Orchestrating ontogenesis: variations on a theme by sonic hedgehog

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Abstract | Embryonic development is an emergent process in which increasing complexity is generated by sequential cellular interactions. Recently, it has become clear that such interactions are mediated by just a few families of signalling molecules; but how does this limited repertoire elicit the diversity of form that is characteristic of multicellular organisms? Here we review the various ways in which a member of one such family, the sonic hedgehog (SHH) protein, is deployed during embryonic development. These examples of SHH function provide paradigms for inductive interactions that should help to inform attempts to recapitulate cellular programming and organogenesis *in vitro*.

Glia
A non-neuronal cell in the CNS. The three types of glia are astrocytes, oligodendrocytes and microglia.

The central challenge in unravelling the molecular and cellular basis of embryonic development is not simply to elucidate the ways in which the diversity of cell types that characterizes an adult organism is generated, but rather to understand how assemblies of differentiated cells are elaborated in their correct spatial arrangements and proportions. For instance, how does an embryo generate the precise spatial arrays of various neurons and glia that are essential for the establishment of functional neural networks? What underlies the specification and patterned distribution of the different types of muscle fibre that are crucial to normal locomotion? How are the digits on our hands and feet specified, and how is the length of the limbs on which they form determined? Remarkably, in all these processes, the same signalling molecule, sonic hedgehog (SHH), has been found to have a principal role, raising a host of questions about the basis of the versatility of such signals. How can the reiterative deployment of a single signalling pathway elicit numerous and diverse outcomes? How can the activity of a signal be harnessed to ensure that cells are produced in the correct temporal and spatial sequence? And how can the same signal coordinate growth with pattern formation?

Studies of SHH signalling over the past 10 years have started to reveal the diverse and intricate ways in which the embryo uses its limited signalling repertoire. In some instances, the distance of a responding cell from the source of an inducing signal is of crucial importance to its specification, whereas, in others, it is the duration of exposure to the signal that determines cell fate. Cells can move into or out of range of the signal as development proceeds, or can differ in their response to the signal as a function of either developmental time or location (FIG. 1). Each of these principles is well exemplified by SHH signalling.

Mechanisms of HH signalling

Although they are notably absent from nematode worms, HH family proteins are widely distributed throughout much of the animal kingdom. They were first discovered in *Drosophila melanogaster,* in which mutation of the single *Hh* gene that is present in this species gives rise to an embryo that is covered in spikey cuticular processes called denticles, inspiring the 'hedgehog' name. In vertebrates, genome duplication has given rise to multiple *Hh* genes: there are three mammalian *Hh* genes and five in zebrafish, the most well studied in both cases being *Shh*. The core components of the HH-family signalling pathway have been highly conserved through evolution. Our understanding of this pathway comes mainly from genetic analyses in *D. melanogaster*, and it has been extensively reviewed[1–3]: a summary overview of the *D. melanogaster* HH pathway is presented in BOX 1. One key feature of the HH proteins is their lipid modification, a property that has an important effect on the propagation of signalling activity from expressing cells[4–7]. Lipid modified HH molecules require the activity of a highly conserved transporter-like protein, named Dispatched, for their efficient secretion from cells[8–10]. Once secreted, the lipid modification influences the movement of HH molecules between cells, a movement that is modulated by interaction with proteoglycans and, at least in *D. melanogaster*, with the secreted protein Shifted[11–13].

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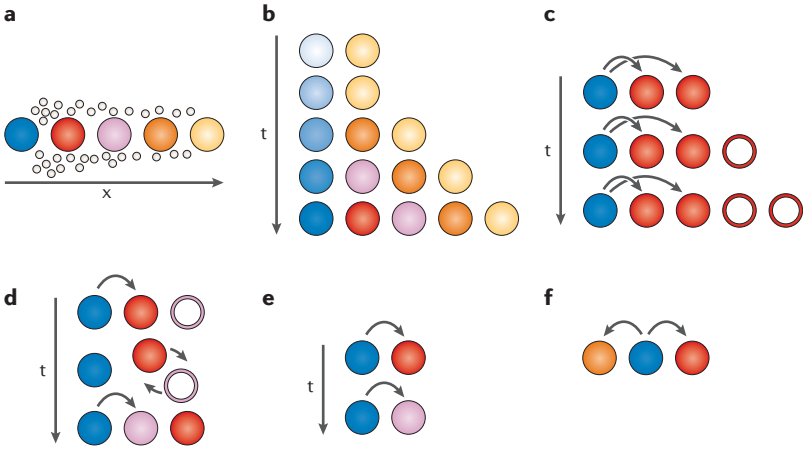


Figure 1 | **Signals can specify cell fate in different ways.** Blue circles represent the signal-secreting cells; other circles represent the responding cells. In **a**, the secreting cell establishes a spatially graded distribution of signal across the adjacent field of cells. Each cell adopts a different identity (indicated by different colours) in response to different levels of the signal. This is the classical morphogen paradigm. In **b**, cell number increases vertically with time. As the secreting cell divides, one of its progeny stops expressing the signal and adopts an identity that is determined by the length of time for which the parental cell has expressed the signal. The integration of signal level over time is represented by the increase in the shading intensity of the secreting cell. Note that the sequence of different cell identities that are generated in this way is the same as in **a**. In **c**, only two different identities are specified in response to the signal (arrows): initially, all cells respond to the signal (red circles), but as the cellular field expands through proliferation, some cells move out of range of the signal and adopt a different identity (white circles). In **d**, the signal acts over a short range to induce two different cell identities; this is accomplished by the movement of one cell away from the source, allowing a second cell that has a different potential or competence to be exposed to the same signal. In **e**, the competence of cells to respond to the same signal changes over time. In **f**, cells in different locations respond differently to the same signal.

