REVIEW

Hedgehog: functions and mechanisms

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The Hedgehog (Hh) family of proteins control cell growth, survival, and fate, and pattern almost every aspect of the vertebrate body plan. The use of a single morphogen for such a wide variety of functions is possible because cellular responses to Hh depend on the type of responding cell, the dose of Hh received, and the time cells are exposed to Hh. The Hh gradient is shaped by several proteins that are specifically required for Hh processing, secretion, and transport through tissues. The mechanism of cellular response, in turn, incorporates multiple feedback loops that fine-tune the level of signal sensed by the responding cells. Germline mutations that subtly affect Hh pathway activity are associated with developmental disorders, whereas somatic mutations activating the pathway have been linked to multiple forms of human cancer. This review focuses broadly on our current understanding of Hh signaling, from mechanisms of action to cellular and developmental functions. In addition, we review the role of Hh in the pathogenesis of human disease and the possibilities for therapeutic intervention.

The origin of the name Hedgehog derives from the short and "spiked" phenotype of the cuticle of the *Hh* mutant *Drosophila* larvae. Mutations in the *Hh* gene were identified by Nusslein-Volhard and Wieschaus (1980) in their large-scale screen for mutations that impair or change the development of the fruit fly larval body plan. *Drosophila Hh* DNA was cloned in the early 1990s (Lee et al. 1992; Mohler and Vani 1992; Tabata et al. 1992; Tashiro et al. 1993). In addition to *Drosophila*, *Hh* genes have also been found in a range of other invertebrates including *Hirudo medicinalis* (leech) and *Diadema antillarum* (sea urchin) (Chang et al. 1994; Shimeld 1999; Inoue et al. 2002). It is important to note that the model organism *Caenorhabditis elegans* (roundworm) has no *Hh* ortholog, even though it has several proteins homologous to the Hh receptor Ptc (Kuwabara et al. 2000).

Hh orthologs from vertebrates—including *Mus musculus* (mouse), *Danio rerio* (zebrafish), and *Gallus gallus* (chicken)—were cloned in 1993 (Echelard et al. 1993; Krauss et al. 1993; Riddle et al. 1993; Chang et al. 1994). Cloning of the first *Rattus rattus* (rat) and human Hh genes were reported shortly thereafter, in 1994 and 1995, respectively (Roelink et al. 1994; Marigo et al. 1995). The vertebrate genome duplication (Wada and Makabe 2006) has resulted in expansion of the *Hh* genes, which can be categorized into three subgroups: the *Desert Hedgehog* (*Dhh*), *Indian Hedgehog* (*Ihh*), and *Sonic Hedgehog* (*Shh*) groups (Echelard et al. 1993). The Shh and Ihh subgroups are more closely related to each other than to the Dhh subgroup, which in turn is closest to *Drosophila* Hh. Avians and mammals have one *Hh* gene in each of the three subgroups, but due to another whole-genome duplication (Jaillon et al. 2004) and further rearrangements, zebrafish has three extra Hh homologs, one in the Shh subgroup: tiggywinkle hedgehog (Twhh) (Ekker et al. 1995), and two others in the Ihh group; echidna hedgehog (Ehh) (Currie and Ingham 1996); and qiqihar hedgehog (Qhh) (Fig. 1A; Ingham and McMahon 2001).

Components of the Hh signal transduction pathway have been identified primarily using *Drosophila* genetics (for example, see Lee et al. 1992; Alcedo et al. 1996; van den Heuvel and Ingham 1996; Burke et al. 1999; Chamoun et al. 2001; Jacob and Lum 2007b). Mechanisms by which the Hh signal is transduced has been further characterized using *Drosophila* and mouse cell culture models (Fig. 1B,C; e.g., see Kinto et al. 1997; C.H. Chen et al. 1999; Chuang and McMahon 1999; Taipale et al. 2000; Lum et al. 2003a; Nybakken et al. 2005; Varjosalo et al. 2006). In both vertebrates and invertebrates, binding of Hh to its receptor Patched (Ptc) activates a signaling cascade that ultimately drives the activation of a zinc-finger transcription factor (Ci in *Drosophila*, GLI1–3 in mammals), leading to the expression of specific target genes (Huangfu and Anderson 2006; Jacob and Lum 2007a; Varjosalo and Taipale 2007).

Although many of the key components are conserved in vertebrates, the mammalian Hh signaling pathway is incompletely understood and harbors some differences and additional pathway components (see below). It was long thought that the main difference between *Drosophila* and mammalian Hh signaling was that mammals had multiple orthologs of many pathway components, including Hh, Ptc, and Ci. However, the roles of mammalian orthologs of two critical components of the

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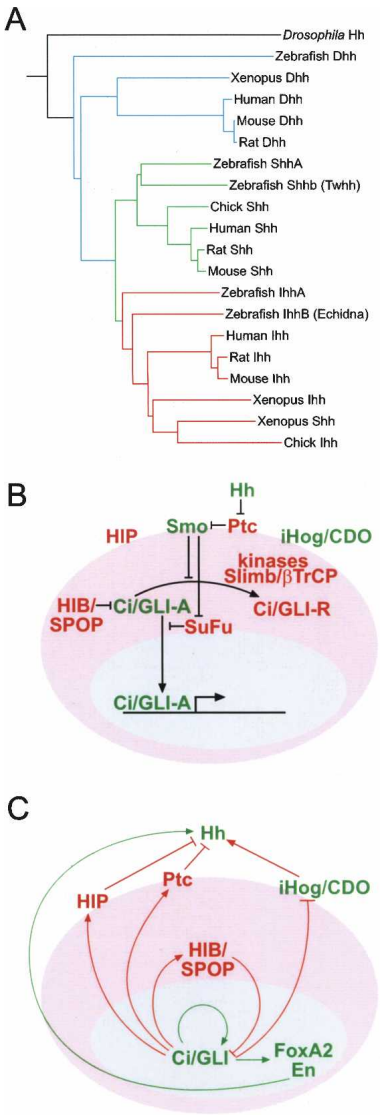

