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Morphogen gradient reconstitution reveals Hedgehog pathway design principles

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Abstract

In developing tissues, cells estimate their spatial position by sensing graded concentrations of diffusible signaling proteins called morphogens. Morphogen-sensing pathways exhibit diverse molecular architectures, whose roles in controlling patterning dynamics and precision remain unclear. Here, combining cell-based in vitro gradient reconstitution, genetic re-wiring, and mathematical modeling, we systematically analyzed the unique architectural features of the Sonic Hedgehog pathway. The combination of double-negative regulatory logic and negative feedback through the PTCH receptor accelerates gradient formation and improves robustness to variation in the morphogen production rate compared to alternative designs. The ability to isolate morphogen patterning from concurrent developmental processes, and to compare the patterning behaviors of alternative, re-wired, pathway architectures offers a powerful way to understand and engineer multicellular patterning.

During development and regeneration, tissue patterning unfolds with astonishing precision in space and time. Diffusible signaling molecules known as morphogens provide a key patterning mechanism. Morphogens are secreted by sender cells and form concentration gradients that are interpreted by cognate signaling pathways in receiver cells to generate distinct cell fate domains (1). Morphogen-sensing pathways have diverse regulatory architectures that actively process intracellular signals and modulate the abundance of extracellular morphogens (2, 3). However, the roles of these features in pattern formation generally remain unclear. To address this question, we developed a system to reconstitute patterning in cell culture, and used it to directly compare the patterning behaviors of natural and re-wired morphogen pathways (Fig. 1A).

We focused on the Hedgehog (HH) pathway, a classic long-range morphogen system that is implicated in developmental diseases and cancer (4). Unlike other morphogen pathways, in

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which ligands positively activate their receptors, the HH pathway uses a unique “double-negative” activation mechanism (Fig. 1B): unliganded PTCH receptors suppress intracellular pathway activity, and this suppression is relieved by HH ligand binding. Furthermore, by sequestering HH, PTCH receptors also modulate the ligand’s extracellular spatial distribution. The combination of these intracellular and extracellular activities makes PTCH effectively bifunctional (5). Finally, HH signaling up-regulates PTCH expression, generating negative feedback through both inhibition of intracellular signaling and sequestration of extracellular ligands (6–8). Despite much work, the functional rationale for this pathway architecture largely remains obscure.

We reconstituted HH signaling gradients in NIH3T3 cells, which transduce HH signals without differentiating and do not naturally express HH ligands (9). We generated an inducible sender line by placing the wild-type Sonic Hedgehog (Shh) coding sequence under the control of a 4-hydroxyta-moxifen (4-OHT) inducible system (Fig. 1C). We also engineered a receiver cell line containing a synthetic construct in which GBS (GLI binding site) sequences recognized by the downstream transcription factor GLI drive the expression of nuclear-localized H2B-Citrine fluorescent protein (10,11). Reporter expression responded to SHH in a dose-dependent manner, and correlated with endogenous SHH pathway targets (fig. S1). Diluting sender cells in a 1000-fold excess of receivers produced radial gradients of SHH signaling extending from single sender cells, reaching a limiting size of ~4–5 cell diameters (80–100 μm) over a timespan of 60 hours (Fig. 1D). Additionally, to mimic quasi-one-dimensional contexts, such as limb buds, neural tubes and *Drosophila* wing discs (12), we used a cell culture insert system to plate senders and receivers in adjacent but contiguous regions (Fig. 1E). In this configuration, gradients extended over ~200 μm . These results show that SHH signaling gradients, with sizes comparable to naturally-occurring gradients, can be generated by co-culturing synthetic senders and receivers in vitro.