Recent studies have revealed that HH proteins bind directly to two related and conserved transmembrane proteins, known as Interference hedgehog (IHOG) and Brother of IHOG (BOI) in *D. melanogaster* and CAM-related 1 downregulated by oncogenes (CDO) and brother of CDO (BOC) in vertebrates[14–16]. This binding facilitates the interaction between HH proteins and the multipass-transmembrane protein Patched[17,18] (PTC in flies and fish, PTCH in mammals), an interaction that is key to the activation of the transmembrane protein Smoothened (SMO), the universal transducer of HH activity[18–20]. The exact mechanism of PTC inhibition of SMO remains unclear, although recent evidence suggests the involvement of PTC-dependent secretion of pro-vitamin D3 (REF. 21). SMO activation initiates an intracellular pathway that culminates in the transcriptional activation of HH target genes (BOX 1). Whereas in *D. melanogaster*, this is mediated by a single member of the Glioma-associated oncogene homologue (GLI) family of transcription factors[22], in vertebrates, three distinct GLI proteins are involved in the transcriptional response to SHH (and other HH proteins[23]). Two of these, GLI2 and GLI3, are structurally similar to the *D. melanogaster* Cubitus interruptus (CI) protein, possessing both repressor and activator domains that flank a DNA-binding domain. In the absence of SHH signalling, GLI3, like CI, undergoes proteolytic cleavage to yield a truncated protein that functions as a repressor of SHH target gene transcription[24]. GLI2 can also be processed to a repressor form, although much of the protein seems to be degraded in cells that do not receive SHH signals[25]. Activation of the pathway results in the suppression of cleavage and/or degradation and the nuclear import of full-length GLI2 and GLI3 proteins. These activate the expression of target genes, while additional target genes become derepressed owing to the depletion of the repressor forms of each protein. By contrast, the GLI1 protein lacks the repressor domain and seems to enhance the activating function of GLI2. Indeed, unlike the other two Gli genes, *Gli1* is itself a target of SHH signalling, which is consistent with it being part of a positive-feedback loop[26].

Multipass-transmembrane protein
An integral membrane protein that spans the membrane more than once and has multiple internal sequences.

Regulating identity and proliferation

During their development, embryos have to accomplish two key tasks: first, they must undergo extensive proliferation and generate pools of progenitor cells that derive from the fertilized egg; and second, they must instruct cells to exit these pools at the appropriate times and places to differentiate into the arrays of specific cell types that characterize different organs. Studies of SHH function in a number of organisms have shown that it has crucial roles in both of these processes.

SHH functions as a long-range morphogen to specify neuronal identity. One of the best known roles of SHH is as a morphogen — a secreted signal that can specify multiple cell identities as a function of its concentration (FIG. 1a). The long-range morphogen-like behaviour of SHH has been especially well described within the vertebrate neural tube where it has a pivotal role in the generation of the diverse array of neuronal subtypes that are required for the assembly and function of neuronal circuits[27].

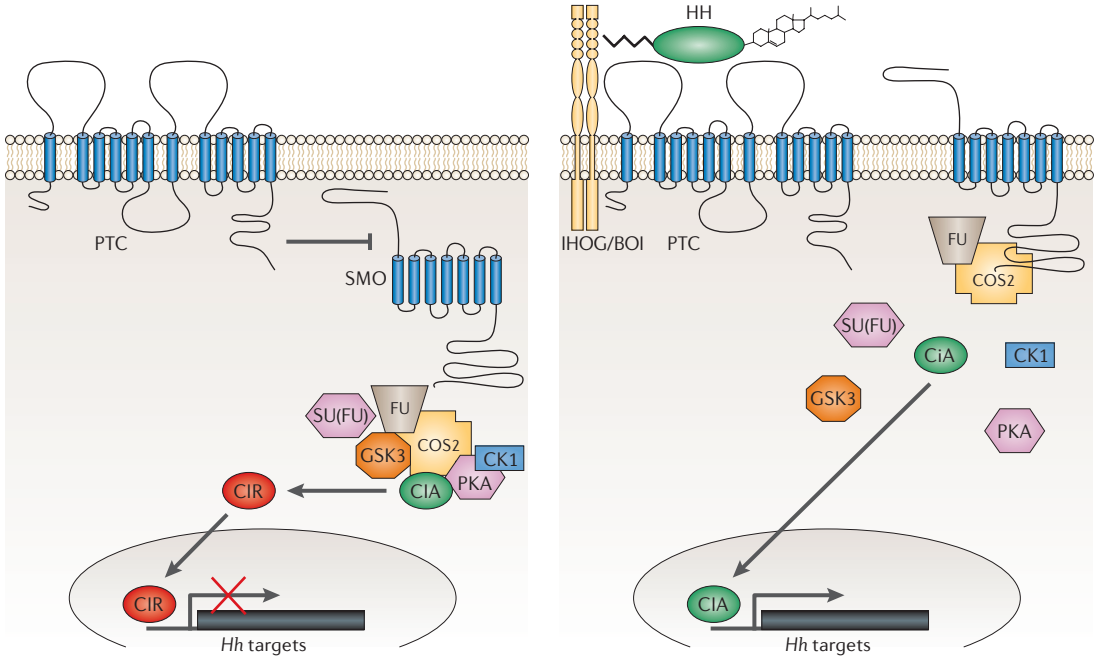
Transcription of SHH is initially confined to the notochord, a rod-like structure that underlies the ventral neural tube, but is rapidly induced within a discrete triangular wedge of cells that is located at the ventral midline of the neural tube, known as the floor plate[28–30]. Whereas *Shh* mRNA is highly localized to these two midline structures, the protein it encodes shows a more widespread and graded distribution, as is expected of a long-range signalling molecule[31]. The first evidence in support of a morphogen-like role for SHH came from *in vitro* studies of explanted chick neural-plate tissue; these showed that incremental two- to threefold changes in SHH concentration, when applied to this tissue, are sufficient to generate distinct neural subtypes characteristic of the ventral neural tube[27,32] (FIG. 2). Various subsequent studies have confirmed that this concentration-dependent activity of SHH operates *in vivo* to organize the formation of discrete cell types. A focal source of ectopically expressed SHH, for instance, can organize the surrounding tissues in the CNS in a manner that is consistent with its concentration-dependent activity[33,34]. And crucially, manipulating the activity of the SHH signal-transduction pathway in cells that are distant from the source of SHH results in cell-autonomous transformations of neuronal fates, indicating that the protein functions directly and at long-range to induce cell identity[35–37].