Figure 1. (*A*) Phylogram illustrating the evolution of the Hh proteins. The different Hh proteins were aligned using Prankster (Loytynoja and Goldman 2005). Hh subgroups are indicated by a color code, as follows: Dhh (blue), Shh (green), and Ihh (red). (*B*) The central conserved components of the Hh signaling pathway and their role in forward signaling. Positively and negatively acting pathway components are in green and red, respectively. Note that most interactions between components are inhibitory. The conserved kinases involved in regulation of Ci/GLI processing from activator forms (Ci/GLI-A) to repressor forms (Ci/GLI-R) are casein kinases (CKs) 1α and 1ε, glycogen synthase kinase-3β (GSK3β), and protein kinase A (PKA). (*C*) The four negative (red) and two positive (green) transcriptional feedback loops of the Hh pathway. Ci/GLI-positive feedback to itself is mediated by GLI1 in mammals. HIP and FoxA2 are only found in vertebrates, and Engrailed (En) has been characterized as a regulator of Hh only in *Drosophila*. Both *Drosophila* and mammalian names of the components are given separated by a slash.

Drosophila pathway, the protein kinase Fused (Fu) and the atypical kinesin Costal2 (Cos2), appear not to be conserved (Chen et al. 2005; Merchant et al. 2005; Svard et al. 2006; Varjosalo et al. 2006). This suggests that the mechanisms of Hh signal transduction from the receptor to the Ci/GLI transcription factors have evolved differentially after separation of the vertebrate and invertebrate lineages approximately 1 billion years ago (Hedges 2002; Varjosalo and Taipale 2007).

Developmental functions and expression of mammalian Hh proteins

The Hh proteins act as morphogens controlling multiple different developmental processes (Fig. 2). All mammalian Hh proteins are thought to have similar physiological effects—the differences in their roles in development result from diverse pattern of expression (McMahon et al. 2003; Sagai et al. 2005).

Dhh expression is largely restricted to gonads, including sertoli cells of testis and granulosa cells of ovaries (Bitgood et al. 1996; Yao et al. 2002; Wijgerde et al. 2005). Consistent with its expression in a very narrow tissue range, Dhh-deficient mice do not show notable phenotypes is most tissues and are viable. However, males are infertile due to complete absence of mature sperm (Bitgood et al. 1996).

Ihh is specifically expressed in a limited number of tissues, including primitive endoderm (Dyer et al. 2001), gut (van den Brink 2007), and prehypertrophic chondrocytes in the growth plates of bones (Vortkamp et al. 1996; St-Jacques et al. 1999). Approximately 50% of $Ihh^{-/-}$ embryos die during early embryogenesis due to poor development of yolk-sac vasculature. Surviving embryos display cortical bone defects as well as aberrant chondrocyte development in the long bones (St-Jacques et al. 1999; Colnot et al. 2005). Homozygous hypomorphic mutations of *IHH* in humans cause acrocapitofemoral dysplasia, a congenital condition characterized by bone defects and short stature (Hellemans et al. 2003).

Shh is the most broadly expressed mammalian Hh signaling molecule. During early vertebrate embryogenesis, Shh expressed in midline tissues such as the node, notochord, and floor plate controls patterning of the left–right and dorso-ventral axes of the embryo (Sampath et al. 1997; Pagan-Westphal and Tabin 1998; Schilling et al. 1999; Watanabe and Nakamura 2000; Meyer and Roelink 2003). Shh expressed in the zone of polarizing activity (ZPA) of the limb bud is also critically involved in patterning of the distal elements of the limbs (Riddle et al. 1993; Chang et al. 1994; Johnson et al. 1994; Marti et al. 1995). Later in development, during organogenesis, Shh is expressed in and affects development of most epithelial tissues (Fig. 2).

Deletion of *Shh* leads to cyclopia, and defects in ventral neural tube, somite, and foregut patterning. Later defects include, but are not limited to, severe distal limb malformation, absence of vertebrae and most of the ribs, and failure of lung branching (Chiang et al. 1996; Litingtung et al. 1998; Pepicelli et al. 1998).

The different Hh ligands often act in the same tissues during development, and can function partially redundantly (Fig. 2). For example, Shh and Ihh act together in