In principle, SHH gradients could form through diffusion of ligands in the liquid medium or through lateral movement of ligands within the cell layer (as by diffusion in extracellular matrix, or transport along filopodia) (13–16). To distinguish between these two possibilities, we analyzed gradient formation on a laboratory rocker, which should disrupt media-based gradients. Strikingly, rocking had no effect on gradients (Fig. 1F). In a second experiment, we cultured senders and receivers on coverslip fragments separated by a 30 μm gap. This gap, which is much shorter than the gradient length, was sufficient to prevent activation of receivers by senders (Fig. 1G). By contrast, when the coverslips were directly adjacent (no gap), the gradient formed normally. Both experiments indicate that, under these conditions, the SHH gradient forms predominantly through movement within the cell layer, requiring cell-cell contact or continuous extracellular matrix (17). In addition, we note that here cell migration and division occur at low rates that have minimal effects on gradient formation, but could play more substantial roles in natural contexts (fig. S2, B to D).

The ability to reconstitute gradient formation in cell culture enabled us to explore the functional implications of the SHH pathway architecture. We first focused on the unusual double negative logic of the core pathway (Fig. 1B) by eliminating feedback on PTCH (table S1) to create a simpler, “open loop” receiver cell line (Fig. 2A). We knocked out both endogenous *Ptchl* alleles, and integrated a copy of *Ptchl* under a Doxycycline (Dox)-

inducible promoter (fig. S3). Co-culturing this open loop cell line adjacent to sender cells enabled us to observe the dynamics of open loop gradient formation across a matrix of SHH and PTCH expression levels (Fig. 2, B to D; fig. S4, A and B; movies S1 and S2). Analysis of the resulting movies revealed that elevating the SHH production rate increased gradient amplitude and extended the lengthscale, while elevating the PTCH production rate had the opposite effect (Fig. 2D and fig. S4C). In fact, gradient lengthscale and amplitude specifically depended on the ratio of SHH and PTCH production rates (Fig. 2E and fig. S4D).

To understand this ratiometric behavior, we constructed a minimal mathematical model of the core pathway (fig. S5A). This model assumes that free PTCH promotes production of the repressor form of GLI (GLIR), which in turn inhibits target gene expression. Binding of PTCH to HH inactivates both proteins, decreasing GLIR production. We fit the model to experimental data for a range of SHH production rates at a single PTCH level (fig. S5, B to D, and table S2) (17). The model recapitulates ratiometric lengthscale and amplitude control across a broad range of PTCH expression levels (Fig. 2F and fig. S5E). Further analysis reveals that double negative regulatory logic is sufficient for ratiometric control (17). Incorporating additional features of the natural pathway, such as the activating form of GLI, preserves this behavior (fig. S6) (18). Together, these results revealed that control of gradient properties is shared by both senders (through the SHH production rate) and receivers (through the PTCH production rate) in the open loop configuration.

Ratiometric control provides robustness to correlated changes in SHH and PTCH expression that preserve their ratio, but implies sensitivity to perturbations in these parameters individually. Deletion of one SHH allele has no obvious phenotype in mice (19). While it is not known whether gene dosage directly affects the level of secreted SHH, this nevertheless suggests that the system may have intrinsic mechanisms to buffer variations in the morphogen production rate.

We hypothesized that negative feedback resulting from the highly conserved, SHH-dependent up-regulation of PTCH expression could provide robustness to SHH expression level (20–22). To represent this feedback in the model, we introduced a GLI-dependent PTCH production term, with a single new parameter for feedback strength, defined as the amount of PTCH expression for a given level of signaling (Fig. 3A and fig. S7). In simulations, PTCH feedback had three effects. First, it enhanced the robustness of gradient amplitude and lengthscale to variations in ligand production rate (Fig. 3B). Second, it accelerated the approach to steady state (Fig. 3C). Third, it preserves the relatively linear gradient shape as HH production rate increases, while the open loop gradients become increasingly plateau-like (Fig. 3, D and E). Linear profiles have been suggested to maximize the extent of the ‘useful’ region for patterning multiple cell fate domains (23).