Serpentine protein
A protein that spans the membrane seven times.

Graded SHH activity establishes distinct domains of homeobox gene expression along the dorsoventral axis of the neural tube[38]. These fall into two distinct classes: genes that encode class I proteins are repressed by SHH activity, whereas genes that encode class II proteins are activated by SHH signalling. Cross-repressive interactions between class I and class II proteins refine and maintain these domains, sharpening their boundaries to define distinct progenitor pools, each of which generates a distinct class of post-mitotic neuron (FIG. 2). It is generally assumed that the homeobox genes are direct targets of the GLI transcription factors, although this remains to be proven.

Interpreting graded SHH signalling through the GLI transcription factors. A key premise of the morphogen concept is that cells have the ability to interpret variations in the levels of signalling molecules to which they are exposed. In the case of SHH, numerous lines of evidence have identified the GLI transcription factors as the key mediators of the cellular response to the signal: the levels of signalling activity that are sensed by a cell are translated into the levels of the activator and repressor forms of the GLI proteins that enter the nucleus. So, for instance, SHH promotes the specification of the p0, p1, p2 and pMN cell identities by antagonizing

Box 1 | **The HH signal transduction pathway**



Hedgehog (HH) proteins signal by binding to the transmembrane proteins Interference hedgehog (IHOG) and Brother of IHOG (BOI), which facilitates the interaction of HH with the multipass transmembrane protein Patched (PTC). This interaction relieves the repressive effect of PTC on the serpentine protein Smoothened (SMO). Like PTC, SMO is an obligate component of the HH pathway, being required for all aspects of HH signal transduction that have so far been described. In *Drosophila melanogaster*, SMO becomes hyperphosphorylated in response to HH signalling and accumulates in the plasma membrane, whereas in vertebrate cells, the protein localizes to primary cilia following exposure to HH ligands. Once activated, SMO modulates the activities of members of the Glioma-associated oncogene homologue (GLI) transcription-factor family. In *D. melanogaster*, there is just one GLI-family protein, named Cubitus interruptus (CI), which is crucial for HH signalling. CI is a bifunctional transcription factor with both repressor and activator domains that flank a central DNA-binding zinc-finger domain. In the absence of HH signalling, CI undergoes proteolytic cleavage, which is primed by its phosphorylation by three kinases, Protein kinase A (PKA), Glycogen synthase kinase 3 (GSK3) and Casein kinase 1 (CK1), and mediated by the ubiquitin ligase pathway; this yields a truncated form of the protein that acts exclusively as a repressor of HH target-gene transcription (CIR). Activation of SMO suppresses CI cleavage and promotes the nuclear import of a full-length CI protein (CIA); the resulting depletion of the truncated form of CI relieves the repression of some HH target genes, and the full-length CI protein further enhances their transcription. CI is present in a complex with the COS2 scaffold protein, which recruits PKA, GSK3 and CK1, facilitating phosphorylation of CI on residues that are crucial for its cleavage. COS2 binds directly to the intracellular C-terminal tail of SMO, thereby providing a physical basis for the regulatory interaction between SMO and CI. Exactly how SMO activation abrogates CI processing is unclear, but one clue comes from the dependence of SMO activation on its hyperphosphorylation. Notably, the phosphorylation sites that are essential for SMO activity resemble those that are phosphorylated by PKA, GSK3 and CK1 in CI, leading to the suggestion that the phosphorylated C-terminal tail of SMO might compete with CI for a binding partner that mediates its cleavage. CI also interacts with the Fused (FU) serine threonine kinase that abrogates its sequestration in the cytoplasm by the Suppressor of fused (SU(FU)) protein. A negative-feedback loop is initiated when the BTB/rdx proteins, targets of HH signalling, degrade CI[93,94].

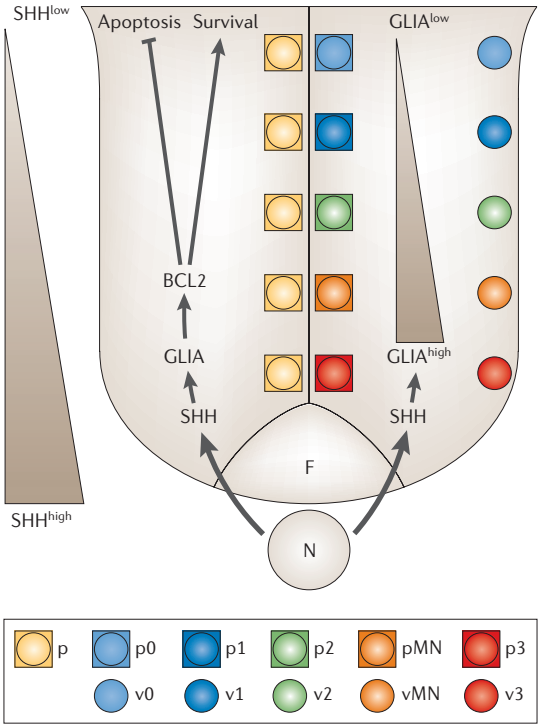


Figure 2 | **SHH governs cell proliferation, survival and fate in the neural tube.** The left-hand side of the figure shows sonic hedgehog (SHH) that is secreted from the notochord (N) and the floor plate (F) working on early neural progenitors to prevent apoptosis and promote survival and proliferation through Glioma-associated oncogene homologue A (GLIA) and B-cell leukaemia/lymphoma 2 (BCL2). The right-hand side shows the establishment of cell fate by the SHH gradient. This is transduced into a gradient of GLIA (high ventrally, diminishing further dorsally), which in turn establishes distinct progenitor domains (p) along the dorsoventral axis of the neural tube. These prefigure and predict defined classes of neurons — V0, V1, V2 and V3 interneurons, and motorneurons.