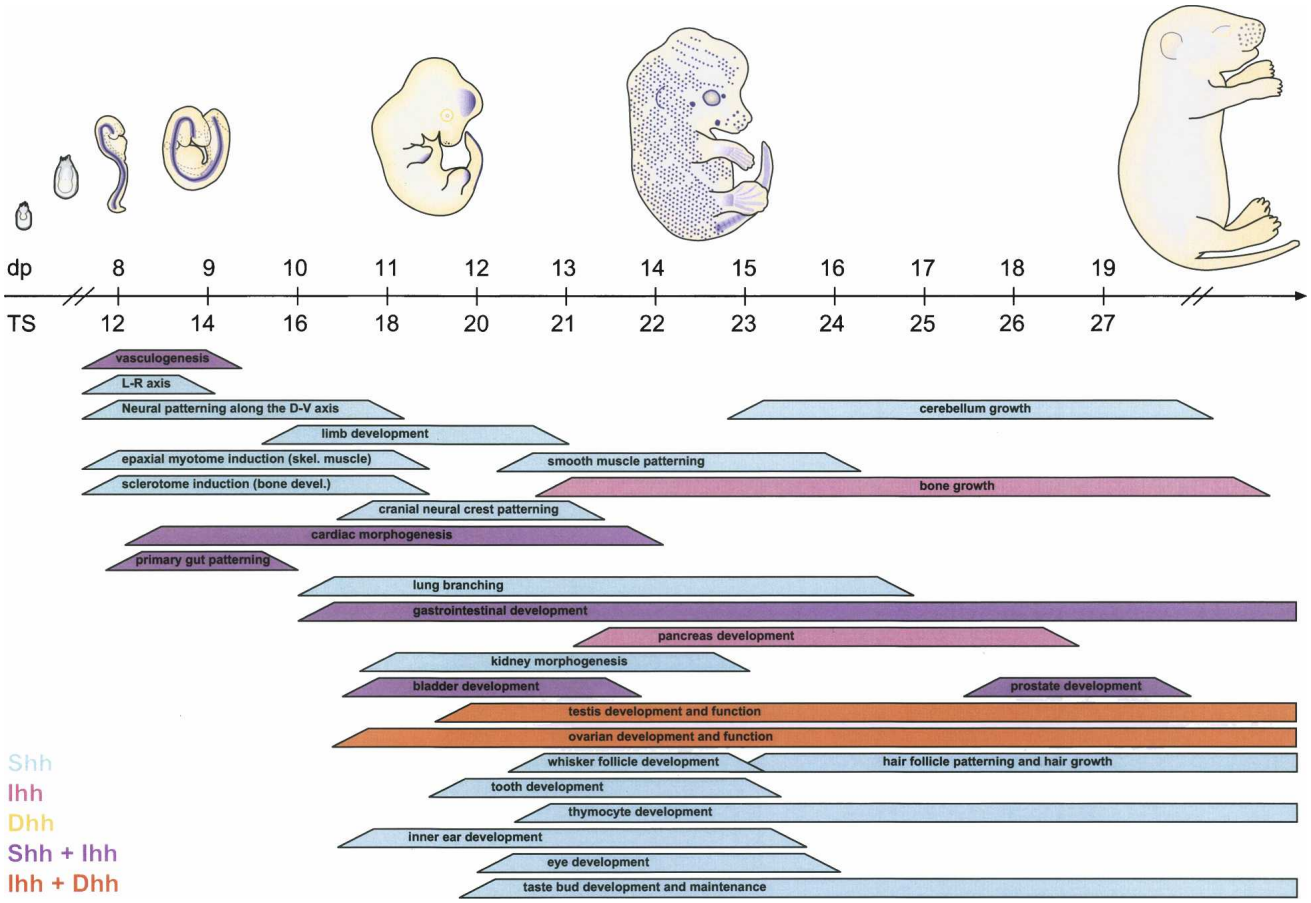

Figure 2. Shh controls mouse development from an embryo to an adult. (*Top*) The embryo cartoons show aspects of expression of the Hh target gene *patched* (blue) during mouse embryonic development. (*Bottom*) Bars show approximate embryonic stages when Shh, Ihh, and/or Dhh (color code in *bottom left*) control developmental processes in the indicated tissues or cell types. The approximate embryonic stage is indicated by dpc and Theiler stage (TS) (Theiler 1989). References: the role of Hh in early embryogenesis prior to TS 15 (Chiang et al. 1996; Zhang et al. 2001; Astorga and Carlsson 2007); limb development (Ahn and Joyner 2004); cranial neural crest (Jeong et al. 2004); cardiac septation (Goddeeris et al. 2008); gastrointestinal system (Madison et al. 2005); bladder (Haraguchi et al. 2007); lung (White et al. 2007); prostate (Berman et al. 2004); pancreas (Hebrok et al. 2000); testis development (Yao et al. 2002); retina (Sigulinsky et al. 2008); kidney (Hu et al. 2006); hair (St-Jacques et al. 1998; Jeong et al. 2004); taste buds (Miura et al. 2001); ear (Riccomagno et al. 2002); ovary (Wijgerde et al. 2005; Pangas 2007); tooth (Cobourne et al. 2001, 2004); bone growth (St-Jacques et al. 1999); cerebellum growth (Hatton et al. 2006; Sillitoe and Joyner 2007).

early embryonic development, and their combined loss phenocopies the loss of the Hh receptor component Smoothened (Smo), leading to early embryonic lethality due to defects in heart morphogenesis and extraembryonic vasculogenesis (Zhang et al. 2001; Astorga and Carlsson 2007).

Regulatory elements affecting mammalian Hh expression

Of the mammalian *Hh* genes, only the mechanisms controlling *Shh* expression have been studied in detail. The expression pattern of *Shh* is the result of the combined action of multiple enhancer-elements, which act independently to control *Shh* transcription in different tissues and expression domains. Both local-acting and very distal elements have been identified (Fig. 3).

Two independent enhancers—Shh floor plate enhancer 1 (SFPE1) and SFPE2, located at –8 kb and in intron 2, respectively—act to direct reporter expression exclusively to the floor plate of the hindbrain and spinal cord (Epstein et al. 1999). A third element in intron 2, Shh brain enhancer 1 (SBE1), directs reporter expression to the ventral midbrain and caudal diencephalon. The more distal elements SBE2, SBE3, and SBE4, which are located >400 kb upstream of the Shh transcription start site (TSS) drive reporter expression in the ventral forebrain. The combined activity of these enhancers appears to cover all regions of Shh transcription along the anterior-posterior axis of the mouse neural tube (Jeong et al. 2006).