To understand what features of PTCH produce these advantageous effects, we considered alternative feedback schemes mediated by hypothetical PTCH-like proteins possessing subsets of its features (Fig. 3A and fig. S7, A and B). Feedback through a protein, denoted I, possessing only PTCH’s intracellular signal inhibition activity provided amplitude robustness but exacerbated lengthscale sensitivity (Fig. 3B and figs. S7, C and D, and S8).

On the other hand, feedback through a protein, denoted E, possessing only PTCH's ligand-binding activity provided lengthscale but not amplitude robustness [consistent with studies of self-enhanced ligand degradation (21)]. Furthermore, simply co-expressing I and E together in a single "uncoupled" model was not sufficient to reproduce the benefits of PTCH feedback (Fig. 3, B to E, and fig. S7, C and D). A model in which I and E are physically tethered, but where the intracellular activity persists even when the extracellular domain is bound to ligand, also does not provide PTCH-like robustness (fig. S9D). Thus, the bifunctional nature of PTCH — specifically, its ability to switch between intracellular inhibition (ligand-free) and extracellular sequestration (ligand-bound) states — is essential for the robustness provided by this feedback mechanism. Interestingly, the coupling of multiple functions in the same protein has similarly been shown to promote robustness in other biological contexts (24).

The differences between PTCH and uncoupled feedbacks can be understood in terms of their divergent responses to high ligand concentrations (fig. S8). In both models, high ligand levels can deplete the extracellular feedback component (E or PTCH). With PTCH feedback, this simultaneously reduces the intracellular activity, which in turn activates the pathway, replenishing PTCH and thereby continuing to limit ligand penetration. By contrast, in the uncoupled model, depletion of E does not directly reduce I; this results in a disproportionate accumulation of I, which blocks replenishment of E by suppressing signaling. In this way, the uncoupled model fails to keep up with increasing ligand expression levels. The benefit of PTCH feedback compared to uncoupled feedback persists even if one independently fine-tunes the I and E feedback strengths (fig. S9, A to C). These qualitative differences among feedback models are preserved in models that incorporate the activator form of GLI, positive feedback on GLI expression, or temporal adaptation through GLI downregulation (figs. S6 and S10 to S12).

To experimentally test the prediction that PTCH feedback improves the speed and robustness of gradient formation, we designed a synthetic feedback (SynFB) pathway in which SHH signaling up-regulates PTCH1 expression (Fig. 4A). We placed the Tet3G activator under SHH signaling control using an additional copy of the GBS promoter (Fig. 1C and fig. S1A). The SynFB design mimics the natural PTCH1 feedback, but allows continuous modulation of feedback strength through Dox concentration, from an open loop regime to a strong closed loop regime exceeding the feedback strength of wild-type 3T3 cells (fig. S13, A to C). Movies of gradient formation in the SynFB cell line revealed that PTCH feedback accelerated the approach to steady state, made both the amplitude and lengthscale of the signaling gradient less sensitive to variations in ligand production rates, and improved the linearity of the gradient (Fig. 4, B to D; fig. S13, D to F; movies S3 to S6). Furthermore, the magnitude of improvement increased with the strength of the feedback (fig. S13D). These results are consistent with model predictions.

As a further test of the model, we constructed a cell line incorporating a synthetic intracellular feedback (Fig. 4E). We substituted a PTCH mutant, PTCH1^{Loop2}, which is unable to bind ligand, for wild-type PTCH1 in SynFB (25) (Fig. 4E and fig. S14). As predicted by the model, these cells produced gradients whose amplitudes were more robust to ligand production rate, and whose lengthscales and shapes were more sensitive (Fig. 4E

and fig. S13F). Together, these results demonstrate that PTCH feedback has the unique capability of both reducing gradient sensitivity to variations in ligand production rates and accelerating the approach to steady state, providing a functional rationale for this highly conserved feature of the HH pathway (6–8).