the repressive activity of the GLI3 protein; in the absence of SHH, these cell types fail to form, but they can be rescued in *Shh* mutants by the simultaneous inactivation of *Gli3* (REFS 23,37,39–41). By contrast, the generation of FP and V3 interneurons, which are the two most ventral cell types that are dependent on SHH signalling, is blocked in the absence of the *Gli2* gene, indicating that, in this case, the response of cells to the signal is mediated by GLI-dependent transcriptional activation[42,43]. Simultaneous inactivation of *Gli2* and *Gli3* cannot restore the cell types that are missing in *Gli2* mutants, but results in the random differentiation of p0, p1, p2 and pMN cells. This indicates that the proper spatial arrangement of these cells depends on the correct balance of GLI activities, generated by the SHH gradient[23,40]. The precise nature of the relationship between extracellular SHH activity and the relative levels of GLI activator and repressor activities in the responding cells is still unclear. However, expression of attenuated GLI proteins encoded by transgenes that have been electroporated into the neural tube of chick embryos has demonstrated, at least in principle, how small changes in the level of GLI activity can recapitulate the specification of neuronal-subtype identity elicited by two- to threefold variation in SHH concentration[44].

Interestingly, the most potent GLI protein can induce the generation of ectopic ventral cell types within 12 hours of the activation of transgene expression, whereas a moderately attenuated GLI protein induces a similar response, but only after 72 hours, indicating that cells can integrate the levels of GLI activity to which they are exposed over time[44].

SHH regulates cell proliferation. SHH not only directs the fate of cells within the neural tube, but also has a direct role in regulating cell numbers by promoting the proliferation of neural progenitor cells. In the anterior neural tube, a number of structures, including the cerebellum, the neocortex and the tectum, depend on the mitogenic effect of SHH signalling[45]. Similarly, the maintenance of neural stem cells in the late-embryonic and adult CNS is also mediated by SHH[46]. In the caudal neural tube, suppression of proliferation ensues following surgical removal of the notochord[47], while blockade of SHH signalling in the neural tube leads to a decrease in the proliferation and survival of progenitor cells[37,39,48]. Conversely, the derepression of the HH signal-transduction pathway, for instance by overexpression of SHH or targeted inactivation of the *Ptch1* gene, causes an expansion of the neural tube[49–51].

Recent studies by Marti and colleagues have provided compelling evidence that SHH controls both proliferation and cell survival in the neural tube through GLI-mediated regulation of cell-cycle and anti-apoptotic gene expression[52]. Notably, these effects are independent of the regional specification of neuronal progenitors that arises from the spatially restricted expression of homeodomain proteins. Indeed, the regulation of proliferation and survival seems to precede the regional specification of cells, implying that SHH itself regulates the size of the cellular field that it subsequently patterns. Intriguingly, growth regulation seems to be less sensitive to the levels of SHH signalling than is the regulation of patterning genes[52], suggesting that SHH is simply permissive for growth.

A role for SHH signalling in promoting progenitor -cell proliferation seems at odds with its role in specifying cell identity, as the restriction in fate that is implied by the second process normally presages withdrawal from the cell cycle and terminal differentiation. Indeed, in some cases it has been demonstrated that HH proteins work directly to promote cell-cycle exit. In the zebrafish eye, for instance, where Shh is required for the differentiation of all the principal retinal cell types, cell-cycle exit by retinal progenitors is triggered by Shh as it transiently activates expression of the cyclin-kinase inhibitor p57Kip2 (REF. 53). Clearly the propensity of SHH to promote or inhibit proliferation is context- and time-dependent. Such versatility underpins the pivotal role of SHH in coordinating growth and differentiation, which is a key feature of morphogenesis.

Interneuron
A neuron that connects with and transmits information only to other neurons.

Cerebellum
The part of the vertebrate hindbrain that modulates the force and range of movements, maintains balance and is involved in motor learning.

Neocortex
The most recently evolved part of the cerebral cortex. It is believed to orchestrate high-level motor, sensory and cognitive functions.

Tectum
The dorsal portion of the midbrain (mesencephalon) that mediates reflexive responses to visual and auditory stimuli.

SHH as a short-range morphogen. The specification of different muscle lineages in the zebrafish embryo provides another example of the dose-dependent action of Shh. Cells lying immediately adjacent to the Shh-secreting notochord give rise to two different types of slow-twitch muscle fibres — muscle pioneers (MPs) and superficial slow fibres (SSFs) — and both of these require Shh signalling for their proper specification[54]. Furthermore, a subset of the fast-twitch muscle fibres, known as medial fast fibres (MFFs), are also induced in response to Shh signalling from the midline tissues[54,55] (FIG. 3). In zebrafish, Shh is just one of three Hh genes that are expressed in the notochord and/or floor plate; and significantly, inactivation of Shh alone selectively eliminates the MPs and MFFs, but not the SSFs. This implies that the different cell types require different levels of combined Hh activity. In line with this, modulation of Smo activity using the small-molecule inhibitor cyclopamine reveals that successively higher levels of Smo, and therefore of Hh signalling activity, are required for the specification of the SSFs, MFFs and MPs[55]. These observations suggest that SHH can specify distinct cell fates even when acting at short range. But, in contrast to the long-range action of SHH in the ventral neural tube, where there is a clear relationship between the level of SHH signalling that is required for a particular cell identity and its location relative to the source of the signal, this is not apparent in the specification of muscle cell identities. Part of the solution to this enigma seems to be that the competence of cells to respond to SHH signalling changes with time, as discussed below. But it is also possible that the strength of the response that is elicited is at least in part a function of the duration of the exposure of a cell to the signal.

