jessell and Lumsden, 1997; Ruiz i Altaba et al., 2002a). Importantly, Shh can pattern tissue during development by forming a concentration gradient, a phenomenon best characterized in the neural tube and spinal cord (Ericson et al., 1995, 1996), and cells sense their position within the gradient and differentiate into distinct cell phenotypes as a function of the concentration.

In addition to patterning cell differentiation, Shh also controls the proliferation of numerous cell populations during development, including cerebellar granule cells and retinal progenitors (Ruiz i Altaba et al., 2002a; Wechsler-Reya and Scott, 1999). Beyond its roles in development, we recently found that Shh also stimulates neural progenitor cell proliferation in the adult brain, and therefore regulates the process of adult neurogenesis in the hippocampus (Lai et al., 2003). In addition, similar to other signaling systems that control cell proliferation, mutations in the Shh system have been associated with cancer in numerous tissues (Ruiz i Altaba et al., 2002b). In different contexts, therefore, the Shh signaling system functions as a modular circuit that can manage or be tasked to different cellular decisions. Very importantly, Shh flips cells between alternate functional states at key threshold concentrations. However, the properties of the Shh gene regulatory network that allow it to function as a switch during cell fate determination, and to malfunction during disease, have not been quantitatively analyzed.

Shh transduces its signal to cells by interacting with its 12-pass transmembrane receptor Patched (Ptc) (reviewed in Ho and Scott, 2002, and Ruiz i Altaba et al., 2002a). In the absence of Shh, Ptc represses the signaling activity of Smoothened (Smo), a seven-pass transmembrane protein, and therefore acts as a repressor of Shh signaling (Fig. 1 *A*). Binding of Shh to Ptc releases its repression on Smo, which then transduces a signal by activating members of the Gli family of transcription factors. In the absence of a Smo signal, Gli3 is constitutively cleaved to generate a transcription factor (Gli3R in Fig. 1 *A*) that represses expression of Shh targets. Activation of Smo reduces the rate of Gli3

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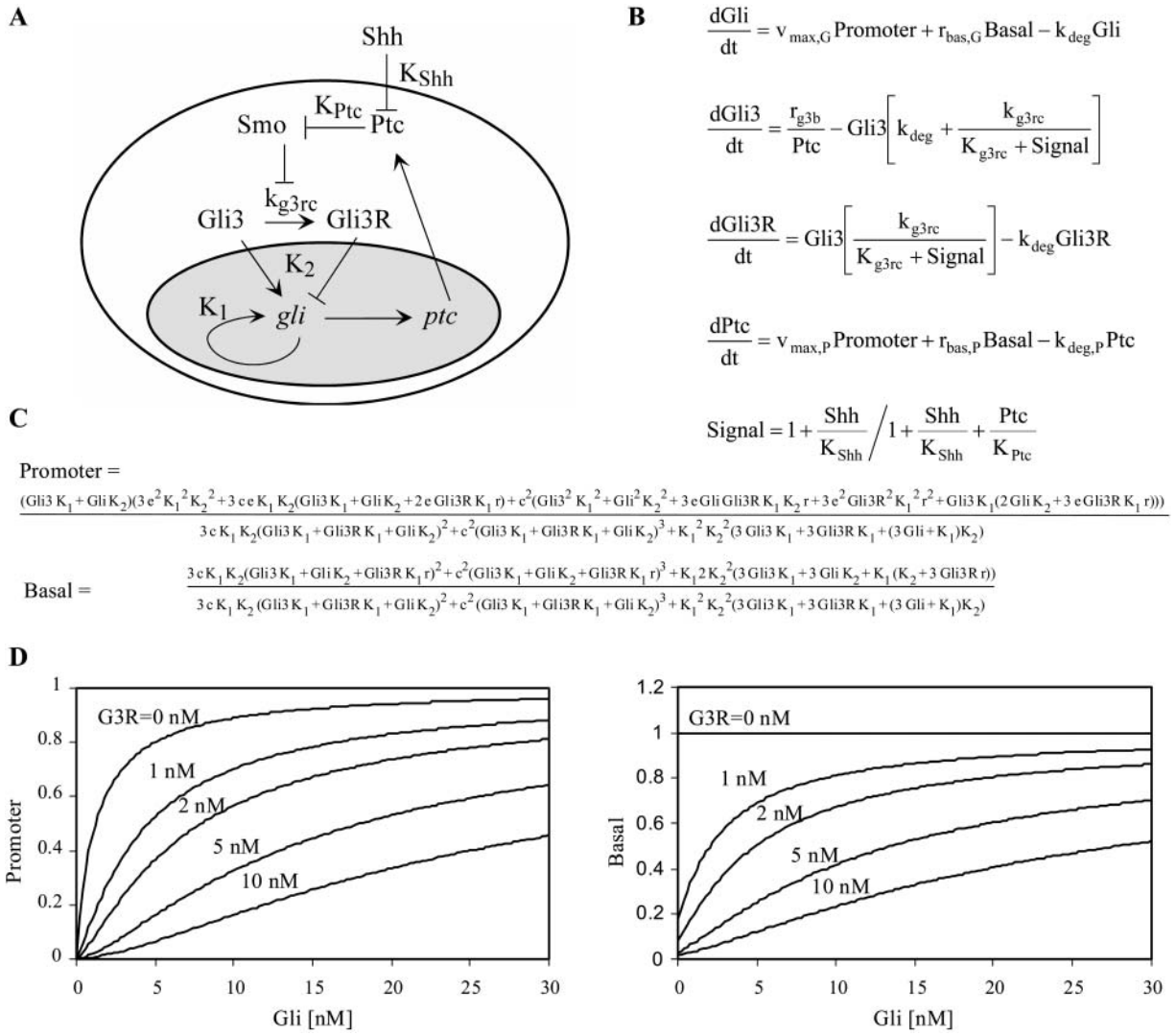