Spatial patterning is an active process in which the dynamics of the morphogen and those of its signaling pathway are intertwined. Compared to analysis of embryos, reconstitution of morphogen gradient formation in vitro provides several advantages: It avoids interference from other processes and pathways (isolation), permits quantitative control of key parameters, allows rewiring of regulatory interactions, and facilitates straightforward analysis of patterning dynamics in space and time. In this case, reconstitution revealed how the unique combination of features in the HH pathway together provide a compact, elegant design solution to the challenge of rapidly generating robust gradients (Fig. 4F and fig. S9C). Future work should help to extend the bottom-up approach developed here to more complex phenomena by incorporating downstream signal interpretation circuits (26) and integrating with additional, concurrent, morphogenetic patterning processes (27).

The HH architecture strikingly contrasts with that of other morphogen pathways, such as BMP or FGF, in which ligands activate receptors, which in turn activate intracellular effectors. These “double-positive” architectures should display a different dependence on ligand and receptor levels (17). Compared to HH, receptor feedback appears to be less pervasive in these systems, provoking the question of whether they possess alternative mechanisms to achieve similar patterning capabilities, or whether they are optimized for distinct spatiotemporal behaviors (28). The approaches developed here should provide general insights into the performance tradeoffs among different morphogen systems, and establish a platform for designing synthetic circuits that genetically program cells to self-pattern into spatially organized tissues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. In vitro reconstitution of morphogen signaling gradients.

(A) Reconstitution enables quantitative analysis of the spatio-temporal patterning dynamics, including lengthscale (ruler) and speed (clock), generated by natural morphogen pathways, as well as minimal and re-wired variants. The sensitivity of circuit variants to perturbations, such as changes in ligand production, can also be determined. (B) Unique combination of architectural features in the Hedgehog (HH) pathway. (C) Sender and receiver cell lines for reconstituting SHH signaling gradient in wild-type NIH3T3 cells. Senders constitutively expressing GAL4 fused to a mutant estrogen receptor (ERT2) and mTurquoise2 (mTurq2) fused to Histone 2B (*H2B*) produce SHH upon induction with 4-hydroxytamoxifen (4-OHT). An 8xGLI binding sequence (GBS) driving H2B-Citrine expression reports pathway activity in receivers. (D and E) Reconstituting SHH signaling gradients in radial and linear geometries. Blue and yellow cells (schematic) represent senders and receivers, respectively. Arrows indicate the direction of gradient propagation. In the radial gradients (D, $n = 13$), all activation was due to a single sender cell (blue, near center of dashed circle that indicates

the gradient outer edge). In the linear gradients (E, $n = 7$), the white dashed line indicates the boundary between sending and receiving fields. (F and G) Ligand transport requires continuous cell or extracellular matrix contact. In principle, ligand transport could involve bulk diffusion through the medium (upper schematic) or lateral movement within the cell layer (lower schematic). Gradient formation is unaffected by rocking that should disturb bulk diffusion (F, $n = 8$), and is blocked by a 30 μm gap between senders and receivers (G, $n = 5$).

Fig. 2. Open loop SHH pathway architecture produces gradients sensitive to variations in key parameters.

(A) Engineering open loop receiver cells. Both *Ptch1* alleles in wild-type receivers were deleted and replaced by ectopic *Ptch1* under Tet-3G control, enabling graded tuning of PTCH1 abundance with Doxycycline (Dox), indicated by coexpression of mCherry (mChr). (B) Time-lapse images of representative radial and linear SHH signaling gradients. (C) Quantifying spatio-temporal dynamics of linear signaling gradients. Total fluorescence (upper plot) reflects the time-integrated pathway activity (mean of $n = 8$). The time derivative of Citrine (lower plot) approximates instantaneous pathway activity over space and time (fig. S1C). (D) Signaling gradient sensitivity to variations in SHH and PTCH1 production rates (α_{HH} and α_{PTC} , respectively). α_{HH} was increased by varying the sender density (upper panel), whereas α_{PTC} was increased in the receivers by varying the Dox concentration (lower panel). (E) The ratio of α_{HH} and α_{PTC} determines gradient lengthscale, defined by the distance at which the signal drops to 1/e of the amplitude. The α_{HH}/α_{PTC} ratio also controls gradient amplitude, defined by the signaling strength in the cells closest to the boundary (fig. S4D). (F) A simple model recapitulates the ratiometric dependence of gradient properties on α_{HH} and α_{PTC} (see also fig. S5E).