The enhancer controlling Shh expression in the ZPA of limb buds, mammals–fish conserved sequence 1 (MFCS1), is located even further upstream of the start

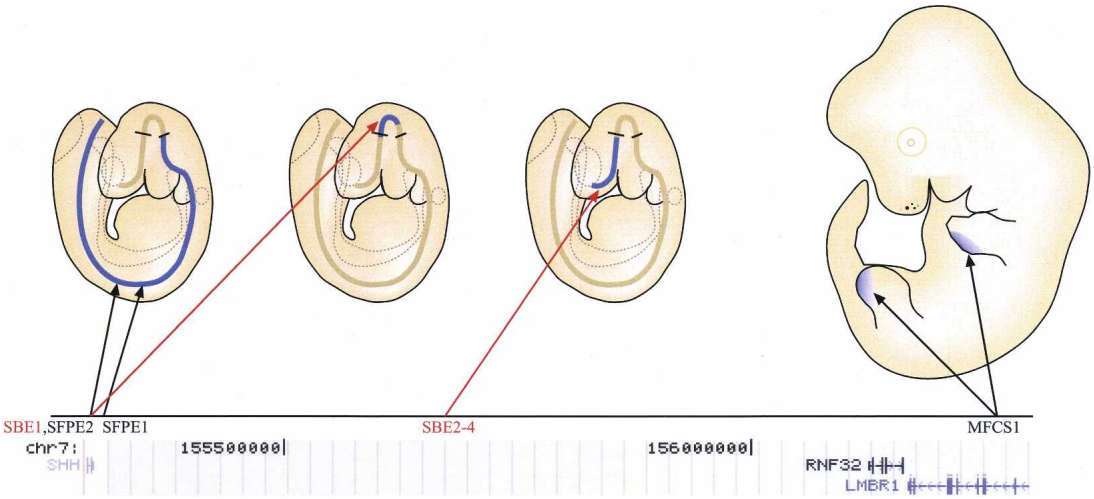

Figure 3. Regulation of mammalian Shh gene expression. (*Top*) Enhancer-elements driving expression of the mouse Shh gene in different neural domains (*left*) and in posterior margin of the embryonic limb buds (*right*). Approximate expression domains of the elements are indicated by blue color. Black lines perpendicular to the neural tube indicate zona limitans intrathalamica (ZLI) and midbrain–hindbrain junction. (*Bottom*) Known genes in the ~1 Mb genomic region upstream of the human Shh gene (University of California at Santa Cruz genome browser, assembly 36). Note that only one transcriptional start site of another gene appears to be between the most distal conserved Shh enhancer (MFCS1) and the Shh gene itself.

site, at –1 Mb in intron 5 of the *Lmbr1* gene (Sharpe et al. 1999; Lettice et al. 2003; Sagai et al. 2004). This element is the only enhancer in Shh that has been analyzed also by loss-of-function studies (Sagai et al. 2005), which conclusively demonstrate that MFCS1 is necessary for Shh expression in mouse ZPA. Consistently in humans, germline mutations within the conserved MFCS1 element cause congenital limb malformations characterized by preaxial polydactyly (Lettice et al. 2003). Interestingly, the MFCS1 sequence is not conserved in limbless vertebrates such as snake, limbless lizard, and newt (Sagai et al. 2005). Although the SBE2–4 and MFCS1 elements are physically far from *Shh*, the TSS of the region upstream of *Shh* contains very few genes, and only one well-described TSS exists between the MFCS1 and the TSS of *Shh* (Fig. 3). Given the diverse expression pattern of Shh, it is likely that a number of other enhancer-elements remain to be identified in this "gene-poor" region.

Although many enhancers that drive Shh expression have been identified, very little is known about the specific transcription factors that control their activity. The temporal and spatial expression pattern of FoxA2 suggests that it could induce Shh expression (Chang et al. 1997; Epstein et al. 1999) in the midline. Consistently, conserved binding sites for FoxA2 and Nkx6 are required for SFPE2 activity (Jeong and Epstein 2003). The Nkx2.1 homeodomain protein has also been suggested as a likely candidate regulating Shh expression in ventral forebrain (Jeong et al. 2006).

No known consensus binding sites for transcription factors are affected by the mutations in the MFCS1 limb enhancer, and the mutations are not clustered close together. However, the severity of the polydactyly phenotype correlates negatively with the conservation of nucleotide at the mutation sites, suggesting that MFCS1 activity is controlled by conserved transcriptional regulators whose DNA-binding specificity is currently not known.

Hh processing and secretion

After translation, Hh undergoes multiple processing steps that are required for generation and release of the active ligand from the producing cell. The mechanisms involved in Hh processing and secretion are evolutionarily conserved (see Burke et al. 1999; Amanai and Jiang 2001; Chamoun et al. 2001; Ingham and McMahon 2001; Caspary et al. 2002; Dai et al. 2002; Ma et al. 2002).