FIGURE 1 (*A*) Schematic of the Shh signaling system, where Shh binding to its receptor Ptc releases Ptc suppression of the activity of the coreceptor Smo. Smo signaling then inhibits the conversion of the transcription factor Gli3 from an activator to a repressor form. The subsequent accumulation of the Gli3 activator activates the genes *gli* and *ptc*. Gli1 binds to and transcriptionally activates its own promoter, as well as that of the signal repressor Ptc. (*B*) Differential equations that describe the rate of change of the network components depicted in the schematic. (*C*) Promoter and Basal functions. (*D*) These functions, which are ratios of polynomials, vary between 0 and 1 and describe how the inducible and basal activities of the *gli* and *ptc* promoters vary functionally with the concentrations of the three transcription factors that bind them: Gli3, Gli3R, and Gli. The sigmoidal curves of Promoter and Basal are shown as a function of Gli (with Gli3 = 0) at different levels of the repressor Gli3R.

cleavage (Wang et al., 2000), however, and the resulting full-length Gli3 then binds to consensus sites within and thereby activates the promoters of a number of Shh targets. Among these are *ptc*, *shh*, *gli1*, and *gli2*. In addition, *gli3* transcription is repressed. Upon expression, Gli1 and Gli2 act as transcriptional activators with somewhat overlapping functions. They bind to the same consensus sites as Gli3 (TGGGTGGTC) and therefore exert positive feedback on their own expression, as well as activate a number of other targets to mediate the downstream cell regulatory effects of Shh signaling. Genes that positively regulate their own expression can be found in a large number of systems (Aota et al., 2003; Ebert et al., 2003; Kuziora and McGinnis, 1988; Murphy and Reiner, 2002), and Gli therefore represents a general autoregulatory motif. Analyzing the properties of autoregulatory developmental transcription factors controlled by extracellular signals is therefore of broad interest. However, the Shh system also exhibits negative feedback, as Gli transcriptionally activates the signal repressor Ptc (Ho and Scott, 2002; Jessell and Lumsden, 1997; Ruiz i Altaba et al., 2002a,b).

The dynamic behavior of this complex signaling and gene regulatory network is not intuitively evident, and a quantitative, systems biology approach can significantly aid in analyzing its signal processing dynamics (Hasty et al., 2001; Rao et al., 2002). Simple models of autoregulatory transcription

factor systems exhibit bistable behavior (Lewis et al., 1977; Savageau, 1974). We have built upon this work and apply deterministic and stochastic modeling to demonstrate that the Sonic hedgehog regulatory network functions as a bistable genetic switch, a property that likely underlies its ability to correctly flip cell fates at precise, threshold Shh concentrations. Furthermore, the network is composed of a positive feedback loop embedded within a negative feedback loop, and this structure has likely evolved to endow the system with several crucial properties. Specifically, the Shh network architecture makes it relatively insensitive to changes in the values of some but not all of its rate constants, as well as to biological noise or fluctuations in the concentrations of its constituent proteins. However, several genetic mutations in the network have been associated with Shh-related cancers (Ruiz i Altaba et al., 2002b). Intriguingly, changing the values of network kinetic constants to model these mutations ''breaks'' the switch, so that the system continually exists in an ''On'' state to potentially initiate the process of cell transformation and tumorigenesis.

MODEL DEVELOPMENT AND RESULTS

The Shh signaling network (Fig. 1) can be represented as a set of differential equations that track the rates of change in the concentrations of the network constituents, and whose individual terms represent rates of protein synthesis and degradation (derived in Supplemental Material). For example, in the first equation for Gli (Fig. 1 *B*), the first term represents the rate of induced synthesis due to *gli* promoter activation and protein translation (derived in Supplemental Material), the second term is the rate of basal synthesis or leakiness from the promoter, and the final term is Gli protein degradation. For simplicity, because their activities appear to be somewhat overlapping (Bai et al., 2002; Bai and Joyner, 2001), the effects of both the Gli1 and Gli2 transcriptional activators are initially lumped into a single term, Gli. Gli and Ptc transcriptional repression and activation are represented within the expressions Promoter and Basal, which are ratios of polynomials and are sigmoidal similar to a Hill function (Fig. 1, *C* and *D*). These terms describe the inducible and basal rates of synthesis due to the presence of multiple Gli binding sites within the *gli1*, *gli2*, and *ptc* promoters (Dai et al., 1999; Marigo and Tabin, 1996; Ruiz i Altaba, 1998). mRNA dynamics are not independently tracked, and both transcription and translation are lumped into the synthesis rates (see Supplemental Material for details of this approximation). Next, it has been experimentally observed that Shh signaling represses Gli3 expression (Wang et al., 2000). We have therefore made Gli3 synthesis inversely related to Ptc levels (Fig. 1 *B*), a nonmechanistic relationship that can be revised in the future as the biology is further elucidated. Finally, the Shh concentration is incorporated into the term Signal in the Gli3 equation, and it acts by reducing the proteolysis of Gli3 into the transcriptional repressor Gli3R.