Fig. 3. Mathematical modeling shows that PTCH feedback improves patterning performance by physically coupling intracellular and extracellular activities.

(A) Negative feedback can act intracellularly by inhibiting signaling (IC feedback) or extracellularly by sequestering ligand (EC feedback). These functionalities can coexist, implemented either through separate molecules (uncoupled feedback) or in a bifunctional molecule like PTCH (PTCH feedback). (B) Steady-state gradient length and amplitude as a function of α_{hh} (marker size) for different models. The feedback strengths for the IC and EC models were finetuned so that the amplitude or lengthscale, respectively, matches that of PTCH feedback at relative $\alpha_{hh} = 0.0625$. Those same feedback strengths were used for the uncoupled model, but the qualitative differences between those models hold across all nonzero feedback strengths (figs. S7, C and D, and S9, A and B). Panels C-E use the same feedback strengths. (C) Time to reach steady state (τ) for each model as a function of α_{hh} and λ_{50} , the position at which steady-state signal activity equals 50% of the amplitude. τ is the first timepoint at which signal activity reaches 90% of its steady-state value at λ_{50} (schematic). (D) Amplitude-normalized signaling gradient profiles for open loop, uncoupled, and PTCH feedback models at different relative values α_{hh} (0.0625, 0.25, 0.50, and 1.0) show distinct trends in lengthscale and shape. (E) PTCH feedback uniquely maintains a constant gradient shape with increasing α_{hh} . The shape factor θ equals the ratio of the width

of the second third of the gradient (L_2) to the width of the first third of the gradient (L_1) (schematic).

Fig. 4. PTCH feedback simultaneously improves gradient speed and robustness.

(A) A synthetic PTCH1 feedback loop (red), whose strength is tunable with Dox, was introduced in *Ptch1*^{-/-} receiver cells to generate the PTCH1-SynFB cell line. At 0 ng/ml Dox, the basal activity of the *TRE* promoter produces sufficient PTCH1 to suppress pathway activity in the absence of SHH. (B) Temporal evolution of PTCH1-SynFB signaling gradients (yellow) with (20 ng/ml Dox) or without (0 ng/ml Dox) PTCH1 feedback. Dotted white line represents the sender-receiver boundary. Note that sender cells (blue) remain throughout experiment but are visually obscured by increasing Citrine expression. (C) PTCH1-SynFB accelerates the approach to steady state at λ_{50} (defined in Fig. 3C). (D) Profile of PTCH1-SynFB signaling gradients, with (right, 20 ng/ml Dox) or without (left, 0 ng/ml Dox) PTCH1 feedback, at 42.5 hours after 100 nM 4-OHT induction. Gradient profiles are normalized to their own amplitudes to show differences in lengthscale (distance

at which the dotted line is crossed) and shape. Bar plots show amplitudes (mean $\pm$ s.e.m., $n = 7$ each). (E) A SynFB circuit was introduced in wild-type receiver cells to generate the PTCH1-ALoop2-SynFB cell line (left). PTCH1- Loop2 lacks the HH binding domain, but has the same capability as PTCH to suppress intracellular signaling (fig. S14). This IC feedback circuit enables robust gradient amplitude at the cost of greatly flattened shape and exacerbated lengthscale sensitivity to α_{hh} (right). (F) Summary of the performance of different feedback architectures (simulation results). The unique, conserved architectural features of the HH pathway combine to enhance speed and robustness of signaling gradient formation. Performance is measured relative to that of the open loop model at relative $\alpha_{hh} = 0.25$, which has a value of 1 in each dimension (see fig. S9C for plots at other α_{hh} values).