After the signal sequence is removed, the Hh molecule undergoes a cleavage catalyzed by its own C-terminal domain that occurs between conserved glycine and cysteine residues (Fig. 4; Lee et al. 1994; Porter et al. 1996). First, the peptide bond between these residues is rearranged to form a thioester. Subsequently, a hydroxyl-oxygen of cholesterol attacks the carbonyl of the thioester, displacing the sulfur and cleaving the Hh protein into two parts, a C-terminal processing domain with no known signaling activity and an N-terminal Hh signaling domain (HhN) of ~19 kDa that contains an ester-linked cholesterol at its C terminus (Porter et al. 1996). The cholesterol modification results in the association of HhN with the plasma membrane. Subsequently, a palmitic acid moiety (Pepinsky et al. 1998) that is required for HhN activity is added to N terminus of Hh by the acyltransferase Skinny hedgehog (Ski, HHAT in humans) (Chamoun et al. 2001; Lee et al. 2001; Buglino and Resh 2008). The resulting fully active HhN signaling molecule is thus modified by cholesterol at its C terminus and palmitate at its N terminus (Chamoun et al. 2001; Lee

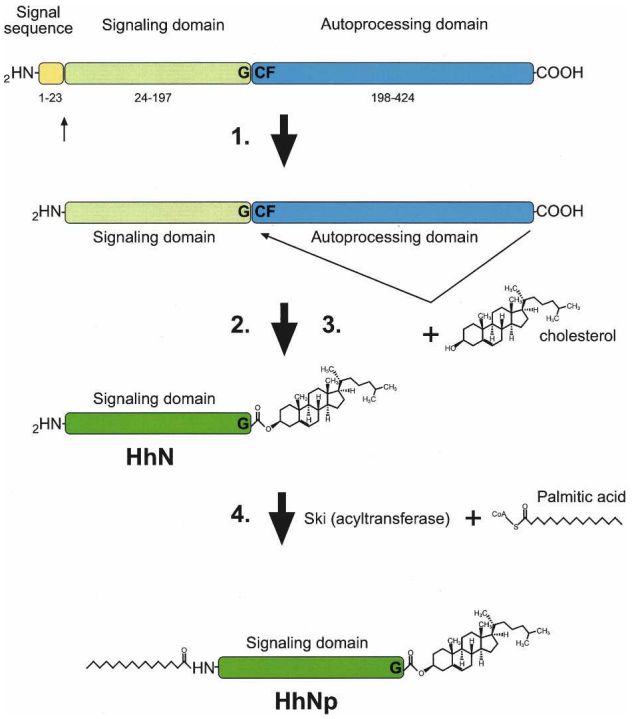

Figure 4. (*A*) Hedgehog protein maturation. Hh protein undergoes multiple processing steps: (1) the signal sequence is cleaved; (2) the C-terminal domain of the Hh polypeptide catalyzes an intramolecular cholesteroyl transfer reaction, resulting in (3) the formation of a C-terminally cholesterol-modified N-terminal Hh signaling domain (HhN). This causes association of HhN with membranes, which facilitates the final modification step 4, the addition of a palmitic acid moiety to the N terminus by the acyltransferase Skinny hedgehog, resulting in the formation of dually modified Hh signaling domain (HhNp).

and Treisman 2001). For clarity, we refer to this protein as Hh hereafter.

Formation of the Hh gradient

Although Hh is tightly associated with the plasma membrane, it is able to act directly over a long range (Roelink et al. 1995; Briscoe et al. 2001; Wijgerde et al. 2002). In both *Drosophila* and vertebrates, the secretion of Hh from the producing cell requires the activity of the 12-span transmembrane protein, Dispatched (Disp). Disp, like Ptc, belongs to the bacterial RND (Resistance-Nodulation-Division) family of transport proteins. Loss of Disp leads to accumulation of Hh in the producing cells and failure of long-range signaling (Burke et al. 1999; Ma et al. 2002).

Distances over which Hh has been shown to act are ~50 µm in *Drosophila* wing imaginal disc and ~300 µm in vertebrate limb bud (Zhu and Scott 2004). How Hh moves over a such a long distance is still not clear, and could involve passive diffusion, active transport, and/or transcytosis. Genetic evidence points to a role of heparan sulfate proteoglycans in this process, as Hh cannot be transported across a field of cells lacking the heparan sulfate synthesizing enzymes of the EXT/tout velu (ttv)/brother of tout velu (botv)/sister of tout velu (sotv) family (Bellaiche et al. 1998; Lin et al. 2000; Bornemann et al. 2004; Han et al. 2004a; Koziel et al. 2004). The substrates of ttv involved in this process appear to be the glypicans (glycosylphosphatidylinositol-linked HSPGs) Dally and Dally-like (Han et al. 2004b). Dally and Dally-like also affect Hh signaling by facilitating binding of Hh to cell surfaces (Nakato et al. 1995; Lum et al. 2003a; Han et al. 2004b).

Whether Hh is transported as individual molecules or assembled into larger particles prior to transport is not clear. Several lines of evidence support the role of large lipid/protein particles in long-range Hh transport. First, Hh staining of receiving cells displays a punctate pattern (Panakova et al. 2005). In addition, soluble Shh multimers that contain lipids and that have strong signaling potency have been described in mammalian cells (Zeng et al. 2001), and it has been reported that *Drosophila* Hh is transported in lipoprotein particles (Panakova et al. 2005; Callejo et al. 2006). Recent genetic evidence also suggests that Hh may be secreted in two different forms, the first of which diffuses poorly and acts at a short range. The second form is "packaged" for long-range transport, and its formation requires the cytoplasmic membrane-scaffolding protein Reggie-1/flotillin-2 (Katanaev et al. 2008).