Kinetic and binding parameter values were either directly taken from literature or estimated based upon analogous biological systems (Table 1 and Supplemental Materials). The equations were initially numerically integrated using Berkeley Madonna software (www.berkeleymadonna.com) to track the system behavior as the Shh concentration is changed, and since Gli1 and Gli2 are key effectors of downstream cellular responses to the Shh signal (Bai et al., 2002; Bai and Joyner, 2001; Ruiz i Altaba 1998), we will report the Gli concentration as the most relevant and important output of the system. Furthermore, since a range of equilibrium dissociation binding constants have been reported for Shh binding to Ptc varying from 0.5 to 2 nM (Fuse et al., 1999; Taipale et al., 2002), the Shh concentration

TABLE 1 Parameter values

Parameter	Description	Value/range
K_1, K_2 (Mizugishi et al., 2001)	Dissociation constant of Gli1 and Gli3 for Gli DNA binding site, respectively	8.3×10^{-10} M
k_{deg} (Chen et al., 1999)	Degradation rate constant for all Gli variants	0.009 min^{-1}
K_{Shh} (Fuse et al., 1999; Taipale et al., 2002)	Dissociation constant for Shh-Ptc binding	0.58–2.0 nM
K_{Ptc} (Taipale et al., 2002)	Half-maximal concentration of Ptc which inhibits Smo signaling	8.3×10^{-11} M
c (Keller, 1995)	Binding cooperativity	1
ε (Keller, 1995)	Transcriptional efficiency	0.5
r	Transcriptional repression	0.2
$k_{deg,\ P}$ (French and Lauffenburger, 1996)	Degradation rate constant for Ptc	0.09 min^{-1}
$v_{max,G}$	Maximum rate of Gli synthesis	2.4×10^{-10} M/min
$r_{bas,G}$	Basal rate of Gli synthesis	$v_{max,G}/100$
r_{g3b}	Basal rate of Gli3 synthesis	1.6×10^{-19} M^2/min
K_{g3rc}	Sensitivity constant of the conversion to signal strength	0.1
k_{g3rc}	Rate constant for the conversion of Gli3 to Gli3R	0.012 min^{-1}
$v_{max,P}$	Maximum rate of Ptc synthesis	7.5×10^{-10} M/min
$r_{bas,P}$	Basal rate of Ptc synthesis	$v_{max,P}/100$

Values initially used for the computations in Fig. 2, *A* and *B*. Parameter sensitivity analysis was subsequently performed for all parameters.

will be reported as its ratio to the Shh dissociation binding constant (Shh/K_{Shh}). Fig. 2 *A* demonstrates that at a low Shh level, the Gli concentration likewise settles to a low value. However, when at $t = 0.5$ h the Shh level is increased to a concentration 15-fold higher than its Ptc binding dissociation constant, Gli rapidly increases to a new, 23-fold higher steady-state value. The system therefore appears to behave as a switch. That is, at low Shh and Gli concentrations, an ''Off'' state is maintained because Gli transcription continually occurs at a low rate. However, as Shh is increased above some threshold concentration, it stimulates Gli production to the point where Gli positively feeds back upon its own expression and rapidly drives the state of the network ''On.'' Positive Gli feedback therefore generates a switch.

Parameter sensitivity/bifurcation analysis

To examine this switchlike behavior in greater detail, we performed parameter sensitivity analysis to determine how the steady-state Gli concentrations changed as parameter values were varied. Specifically, bifurcation analysis was performed (using XppAuto) first to investigate how steady-state Gli concentrations shifted as a function of Shh. Fig. 2 *B* demonstrates that the system exhibits hysteresis, i.e., Gli switches from a low/Off to a high/On state at a sharp threshold Shh concentration, and the switching point differs depending upon whether Shh is being increased or reduced. Hysteresis, which has been experimentally observed in two important systems (Bagowski and Ferrell, 2001; Pomerening et al., 2003), serves two important functions: it provides an unambiguous threshold switching mechanism, and it acts to filter noise from a system (below). Important early theoretical work demonstrated that hypothetical autoregulatory transcription factors could function as hysteretic switches (Savageau, 1974), but the Shh network imposes the additional intricacies of complex regulation by an external signal, multiple transcription factors, and negative feedback via Ptc.