Duration of exposure to SHH governs cell fate. A striking precedent for such an effect is provided by the activity of SHH in the vertebrate limb. Here, SHH is expressed in a subset of mesenchymal cells at the posterior margin of the limb bud[56], a region that is known as the zone of polarizing activity (ZPA). Expression of SHH in the ZPA specifies the identity of cells along the anteroposterior axis of the limb: in SHH mutants, only a single digit forms at the end of a foreshortened limb[4]. By contrast, ectopic expression of SHH at the anterior margin of the limb gives rise to supernumary digits[56]. In principle, digit identity could be specified by a graded distribution of SHH activity across the anteroposterior axis of the limb. Indeed, the protein exhibits such a distribution, extending anteriorly from the ZPA[31]. Elegant lineage analyses of cells that either respond to or express SHH indicate, however, that digit identity is a function of the duration of exposure to SHH activity as well as the level to which cells are exposed. Using transgenic mice that express an inducible form of the Cre-recombinase under the control of either the *Gli1* or *Shh* promoters, cells that respond to or express SHH can be indelibly marked at different stages of development[57,58]. Surprisingly, these analyses show that, although all digits except digit 1 are derived from cells that have responded to SHH, digits 3, 4 and 5 derive from cells that have also expressed SHH. Moreover, the longer a cell has expressed SHH, the more posterior it will become. This implies that cells might not simply respond to spatial differences in SHH activity, as exemplified in the neural tube, but can also integrate the level of SHH signalling to which they are exposed over time (FIGS 1b,4a,b).

Regulating SHH signalling in time and space

As discussed above, a key determinant of the effects of any signalling molecule is its effective range. Accordingly, organisms have evolved various strategies to modulate the distribution of inductive signals, a number of which are exemplified by SHH. Modulation can be achieved by regulating the spatial distribution of SHH activity itself — either at the level of the protein or at the level of gene expression. It can also be achieved by cellular movement or the expansion of cellular fields, such that cells move into or out of range of SHH activity.

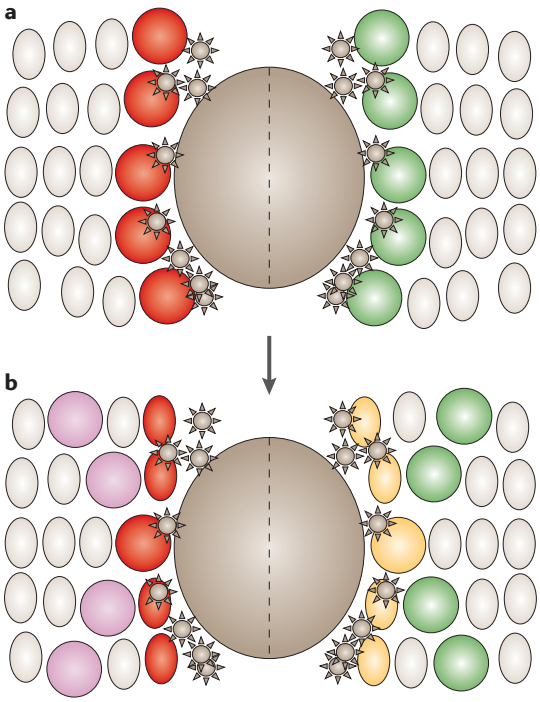


Figure 3 | **Specification of muscle cell identity in the zebrafish embryo by Shh.** The notochord (brown circle) secretes sonic hedgehog Shh (stars). The two sides of the figure represent equivalent cells: *patched* (*ptc*) expression is depicted on the left-hand side and *PR-domain-containing protein 1* (*prdm1*) expression is depicted on the right-hand side. **a** | The paraxial mesodermal cells that lie closest to the notochord receive the signal and upregulate transcription of *ptc* (red) and *prdm1* (green). The activation of Prdm1 commits cells to the slow-muscle lineage. Upregulating Ptc results in the sequestration of Shh protein by these cells, preventing its spread to more lateral cells (open ovals). **b** | Once specified, most of the slow-muscle cells migrate away from the notochord, downregulating *ptc* transcription as they go (pink), but not downregulating *prdm1*. This movement displaces other muscle progenitors towards the notochord, resulting in their exposure to the Shh protein. These cells also upregulate *ptc* transcription but are not competent to activate *prdm1* transcription. Instead, they activate transcription of the engrailed (*eng*) gene (yellow).