Multiple studies have analyzed the role of cholesterol modification in Hh transport in vivo, with conflicting results suggesting that cholesterol either aids or hinders Hh transport (for example, see Lewis et al. 2001; Dawber et al. 2005; Gallet et al. 2006; Li et al. 2006). These studies are complicated because the protein expression levels of the different mutant forms of Hh need to be constant in order to rule out dose effects. In addition, interpretation of the results is made even more difficult by the fact that Hh protein lacking cholesterol modification is soluble, and thus its secretion does not require Dispatched and it can escape the producing cell without being palmitoylated (Mann and Beachy 2004) and could even become palmitoylated later during transport or at the receiving cell. Thus, genetic experiments alone cannot conclusively determine the role of cholesterol modification in Hh activity and transport. In contrast, analysis of the role of the palmitate modification in Hh transport is more straightforward, as palmitoylation can be selectively prevented either by mutation of Ski, or mutation of the palmitoylated N-terminal cysteine of the Hh proteins. Such experiments indicate that palmitoylation is required for Hh activity in *Drosophila* (Burke et al. 1999), and for generation of soluble multimeric Hh protein complexes and long-range signaling in vertebrates (Chen et al. 2004).

Several mechanisms are used to control the shape and size of the Hh gradient (for review, see Teleman et al. 2001). Very high levels of Hh can induce Hh expression in responding cells both in *Drosophila* and in mammals (Tabata et al. 1992; Roelink et al. 1995; Methot and Basler 1999). This increases the local concentration of

Hh near the original source. Hh also induces the expression of its receptor Ptc, which internalizes Hh and targets it to the lysosomes for degradation (Chen and Struhl 1996; Incardona et al. 2000; Gallet and Therond 2005). This negative feedback loop restricts the spreading of the Hh signal through tissues. Vertebrates also have an additional transmembrane protein, Hedgehog-interacting protein (HIP), which is also induced by Hh signaling and binds to and further reduces the range of movement of Hh (Chuang and McMahon 1999; Jeong and McMahon 2005).

Hh signal transduction

Hh receptor

In addition to the glypical dally-like, which acts both in Hh transport and as an accessory receptor, the binding of Hh to responding cells is facilitated by the transmembrane proteins Cdo and Boc (iHog and boi in *Drosophila*) (Lum et al. 2003a; Tenzen et al. 2006; Yao et al. 2006). These proteins act positively in the pathway, binding to Hh via conserved fibronectin repeats (Yao et al. 2006) and increasing Hh association with its signaling receptor Ptc (Tenzen et al. 2006; Yao et al. 2006). The expression levels of Cdo and Boc are down-regulated in response to Hh signaling, resulting in yet another negative feedback that limits pathway activity (Fig. 1C).

In the absence of Hh ligand, Ptc catalytically inhibits the activity of the seven-transmembrane-span receptor-like protein Smo (Taipale et al. 2002). Binding of Hh to Ptc results in loss of Ptc activity, and consequent activation of Smo. Smo then transduces the Hh signal to the cytoplasm (Stone et al. 1996; Taipale et al. 2002). This general model is based on the genetic observations that loss of Hh or Smo cause similar phenotypes, and that Ptc loss results in a phenotype that is similar to strong overexpression of Hh. Epistasis analyses in turn indicate that Ptc acts downstream from Hh and upstream of or parallel to Smo (Ingham et al. 1991; Alcedo et al. 1996; van den Heuvel and Ingham 1996). Binding of Hh to Ptc, in turn, was determined using purified Shh and cultured cells overexpressing Ptc (Stone et al. 1996; Fuse et al. 1999).

By inferring the protein levels of ligand-bound and unbound Ptc from gene expression, Casali and Struhl (2004) suggested that the activity of the pathway depends on the ratio between these two forms. However, the fact that increasing the level of Ptc protein decreases cellular responsiveness to Hh (see Bailey et al. 2002; Taipale et al. 2002) indicates that it is the absolute amount of unliganded Ptc in a cell that controls pathway activity. This mechanism, together with the induction of Ptc by Hh results in gradual desensitization of cells to Hh and allows cells to accurately interpret the wide range of Hh concentrations present in morphogenetic gradients.

In vertebrates, Ptc exists as two isoforms, Ptc and Ptc2. Mice deficient in Ptc2 are viable, but develop alopecia and epidermal hypoplasia and have increased tumor incidence in the presence of one mutant allele of Ptc (Lee et al. 2006; Nieuwenhuis et al. 2006). Loss of Ptc, in turn, results in complete activation of the Hh pathway (Goodrich et al. 1997), suggesting that Ptc is the functional ortholog of *Drosophila* Ptc. Ptc has been proposed to function as a permease to affect the transmembrane movement and/or concentration of small molecules that then either agonize or antagonize Smo (Taipale et al. 2002). Supporting this hypothesis, Smo activity can be modulated by many synthetic small molecules (Chen et al. 2002b; Frank-Kamenetsky et al. 2002) and natural products, including the steroidal alkaloids cyclopamine and jervine (Chen et al. 2002a). These compounds were identified by Keeler and Binns (1966) as active ingredients in *Veratrum californicum*, a plant whose ingestion by sheep led to an outbreak of cyclopia in US midwest in the 1950s. The clue that these compounds antagonize Shh signaling came from the observation that the stillborn lambs have a phenotype that is strikingly similar to that of Shh mutant mouse embryos (Chiang et al. 1996).