It has been observed that certain mutations or gene amplifications in Shh network components underlie disease. For example, *gli1* gene amplification, inactivating Ptc mutations, mutations leading to constitutively active Smo, and Gli3 truncations have all been associated with cancer (Ruiz i Altaba et al., 2002b). These mutations can be mathematically represented as an increase in the *gli* promoter strength ($v_{max,G}$ in Table 1), a decrease in the ability of Ptc to inhibit Smo (lower $1/K_{ptc}$), or an increase in the Gli3 dissociation constant for its target DNA binding site (K_2). We utilized bifurcation analysis to examine the system sensitivity to these parameters. Fig. 2 *C* depicts how $v_{max,G}$ interacts with Shh to modulate the bifurcation points at which Gli switches between two steady-state values, represented by the dark and light curves. The data in Fig. 2 *B*,

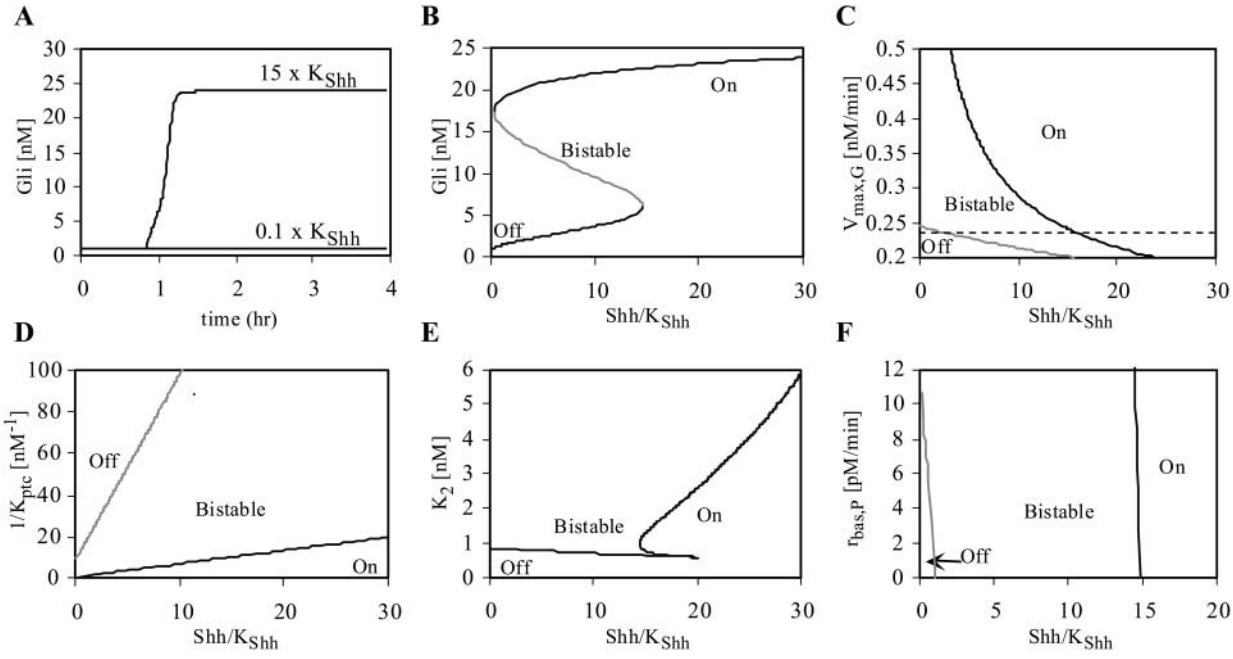

FIGURE 2 (*A*) Gli trajectories as a function of time at Shh concentrations equal to 0.1 or 15 times its binding constant to Ptc. For the upper curve, the concentration was initially low but was then increased at 0.5 h. (*B*) Hysteresis in the Shh network, where the network output Gli can attain two possible steady states for an intermediate range of Shh concentration. The point at which switching occurs depends upon whether Shh is increasing or decreasing. (*C*) Bifurcation, or parameter sensitivity, analysis of how the switching points vary when the *gli* promoter strength, denoted by $v_{max,G}$, is changed. A very small change in this parameter has major effects on the Shh levels at which switching occurs. (*D*) Bifurcation analysis of how the switching points vary with $1/K_{Ptc}$, the potency of Ptc inhibition of Smo. (*E*) Bifurcation analysis of K_2, the binding affinity of Gli3 for its DNA site, shows that the system is also highly sensitive to this parameter. (*F*) By contrast, the system or the values of Shh at which switching occurs are not as sensitive to parameters such as $r_{bas,P}$, the basal rate of Ptc expression.

calculated for $v_{max,G} = 0.24$ nM/min, are represented by the horizontal dashed line in Fig. 2 *C*. As Shh is increased from a low initial concentration, Gli stays in the lower steady state until it reaches the dark bifurcation curve, where it switches to its upper state. To switch back to the lower state along the $v_{max,G} = 0.24$ nM/min line, however, Shh must be reduced until the system hits the lighter curve. If the *gli1* gene were amplified, $v_{max,G}$ would be increased, and at a critical $v_{max,G}$ value, the light curve intersects with the axis. Biologically speaking, this result indicates that when the switch is turned On, it cannot return to the Off state. Therefore, if *gli1* is amplified in a cell where Shh signaling regulates cell proliferation, this irreversible or broken switch would attempt to continually drive mitosis and could initiate the path to cell transformation and cancer.

Next, the potency of Ptc repression of Smo activity is represented by $1/K_{ptc}$, where K_{ptc} is the active Ptc concentration required for half maximal inhibition. Fig. 2 *A* was generated for a $1/K_{ptc}$ value of 12 nM^{-1}. For lower values of Ptc potency $1/K_{ptc}$, the light curve in Fig. 2 *D*, which represents the Shh concentration at which the system turns from On to Off as Shh is reduced, ends. Therefore, Ptc and Smo mutations can also break the switch and initiate cell transformation. In addition to the key parameters $v_{max,G}$ and $1/K_{ptc}$, sensitivity analysis revealed that the affinities of Gli and Gli3 for their DNA consensus binding site were an important determinant of system behavior. For example, Fig. 2 *E* shows that even a slight increase in the dissociation constant K_2, i.e., weaker binding of the repressor Gli3R, can also yield an irreversible switch. This result is consistent with clinical observations that some cancers are caused by Gli3 mutations that result in a truncated protein (Ruiz i Altaba et al., 2002b).