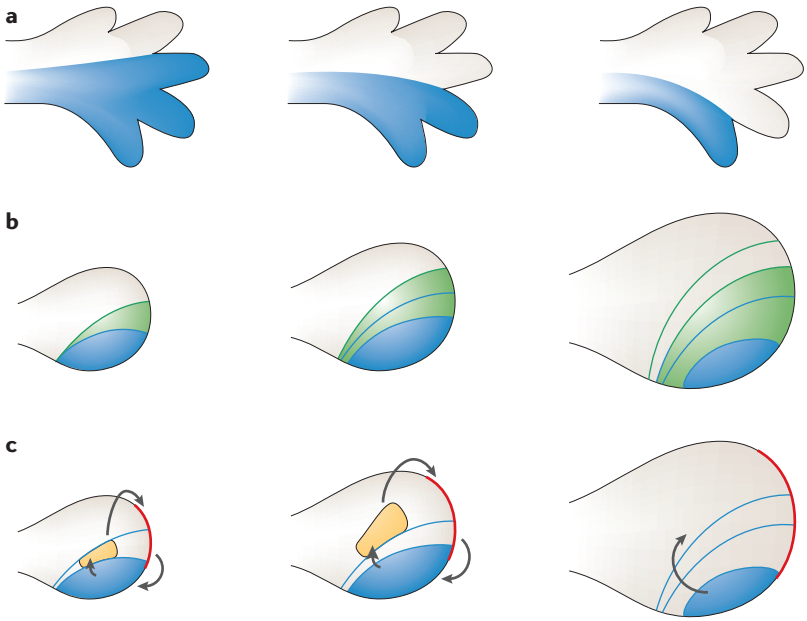


Figure 4 | **Control of identity and growth by SHH in the developing vertebrate limb.** Part **b** depicts three limb buds at successively later stages of development: cells in the zone of polarizing activity (ZPA) (blue) express and secrete sonic hedgehog (SHH) which spreads anteriorly to activate the expression of target genes (green). At the earliest stage shown, cells in the ZPA will contribute to digits 3, 4 and 5, as shown by the later expression of a *lacZ* reporter gene, which is activated in the ZPA at this stage (upper row). As the limb bud grows, proliferating cells exit the ZPA but continue to respond to SHH. By this stage, cells that remain in the ZPA are destined only to give rise to digits 4 and 5. Eventually, five distinct zones, corresponding to the primordia of the five digits, are established, based on the duration of expression and the levels of SHH to which they have been exposed. By this stage, cells in the ZPA are destined only to give rise to digit 5 as shown. Only digit 1, being the most anterior, is composed of cells that have neither expressed nor received SHH. Digit 2 comprises cells that have only received SHH. Digits 3 and 4 derive from the progeny of the ZPA that have continued to receive SHH after exiting the ZPA. Digit 5 is composed of cells that have expressed SHH continuously. Part **a** depicts limbs in which cells that are resident in the ZPA have been marked at successively later stages. Part **c** depicts a similar temporal sequence of limb-bud development, but in this case showing the expression of *Shh* (blue), gremlin (yellow) and fibroblast growth factor 4 (*Fgf4*) (red). At early stages, gremlin transcription is activated by SHH in a broad domain that abuts the ZPA. Gremlin maintains *Fgf4* expression in the apical ectodermal ridge by inhibiting the action of bone morphogenetic proteins (not shown). *Fgf4* expression maintains proliferation by promoting SHH activity. As the limb grows, the gremlin domain becomes separated from the ZPA, because descendants of the ZPA are not competent to activate gremlin transcription. Ultimately, the ZPA descendants expand to such an extent that cells that are competent to activate gremlin in response to SHH are no longer within range of the protein. The loss of gremlin leads, in turn, to a loss of *Fgf4*, and therefore to the loss of *Shh* itself, and the termination of growth. By this mechanism, proper size is ensured.

Negative-feedback loops modulate SHH distribution. One way in which the activity of a signal can be regulated is through its sequestration by responding cells. In the case of SHH, sequestration is mediated in part by binding to the receptor protein patched homologue (PTCH), an effect that is potentiated by the upregulation of *Ptch1* expression in response to SHH signalling. Such upregulation can produce an effective barrier to the spread of the signal beyond these cells, as first demonstrated by experiments in the wing primordia of *D. melanogaster*[59]. The negative-feedback loop that SHH establishes through activation of its own inhibitor, PTCH1, has an essential role in shaping the gradient of SHH activity in the vertebrate neural tube. This role can be observed by blocking activation of the pathway in patches of cells in the chick neural tube (achieved by electroporating cells with a dominant active form of PTCH1 that is incapable of binding SHH)[36]. As expected, the cells in which the pathway is blocked assume a dorsal identity; but those cells located immediately dorsal to them exhibit an opposite dorsal-to-ventral switch in cell identity, which is indicative of the abnormal spread of SHH through the non-responding cells. Notably, this SHH sequestering activity does not depend solely on PTCH1, but is also mediated by a SHH-binding protein called hedgehog interacting protein (HHIP), which is found only in vertebrates.

The range of SHH signalling is increased within mutant mice that lack the feedback activities of PTCH1 and HHIP, extending to the dorsal limit of the neural tube[51]. Cells in mutant mice adopt more ventral fates than would be predicted on the basis of their relative dorsoventral position, indicating that HH signalling is abnormally increased in these cells. Therefore, an SHH-dependent antagonism, which is mediated by PTCH1 and HHIP, is crucial in the normal embryonic neural tube, and reduces or limits both the magnitude and range of SHH morphogen signalling.

Termination of a positive-feedback loop limits SHH signalling. Controlling the range of a signalling molecule can be crucial, not only to ensure the correct spatial pattern and temporal order of cell-type induction, but also for the control of organ size. A striking example of the second principle is provided by the interplay between SHH and fibroblast growth factor (FGF)-signalling in the developing limbs of tetrapods. Outgrowth of the limb along its proximal–distal axis is driven by a specialized population of ectodermal cells that form the apical ectodermal ridge (AER) at the distal tip of the growing bud[60]. These cells express and secrete another signalling molecule, FGF4, which stimulates proliferation of cells in the underlying mesenchyme[61]. The expression of the *Fgf4* gene in the AER is maintained by the activity of SHH in the ZPA, and transcription of the *Shh* gene in the ZPA is in turn maintained by FGF4, which emanates from the AER. Clearly, such a positive-feedback loop, if uninterrupted, could lead to the unlimited extension of the growing limb. To prevent this happening, the embryo exploits the finite range of the SHH protein to terminate the feedback loop and control the size of the limb[62]. How is this achieved? The maintenance of *Fgf4* expression is not mediated directly by SHH, but by the intermediary action of a protein called gremlin, which inhibits the activity of bone morphogenetic proteins (BMPs) that in turn inhibit *Fgf* transcription. Gremlin expression is activated by SHH, but only in a subset of mesenchymal cells that have the ability, or competence, to express the gene. Crucially, cells that originate in the ZPA are not competent to express gremlin, even after they have stopped expressing SHH and left the ZPA. So, the descendants of the ZPA generate a zone of cells that is refractory to gremlin

expression and must be traversed by SHH in order to activate gremlin in the competent cells that are located closer to the AER. As the ZPA descendants proliferate, the refractory zone grows until the competent cells are displaced out of range of the SHH protein. At this point, the inhibition of the BMPs is released, leading to the repression of *Fgf4* transcription and the termination of proliferation (FIGS 1c,4).

Cell migration modulates accessibility to SHH. A rather different example of how the exposure of cells to SHH is modulated comes from the developing myotome of the zebrafish embryo. In this case, migration of one group of cells away from the source of Shh seems to facilitate the exposure of other cells to the signal (FIGS 1d,3). The migrating cells are the progenitors of the slow-twitch muscle fibres that initially flank the notochord in the presomitic mesoderm. These adaxial cells upregulate Ptc expression in response to Shh signalling from the notochord[63], which, as we have described, creates a barrier against the further spread of the signal, thereby protecting the remaining muscle progenitors in the adjacent mesoderm from the inducing effects of Shh[64]. Migration of the adaxial cells away from the notochord commences only after they have activated expression of the *prdm1* (*PR-domain-containing protein 1*) gene, which commits them to follow the slow-twitch muscle pathway of differentiation[64]. This morphogenetic movement results in the displacement of other as yet uncommitted myoblasts towards the notochord, so that they now come into range of the Shh signal, facilitating a second phase of Shh induction[55]. Significantly, however, this second phase does not result in the activation of *prdm1* expression; instead, these cells respond to Shh by activating expression of the engrailed genes, and differentiate into MFFs[55]. So, in this case, cell migration seems to elicit a temporal sequence of Shh-driven inductive interactions, a sequence that results in the specification of distinct cell types by the same signal.