The structural similarity between cyclopamine and sterols (Cooper et al. 1998) suggests that endogenous sterols might regulate Smo activity. This hypothesis is also supported by genetic evidence, as disruption of embryonic cholesterol synthesis leads to developmental malformations that strikingly mimic Hh mutants (Kelley et al. 1996; Cooper et al. 1998). Oxysterols (Corcoran and Scott 2006) and vitamin D3 derivatives (Bijlsma et al. 2006) have been suggested to be the endogenous metabolites that modulate Smo activity. Of these, vitamin D3 appears to bind to Smo (Bijlsma et al. 2006) based on its ability to compete against binding of labeled cyclopamine (Chen et al. 2002a).

Based on the fact that increased activity of oncogenically activated Smo proteins correlates with their increased resistance to cyclopamine, it was suggested that Smo exists in active and inactive conformational states (Taipale et al. 2000). Similarly, experiments in *Drosophila* suggest that dSmo can exist in two conformational states (Zhao et al. 2007). However, the activity of all small molecules found to activate or inhibit Smo appear to be specific for vertebrate Smo proteins, suggesting that mechanisms of action of *Drosophila* and mammalian Smo may be different. Stronger evidence for this comes from both structural and functional analyses, which indicate that Smo C-terminal domain has evolved differentially in vertebrates and invertebrates.

Several lines of evidence suggest that the cytoplasmic components and the mechanism of Hh signal transduction have diverged between *Drosophila* and mammals. In the following section, we will first discuss the mechanism of intracellular Hh signal transduction in *Drosophila*, which is fairly well understood. We will then discuss the evidence suggesting that *Drosophila* and mammals appear to use different components and mechanisms in transducing the Hh signal between Smo and the Ci/GLI transcription factors.

Intracellular Hh signaling in Drosophila

In the absence of Hh, Ptc keeps *Drosophila* Smo in an unphosphorylated state. Unphosphorylated Smo is

cleared from the cell surface via endocytosis and is degraded in lysosomes (Jia et al. 2004; Zhang et al. 2004). After Hh stimulation, Smo is hyperphosphorylated and its endocytosis and degradation are blocked. Phosphorylation can be mimicked by mutation of the phosphorylation sites to negatively charged residues or by mutating adjacent positively charged arginine clusters to alanine. Based on these observations, Zhao et al. (2007) suggested that phosphorylation neutralizes the positive charge of the dSmo C terminus and induces a conformational switch in the C-terminal cytoplasmic tail and consequent dimerization or multimerization of dSmo. How these events lead to activation of downstream signaling pathway components is not understood (Zhao et al. 2007).

dSmo C terminus binds directly to the kinesin-like protein Cos2, which acts as a scaffolding protein, bringing together multiple cytoplasmic components of the pathway (Jia et al. 2003; Lum et al. 2003b; Ogden et al. 2003; Ruel et al. 2003). These include the full-length transcriptional activator form of Ci, CiA (155 kDa) (Robbins et al. 1997), and multiple serine–threonine kinases, including a kinase that specifically acts on the Hh pathaway, Fused (Fu) (Therond et al. 1996) and the multifunction kinases PKA, GSK3β, CKIα, and CKIε (for review, see Aikin et al. 2008).

In the absence of Hh, CiA is hyperphosphorylated by the combined action of PKA, which acts as a priming kinase, and GSK3β and the casein kinases, which further phosphorylate the primed substrate (Fig. 1B). The hyperphosphorylation promotes recognition of CiA by the ubiquitin E3 ligase Slimb (β-TrCP in vertebrates) (Jiang and Struhl 1998), leading to the generation of a truncated transcriptional repressor form of Ci, CiR (75 kDa) (Y. Chen et al. 1999; Price and Kalderon 1999, 2002; Wang et al. 1999; Jia et al. 2002, 2005). In addition to promoting CiR formation, Cos2 regulates Ci by tethering it to the cytoplasm and preventing its nuclear translocation (C.H. Chen et al. 1999; G. Wang et al. 2000).

In the presence of Hh, Sno accumulates and the binding of Cos2 to Smo prevents conversion of CiA to CiR (Hooper 2003; Jia et al. 2003). However, this mechanism alone is not sufficient to fully activate the pathway, as some CiA is still retained in the cytoplasm by another protein, Supressor of Fused [Su(Fu)] (Pham et al. 1995; Methot and Basler 2000). Genetic evidence from *Drosophila* indicates that full activation of the pathway in response to Hh requires the Fu protein kinase, which blocks the negative influence of Su(Fu) on Ci (Ohlmeyer and Kalderon 1998; Lefers et al. 2001; Lum et al. 2003b). Upon entering the nucleus, CiA binds to specific sequences (Kinzler and Vogelstein 1990; Hallikas et al. 2006) in promoter and enhancer regions and controls the transcription of the Hh target gene(s).

In *Drosophila*, cellular responsiveness to Hh is controlled by modulating the expression of Ci. In the posterior compartment of the wing disc, Hh and its receptor components are expressed, but target genes are not activated, as Ci mRNA expression is repressed by Engrailed (Eaton and Kornberg 1990). Cells posterior to the morphogenetic furrow of *Drosophila* eye, in turn, fail to respond to Hh because Ci levels are post-transcriptionally down-regulated due to the expression of hib (Hh-induced MATH and BTB protein; SPOP in vertebrates), a protein that acts as a substrate recognition subunit for the Cul3 E3 ubiquitin ligase. In contrast to Slimb-mediated ubiquitinylation, which leads to partial Ci degradation, the hib/Cul3-mediated ubiquitinylation causes complete degradation of Ci (L. Zhang et al. 2006). Expression of hib increases in response to Hh, providing another negative feedback mechanism to this pathway (Fig. 1C; Kent et al. 2006; Q. Zhang et al. 2006).