To quantify the system sensitivity in more detail, the linear slopes of the left bifurcation curves in Fig. 2, *C–F*, the points at which the system switches from On to Off, were evaluated at the parameter values utilized in Fig. 2, *A* and *B*. It was found that at this point a 10% change in the value of $v_{max,G}$ resulted in a >1000% change in the value of Shh at which On to Off switching occurs, indicating that the system is exceedingly sensitive to this parameter. By comparison, a 10% change in K_2 and $1/K_{ptc}$ results in moderate 60% and 32% changes, respectively, in the Shh level at which switching occurs. However, the system was less sensitive to the values of all other parameters. For example, the switching points do not shift significantly with $r_{bas,P}$, the parameter that describes the basal or leaky expression of the *ptc* promoter in the absence of transcription factor binding (Fig. 2 *F*). Specifically, a 10% change in this parameter yields only a 2.4% change in the Shh switching point.

Shh network sensitivity to biological noise

This deterministic analysis successfully demonstrates how the steady-state values of the bistable system shift as parameter values change. However, numerous studies have revealed the importance of stochastic or probabilistic effects in biological systems where the number of molecules is low enough for noise to be important, and the deterministic chemical kinetic descriptions are limited (Hasty et al., 2001; Rao et al., 2002). To examine whether stochastic fluctuations in the value of Gli and other network components can significantly alter the switch's behavior, with potentially important implications for stem cell fate choice, we implemented the Gillespie stochastic simulation algorithm (Gillespie, 1977) in C++ to simulate the individual molecular reactions of Fig. 1. Fig. 3 *A* shows several Gli time trajectories. At the low initial Shh concentration (1 nM), Gli fluctuates around a mean value due to network noise. At 33 h, the Shh concentration was increased to 14.3 nM, a value just below the concentration at which deterministic analysis predicts the system would switch (Fig. 2 *B*). Stochastic analysis reveals that at this point noise can cause the system to spontaneously switch states, consistent with the observation in other physical systems that noise can induce spontaneous switching in a bistable system (reviewed in Hangii et al. (1990)). One Gli concentration trajectory stays at the low steady state, whereas two others achieve a sufficiently high concentration to slip away from the lower concentration and flip to the higher state at random times (Fig. 3 *A*). That is, noise can undermine the Shh genetic switch by causing spontaneous leaking between states at random times, a result previously observed, for example, in a simple gene network (Hasty et al., 2000) and in the lambda phage lysis/lysogeny switch (Arkin et al., 1998).

To further explore the biological implications of this result in the Shh network and in stem cell fate choice, we analyzed the first pass time (FPT) or the time interval at which the system initially passes from the lower to the upper state. This is an important quantity, as a genetic switch must be able to execute a decision in response to an extracellular stimulus within a biologically relevant duration. The average FPT for 20 simulation runs was computed for a range of Shh concentrations (Fig. 3 *B*). At high Shh concentrations predicted to flip cells to the upper Gli state (Fig. 2 *B*), the FPT was low (~30 h). By contrast, at very low concentrations, the FPT was sufficiently high that the circuit would stay in the Off state for an extremely long period of time (>300 h), indicating that for timescales over which Shh would be acting upon cells (such as spinal cord patterning (Ericson et al., 1996)), they would remain Shh nonresponsive. At intermediate concentrations, where Fig. 3 *A* showed that noise-induced switching would occur, the FPT increases very sharply as Shh concentration decreases. Therefore, although noise can induce a degree of spontaneous switching, it may occur within a sufficiently narrow range of Shh concentrations that the sharp transition in cell state as a function of increasing Shh concentration could be maintained.

To further explore the sharpness of this transition, the average value of Gli at each concentration was averaged over five simulations (each run for 200 h of simulated time). Fig. 3