Cell competence modulates the response to SHH

The zebrafish myotome shows how temporal changes in the competence of myoblasts results in differential responses of cells to the Shh signal. Such variation in cellular competence — both in time and in space — is a critical determinant of the repertoire of cell-fate decisions that are orchestrated by SHH, and understanding its molecular basis is an important goal of current research. In this section, we consider some instances in which the molecular basis of competence is at least partially understood.

Temporally regulated competence in motor-neuron versus oligodendrocyte fate. In the spinal cord, SHH is required for the differentiation of not only motor neurons (MNs) and interneurons, but also of glial cells known as oligodendrocytes. Cell-lineage tracing experiments in the zebrafish embryo show that all three cell types can be derived from a common progenitor cell that is located in the ventral neural tube[65]. Shh activity emanating from the floor plate and notochord specifies the pool of progenitors by activating expression of a basic helix–loop–helix (bHLH) transcription factor, oligodendrocyte transcription factor 2 (Olig2), the sustained expression of which is required both for MN and oligodendrocyte differentiation[66]. So, in the absence of Hh pathway activation, both MNs and oligodendrocytes fail to differentiate. Blocking the Hh pathway after the progenitor pool is specified has no effect on MN differentiation, but blocks oligodendrocyte differentiation. This indicates that Shh is required not only to specify the progenitors, but also to induce the differentiation of the oligodendrocytes, which implies that the progenitors must undergo a temporal switch in their response to Shh (FIG. 1e), after which they are biased towards the oligodendrocyte differentiation pathway[65]. A key component in this process is another bHLH transcription factor, neurogenin 2 (Ngn2), the activity of which is essential for MN specification. *ngn2* expression is induced by Olig2, and antagonizes its role in maintaining the precursor pool by activating the transcription of MN differentiation genes that are repressed by Olig2, thereby driving precursor cells into the MN differentiation pathway[67]. Therefore, whether or not a cell remains in the precursor pool long enough to become an oligodendrocyte depends on a fine balance between the levels of *olig2* and *ngn2* expression. Forced co-expression of Olig2 and the Nkx2.2 transcription factor has been found to promote the precocious and ectopic generation of oligodendrocytes, indicating that the combined action of Olig2 and Nkx2.2 determines oligodendrocyte fate[68,69].

Spatially regulated competence defines the response to SHH signalling. Just as cells seem to be programmed to respond differently to SHH over time, the response of cells to SHH can vary in space, a principle that was first established through studies of HH signalling in *D. melanogaster*[17]. During *D. melanogaster* embryogenesis, the *hh* gene is expressed in a strip of ectodermal cells at the anterior margin of each segmental unit (known as parasegments) from which it signals bidirectionally to adjacent cells, revealed by the activation of *ptc* transcription in pairs of stripes that flank each HH domain. By contrast, expression of the *wingless* (*wg*) gene, another HH target, is restricted to cells that are anterior to those that express *Hh* (FIG. 5a). Moreover, ubiquitous expression of *Hh*, driven by a heat-shock-inducible transgene, results in the ectopic expression of *wg*, but only in the posterior half of each parasegment[70]. These findings led to the proposal that the parasegments are subdivided into domains that differ in their competence to respond to HH (FIG. 1f). Subsequent studies have revealed that this competence is mediated by the activities of transcription factors that are encoded by genes at the *sloppy paired* (*slp*) locus[71] and the T-box genes *midline* (*mid*) and *H15* (REF. 72). The products of the T-box genes repress *wg* transcription in cells that lie posterior to the *Hh* domain, thereby preventing its induction by HH activity. The expression of *slp* anterior to each *Hh* domain represses *mid* and *H15* transcription, and in this way defines the domain that is competent to activate *wg* transcription in response to HH.

Myotome
Tissue formed from somites that develops into the body wall muscle.

Presomitic mesoderm
Precursor unsegmented mesoderm, which generates somites on segmentation.

Motor neuron
A nerve cell that innervates muscle cells.

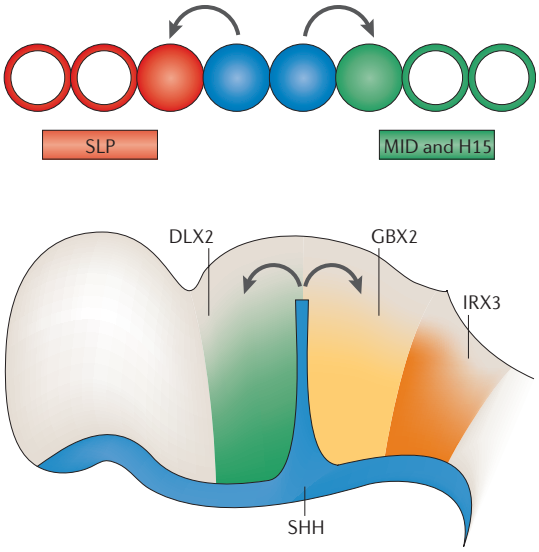


Figure 5 | **Spatial variation in competence to respond to SHH signals underlies the patterning of *Drosophila* segments and the vertebrate brain. a** | In the *Drosophila melanogaster* embryonic segment (upper panel), cells at the anterior boundary of each parasegment express *Hedgehog* (*Hh*) (blue). HH signals to the flanking cells, as evidenced by the upregulation of *Patched* (*Ptc*) transcription (not shown); other responses, however, are asymmetrical owing to differences in cellular competence. Anterior cells express the transcription factor Sloppy paired (SLP), which represses expression of the T-box genes *midline* (*mid*) and *H15*, which act as repressors of *wingless* (*wg*) transcription. So, HH activates *wg* (red) in anterior cells but not in posterior cells (green). **b** | Sonic hedgehog (*Shh*) (blue) is expressed in a narrow strip of cells in the vertebrate diencephalon, known as the zona limitans intrathalamica (ZLI). Cells that lie posterior to the ZLI express the transcription factor iroquois-related homeobox 3 (*Irx3*) (orange), which is permissive for induction of gastrulation brain homeobox 2 (*Gbx2*) (yellow) by SHH. By contrast, anterior to the ZLI, there is no *Irx3* expression and cells respond to SHH by activating expression of distal-less homeobox 2 (*Dlx2*) (green).