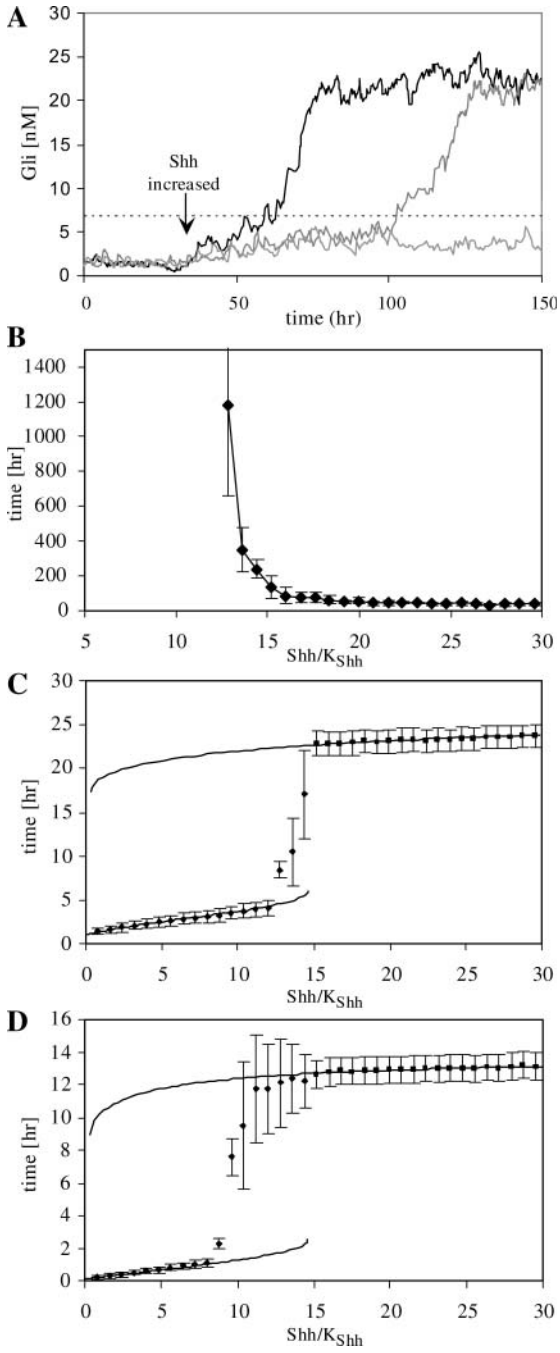

FIGURE 3 (*A*) Stochastic simulations of Gli trajectories just below the Shh concentration at which the system would deterministically switch from Off to On. When the Gli concentration reaches a threshold value (the unstable, intermediate state in the hysteresis curve of Fig. 2 *B*, denoted by the *dotted line*), spontaneous switching occurs. However, one trajectory does not ever switch during the simulation time. (*B*) The first passage time (FPT) for passage from the Off to the On state as a function of Shh. The mean and standard deviation of 20 simulations are plotted. (*C*) Stochastic simulations of Gli as a function of Shh. Each point represents the mean and standard deviation of five simulations, each run for 200 h of simulated time. The underlying solid curves are the stable steady states of the deterministic solution from Fig. 2 *B*. (*D*) Stochastic simulations of Gli as a function of Shh when Ptc is held constant, eliminating the negative feedback loop. The underlying solid curves are the stable steady states of the corresponding deterministic solution for fixed Ptc. Despite the fact that the Shh concentration ranges over which bistability is deterministically predicted are similar to those in Fig. 3 *C*, stochastic switching occurs at a much lower Shh level.

C overlays the Gli average and standard deviations for these stochastic simulations on top of the deterministic solution from Fig. 2 *B*. For the majority of the concentration range, the mean of the stochastic solutions precisely matches the deterministic values, though the error bars indicate that Gli continually fluctuates within a narrow concentration range. At an intermediate Shh concentration near the switching point, however, noise-induced flipping yields a large error in the value of Gli, since some trajectories within this range flip On while others remain in the Off state (Fig. 2 *A*). However, because this uncertainty occurs only within a narrow concentration range, the overall sharp, robust nature of the switch is preserved.

Positive Gli autoregulation enables a bistable switch, but positive feedback also amplifies noise within a system. Negative feedback (in the form of Gli upregulation of the signal suppressor Ptc, Fig. 1 *A*) could potentially serve the role of counteracting this noise amplification. To test this possibility, simulations were performed in the absence of negative feedback, that is, at a constant Ptc concentration not regulated by Gli. One result of this modification was that the value of the Gli promoter strength $v_{max,G}$ had to be reduced nearly twofold to prevent the system from existing in a continually high Gli concentration state, since removing the negative feedback favors the On state. However, the second result was that in the absence of this outer negative feedback loop, the system was far noisier. In contrast to Fig. 3 *C*, there were significant fluctuations in Gli concentration and uncertainty in the cell state over a wide Shh concentration range (Fig. 3 *D*), despite the fact that the Shh concentration ranges over which bistability is deterministically predicted were very similar. This result indicates that negative feedback reduces system noise and enables a robust transition in cell state within a narrow Shh signal concentration range near the deterministically predicted switching point.

DISCUSSION

The Shh network functions as a stem cell fate switch in many contexts, from the developing spinal cord (Jessell and Lumsden, 1997) to the adult hippocampus (Lai et al., 2003). It is therefore effective as a model system to study the structure of regulatory networks controlling cell fate choices. In many systems, particularly for tissue patterning, Shh switches the cell state at a critical threshold level (Ericson et al., 1995; Hynes et al., 1995; Jessell and Lumsden, 1997; Ruiz i Altaba et al., 2002a; Wechsler-Reya and Scott, 1999); however, the properties of the Shh network that endow it

with this capability have not been quantitatively analyzed. We have examined the Shh signaling and gene regulation system using both deterministic and stochastic descriptions, to take into account the low numbers of key molecules present in many biological systems.

This first detailed model of the Shh signaling network reveals that the transcription factor Gli is the heart of the network. That is, its autoregulatory positive transcriptional feedback allows the system to function as a hysteretic switch, with either a low basal Gli expression level or a fully induced expression state. In addition, the extracellular Shh concentration controls switching between these two states, i.e., the network converts the analog Shh input into a binary system output to control ''all-or-none'' stem cell functional choices. Also, hysteresis provides an unambiguous switch-like mechanism insensitive to small fluctuations in the level of external signal once the state switch has occurred (Rao et al., 2002). Furthermore, hysteresis has been experimentally observed in the JNK signaling cascade and in Cdc2 cell cycle regulation, indicating that it may be a broadly important phenomenon in the control of cell function (Bagowski and Ferrell, 2001; Pomerening et al., 2003).