In an analogous manner, the regional differences in the response of cells to SHH have a crucial role in the generation of complexity in the CNS of vertebrates. In the forebrain, for instance, the diencephalon is bisected by a narrow wedge of SHH-expressing cells known as the zona limitans intrathalamica (ZLI). Cells that lie posterior to the ZLI will form the thalamus, whereas those that lie anterior to the ZLI give rise to the prethalamus[73]. Two targets of SHH, *Ptch1* and *Nkx2.2*, are expressed in cells on either side of the ZLI, with their expression being strongest in the cells that are close to those expressing *Shh*. By contrast, two other genes that are activated in response to SHH are expressed asymmetrically with respect to the ZLI; gastrulation brain homeobox 2 (*Gbx2*) is expressed exclusively in cells that lie posterior to the ZLI, whereas distal-less homeobox 2 (*Dlx2*) is expressed exclusively in the prethalamic primordium, which is anterior to the ZLI[74] (FIG. 5b). This asymmetrical activation of gene expression by SHH recalls the asymmetry of *wg* activation in the *D. melanogaster* embryo (FIG. 5a). And, as in *D. melanogaster*, the differential competence of cells to respond to SHH depends on the spatially limited expression of transcription factors. The homeobox gene, *Iroquois-related homeobox 3* (*Irx3*), an orthologue of the iroquois family of pre-pattern genes in *D. melanogaster*, is expressed in the thalamic primordium, which is immediately posterior to the ZLI, but it is not expressed in the prethalamic region. Misexpression of *Irx3* in cells that lie anterior to the ZLI is sufficient to change the competence of the prethalamic precursors, such that they now activate *Gbx2* in response to SHH signalling, while the activation of *Dlx2* is concomitantly inhibited[74,75].

Diencephalon
The region of the brain that includes the thalamus, hypothalamus, epithalamus and subthalamus; derived from the prosencephalon.

Prethalamic primordium
The early developing prethalamus.

Concluding comments

The examples in this review describe the various ways in which SHH is repeatedly deployed to orchestrate development in many animal species. As we have described, a key attribute of the HH signalling pathway is its capacity to translate varying levels of extracellular ligand into different intracellular responses. This property is by no means unique to SHH: distinct threshold responses to the TGFB-family protein activin were described in *Xenopus laevis*[76] well before the characterization of the HH family, and subsequent studies have revealed the morphogen properties of other TGFB-superfamily members[77–79], as well as other secreted signals, such as WNT- and FGF-family proteins[80–82]. Nevertheless, the concentration-dependent effects of SHH probably represent the most extensively characterized example of morphogen activity to date, and provide an excellent paradigm for understanding how cells respond to variation in extracellular signalling activities in both space and time.

A second important feature of HH signalling is the feedback control of its activity, and similar processes also operate to control the activities of other signalling pathways[83–87]. The response to SHH can also be modulated by moving cells into or out of range of the signalling source. Again, this phenomenon is not unique to HH signalling: in the *D. melanogaster* testis, for instance, germline stem cells divide to give two daughters, one of which is maintained as a stem cell by virtue of its close proximity to the source of the Unpaired ligand, whereas the other is displaced from the source, thereby escaping the influence of the signal and entering the spermatocyte differentiation pathway[88]. Similar principles are likely to underpin the operation of other stem-cell niches, and to be of key importance in embryonic development and tissue homeostasis.

Finally, we have considered how variation in the competence of cells to respond to SHH expands its repertoire still further, allowing the same signal to elicit distinct cellular identities as a function of the spatial or temporal differences that are intrinsic to the responding cells. Again, this property is not the preserve of HH proteins: on the contrary, the modulation of cellular competence is fundamental to the operation of most, if not all, inductive signals, and has been characterized in a variety of contexts: for example, in the forebrain, where cells on either side of the ZLI differ in their response to FGF signalling[75]; or in the neural tube, where the competence of cells to

respond to BMP signalling changes over time[78,89]. How the competence of cells is modulated in space and time is still poorly understood, but in at least some instances it is likely to involve epigenetic processes. In the developing neocortex, for example, the competence of cortical progenitor cells to differentiate into astrocytes in response to the secreted signal CNTF (ciliary neurotrophic factor) is modulated by histone methylation at the promoters of astrocyte-specific genes, a modification that is itself induced in response to the activity of the secreted signalling protein FGF2 (REF. 90). This example also shows the importance of inputs from more than one signalling pathway in the specification of cell fate. We have focused on the effects of a member of just one signalling family, but many developmental processes integrate inputs from multiple signals. In the neural tube, for instance, recent studies have revealed that the transcriptional response of ventral cells to SHH signalling is modulated by two inhibitors of WNT signalling, secreted frizzled-related protein 2 (SFRP2) and transcription factor 4 (TCF4) (REF. 91).

A major challenge that remains in the field is to unravel these complex networks of signalling interactions and to explain the sequences of molecular events that direct cells to do the right thing at the right time and place. Perhaps the ultimate goal for developmental biology, however, is to understand how growth and patterning are coordinated to make the diversity of shapes and sizes that characterize the living world. We have seen how SHH not only specifies the identity of cells, but also regulates the expansion of progenitor populations. Again, such a dual role is not uncommon among inductive signals (for example, REF. 92), but exactly how these activities are coordinated during morphogenesis remains to be determined.

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