Hysteresis in the Shh network is maintained despite variation in the values of numerous parameters, including promoter basal expression rates, Shh binding affinity, and the Gli3 proteolysis rate. By contrast, the network is highly sensitive to the values of several key parameters, including the Gli expression rate ($v_{max,G}$) and the potency of Ptc inhibition of Smo activity ($1/K_{ptc}$) (Fig. 2). Specifically, an increase in $v_{max,G}$ or decrease in $1/K_{ptc}$ leads to an irreversible switch, where once the Shh signaling network is turned On, it is incapable of accessing the Off state again. This result is particularly interesting since in tissues where Shh regulates mitosis, *gli1* amplification, mutations leading to constitutively active Smo, and inactivating Ptc mutations have been implicated in cancer, an irreversible state of cell proliferation.

In addition to correlating well with experimental data, the model can make several new predictions. First, the switch behavior is also highly sensitive to the DNA binding affinities of Gli and Gli3, such that increasing Gli DNA affinity or decreasing Gli3 binding also ''breaks'' the switch. This necessity for carefully balanced Gli and Gli3 affinity is interesting, particularly in light of the fact that the DNA binding domains of Gli1, 2, and 3 are highly conserved and share 88% amino acid identity (Lee et al., 1997), suggesting that the relative affinities of the three domains are similar. Gli3 mutations that yield a truncated protein have been associated with cancer (Ruiz i Altaba et al., 2002b). However, no *gli* point mutations have yet been observed to be associated with cancer, and this analysis suggests that such mutations may be revealed in the future. Furthermore, such sensitivity analysis of how the Shh switch can malfunction may elucidate which network locus to target/inhibit to maximize cancer therapy efficacy. The parameter that most sensitively affects system stability is the *gli1* promoter strength, so dominant negative Gli1 or RNA interference directed against Gli1 mRNA would be predicted to have the most therapeutic efficacy for cancer therapy.

A nanomolar Gli concentration range translates into hundreds to thousands of Gli molecules per cell, a level where random fluctuations in gene and protein expression can make system noise significant. In situations where cell fate must be precisely controlled, such as in Shh regulation of stem cell function and tissue patterning (Jessell and Lumsden, 1997; Lai et al., 2003), cells have likely evolved mechanisms to neutralize noise and uncertainty in signaling networks (Rao et al., 2002). The Shh network is composed of a positive Gli transcriptional feedback loop embedded within a negative Ptc signaling loop. In the absence of the negative feedback, the system is extremely noisy, since small initial fluctuations in Gli are amplified by the positive feedback, leading to uncertainty and randomness in the Shh concentration and the time at which switching between states occurs (Fig. 3 *D*). However, the recursive loop structure with external negative feedback acts to dampen Gli fluctuations to reduce system noise and lead to a more ''deterministic'' switch in cell state within a narrow Shh concentration range (Fig. 3, *B* and *C*).

This result is consistent with experimental observations of cell fate patterning by a Shh concentration gradient in the developing neural tube (Ericson et al., 1995, 1996). High Shh concentrations induce expression of the transcription factors Isl1/Isl2 and a subsequent motor neuron fate, whereas lower levels stimulate Lim1/Lim2 expression and an interneuron fate choice. Intriguingly, just at the interface between these two domains, i.e., at the switching point, a number of cells were observed to be positive for both Isl1/Isl2 and Lim1/Lim2 (Fig. 7 in Ericson et al., 1996). This is consistent with the hypothesis that these cells began to commit to Lim expression and an interneuron fate, but stochastic switching in Gli expression to an On state (as in Fig. 3 *A*) subsequently pushed the cells to the motor neuron fate midway through their fate decision. Coexpression of these two transcription factors is not observed at later time points, indicating that the cells finally did commit to a single fate, or were eliminated. At any rate, this result indicates that the interface may be noisy, but that the structure of the Shh network is able to confine that noise to a narrow Shh concentration window and maintain a sharp boundary between the two cell fate domains.

Therefore, the Ptc feedback serves several potential purposes. It makes the system less sensitive to the potent transcription factor Gli (Fig. 2 *D*), and it buffers the system to noise (Fig. 3). A third possible function of Ptc is that its upregulation may play a role in modulating Shh transport through tissue during the establishment of a Shh gradient (Chen and Struhl, 1996).

It has been shown in yeast that a minimal, synthetic, positive autoregulatory system based on the engineered tetracycline transactivator yields a bistable system (Becskei

et al., 2001). Furthermore, computational work indicates that noise can induce random switching between the two states in such simple positive feedback loops (Hasty et al., 2000) and in fate choices such as the role of lambda repressor in the lambda bacteriophage lysis/lysogeny decision (Arkin et al., 1998). By contrast, negative feedback has long been recognized in control theory for its ability to stabilize systems (Morari and Zafiriou, 1997), and experimental analysis in bacteria of a synthetic repressive autoregultory transcription factor has confirmed that negative feedback in a system acts to dampen noise (Becskei and Serrano, 2000). It is therefore intriguing that the Shh network has evolved and exploited positive feedback to create a switch and negative feedback to dampen noise and thereby maintain the robust properties of the switch.

This Shh network structure is representative of a large class of signaling systems that control fate decisions. Positive autoregulatory transcription factors are ubiquitous in biology in systems that control critical cell fate choices, from stem cell fate determination and tissue patterning in *Drosophila* development (Kuziora and McGinnis, 1988) to the GATA transcription factors in hematopoietic stem and progenitor cell differentiation (Murphy and Reiner, 2002). Interestingly, there are also ample examples of gene regulatory and signaling systems in which there is a complex interplay between positive and negative transcriptional feedback loops, similar to the architecture of the Shh system. For example, in a system crucial to the development of the eye and nervous system, Pax6 is autoregulatory, upregulates a second transcription factor, Six3, and is itself upregulated by Six3 (Aota et al., 2003; Goudreau et al., 2002). However, Six3 also negatively regulates its own transcription (Lengler and Graw, 2001). As a second example, the ubiquitous cell fate regulator TGF-β1 upregulates its own expression via the AP-1 transcription factor (Kim et al., 1990), but negatively autoregulates itself via the repressive transcription factor Smad6 (Cutroneo and Phan, 2003). As a final example, BMPs activate Mash1, an autoregulatory, proneural bHLH transcription factor crucial for neural development. However, BMP signaling also activates Zic1, a transcriptional repressor of Mash1 (Ebert et al., 2003). Shh is therefore representative of a common network motif, and future dynamic analysis of other systems may elucidate similarities and differences in the evolved design principles between these key developmental control networks, particularly in how the interplay between positive and negative feedback yields robust switches.

Stem cells are faced with a number of critical fate decisions throughout their lives, including proliferation versus differentiation as well as the many choices faced during lineage commitment. The life of a stem cell can therefore be viewed as a branching decision process controlled by a series of genetic switches. Therefore, stem cell control can be achieved by providing the correct signals as a function of time to flip fate switches and guide the cells down a particular developmental trajectory. Shh can serve as one of these switches that can, in different contexts, be tasked to control different stem cell decisions, including both self-renewal (Lai et al., 2003) and lineage commitment (Jessell and Lumsden, 1997). Therefore, computational analysis of such regulatory switches can aid both basic and applied efforts to understand the mechanisms of stem cell fate choices, as well as to elucidate the extent to which regulatory network noise could in some situations impose inherent limitations on the ability to deterministically or precisely control stem cell fate in vitro or in vivo.

A number of simplifying assumptions were made in this analysis (see Supplemental Materials). For example, the proteins that interact with and tether Gli3 in the cytosol (Fused, SuFu, and Costal-2) are not explicitly incorporated into the model (Ruiz i Altaba et al., 2002a), but are implicitly accounted for in the release and activity of full-length Gli3 upon Shh signaling. We also assume that on the timescales of protein synthesis, rapid intracellular transport eliminates protein concentration gradients, an assumption that could be relaxed to consider separate nuclear and cytoplasmic compartments. Moreover, we represent the repression of Gli3 synthesis by Shh signaling as an inverse relationship between Ptc and Gli3 synthesis (Fig. 1 *B*), a formulation that can be revised as the mechanism for this repression is elucidated. In addition, there is evidence that Shh is transcriptionally upregulated upon Shh signaling (Epstein et al., 1999), but incorporating Shh upregulation and transport into a three-dimensional model of Shh signaling during tissue patterning reveals that it actually assists in inducing sharp spatial regulation of cell state (unpublished).

Furthermore, this analysis indicates the network can specify up to two cell fates, but limb and spinal cord tissue patterning involve multiple cell types. The incorporation of gradients in other factors, such as BMPs in the spinal cord (Jessell and Lumsden, 1997), may provide the additional spatial information necessary to induce multiple cell fates. For example, BMPs may push Shh signaling and the Gli concentration down to zero, yielding a three-state cell differentiation switch similar to the manner in which each of three concentrations of the transcription factor Oct3/4 specify a different embryonic stem cell fate (Niwa et al., 2000). It will also be interesting to analyze whether the BMP signaling system also behaves as a binary switch. Finally, including additional factors, such as tracking both mRNA and protein synthesis, separately analyzing Gli1 and Gli2 (Ruiz i Altaba et al., 2002a), and inclusion of receptor-ligand trafficking dynamics (Denef et al., 2000), could shift the values of kinetic parameters for which switchlike behavior is observed, but would not affect the fundamental conclusions of this work.

In summary, we have computationally and theoretically analyzed the Shh network, a crucial signaling system that controls cell survival, proliferation, and differentiation in different contexts, such as the eye, skin, heart, spinal cord, and nervous system. We have found that the network

architecture, composed of recursive positive transcriptional and negative signaling feedback loops, endows this network with the ability to function as a bistable cell fate switch despite many potential mutations as well as biological noise. However, the system behaves as an irreversible or broken switch when the values of several key parameters are changed even modestly, changes that would correspond to mutations and gene amplifications that underlie genetic disease and cancer. This work illustrates that theoretical analysis is a powerful tool to understand the functions, and malfunctions, of highly interconnected genetic networks that control and execute stem cell behavior.

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