Divergence of pathway components and mechanisms

Despite the conservation of the Hh signaling pathway and many of its roles in development between invertebrate and vertebrate species (Ingham and McMahon 2001; Taipale and Beachy 2001), the mechanisms by which Smo regulates the Ci/GLI transcription factors appears to be distinct between *Drosophila* and mammals (Huangfu and Anderson 2006; Varjosalo and Taipale 2007).

The relatively rapid evolution of some components of the Hh pathway, including Smo, Cos2, and Fu, is apparent at sequence level. The C-terminal domains of vertebrate Smo proteins are significantly shorter than those of invertebrates and lack the main phosphorylation regions described below. In addition, the two mammalian orthologs of Cos2, Kif27, and Kif7 have none of the unique sequence characteristics of Cos2 that differentiate Cos2 from the kinesin family of motor proteins. Based on sequence, Kif7 and Kif27 appear to be functional molecular motors, whereas Cos2 has apparently lost its ability to bind ATP and function as a motor protein. The closest mammalian homolog of *Drosophila* Fu is also highly diverged, and significant homology between these proteins can be seen only in the protein kinase domain itself (Murone et al. 2000).

Drosophila Smo activation is coupled to the hyperphosphorylation of 26 serine/threonine residues located within the C-terminal cytoplasmic tail by PKA and CKI (Jia et al. 2004; Zhang et al. 2004; Apionishev et al. 2005). None of these PKA or CKI phosphorylation sites are conserved in vertebrate Smo. The vertebrate Smo C termini lacks one of the two known Cos2-binding domains (Jia et al. 2003), and the region homologous to the other domain (Lum et al. 2003b) is dispensable for mouse Smo (mSmo) function (Varjosalo et al. 2006). *Drosophila* Cos2, or mouse Kif7 or Kif27 do not appear to bind to mSmo or GLI proteins or affect Shh signaling when overexpressed in NIH-3T3 cells (Varjosalo et al. 2006). Furthermore, loss of the Fu protein kinase—which forms a tight complex with Cos2 and is required for Hh signaling in *Drosophila*—appears not to impair Hh signaling in mice (Chen et al. 2005; Merchant et al. 2005). Taken together, this evidence suggests that the Cos2–Fu complex, which is centrally important in *Drosophila*, plays little or no role in mammalian Hh signaling. Instead, it appears that mammalian Hh signaling critically depends on Su(Fu) (Svard et al. 2006)—which has a minor role in *Drosophila*

(Ohlmeyer and Kalderon 1998)—and on several components involved in formation of the primary cilia, which either do not have *Drosophila* orthologs or whose orthologs appear not to function on the *Drosophila* Hh pathway (Nybakken et al. 2005).

Primary cilium is an organelle that protrudes from the surface of most vertebrate cells. Genetic evidence suggesting a role for primary cilium in mammalian Hh signaling comes from studies that found that mutations of several proteins required for its formation, including Kif3a, Ift88, and Ift172, result in embryonic phenotypes characteristic of the loss of Shh signaling (Huangfu et al. 2003; Park et al. 2006; Caspary et al. 2007; Vierkotten et al. 2007). Subsequent studies have linked these proteins to the processing of the GLI transcription factors (May et al. 2005; Caspary et al. 2007). Some experiments suggest that primary cilium would act as a "signaling center" where the biochemical events of signal transduction take place. It has been reported that activated mammalian Smo accumulates to primary cilia in response to Shh treatment (Corbit et al. 2005); in the absence of Shh, this accumulation is prevented by Ptc (Rohatgi et al. 2007). Other components involved in Hh signaling, including Su(Fu) and unprocessed GLI proteins, have also been localized to the primary cilium (Haycraft et al. 2005).

Drosophila lacking centrioles, and all microtubule-based structures derived from them, including centrosomes, cilia, and flagella develop almost normally, indicating that cilia are not required for *Drosophila* Hh signaling (Basto et al. 2006). In contrast, the genetic studies described above have clearly established that mammalian Hh signaling depends on a process that requires components involved in formation of primary cilia. However, this evidence is also consistent with a model where some other microtubule-linked process that is critical for Hh signaling is disrupted by loss of these proteins. In addition, the fraction of cellular Hh pathway components found in the primary cilium at any given time appears small. Thus, it remains to be established what role cilia play in mammalian Hh signaling and whether localization of the pathway components to cilia is required for signaling.

The lack of effect of the closest mammalian homolog of *Drosophila* Fused on Hh signaling suggests that other—mammalian-specific—kinases act on this pathway. We recently identified two such kinases, DYRK2 and MAP3K10, which are required for Shh signaling in NIH-3T3 cells (Varjosalo et al. 2008). Of these, DYRK2 directly phosphorylates GLI2 and GLI3 and induces their degradation. MAP3K10, in turn, appears to act in a more indirect fashion, binding to and phosphorylating multiple other proteins that regulate the Hh pathway, including GSK3β, DYRK2, and Kif3a (Nagata et al. 1998; Varjosalo et al. 2008). Because of the many connections of MAP3K10 to different pathway components, its mechanism of action is likely to be complex, and requires further study. In addition to DYRK2 and MAP3K10, it has been reported that other vertebrate-specific kinases regulate Shh signaling. These include protein kinase C-δ (PKCδ), mitogen-activated protein/extracellular signal-regulated kinase-1 (MEK-1), Akt, and DYRK1 (Mao et al. 2002; Riobo et al. 2006a,b). From our studies and previous analyses of the Hh pathway, it appears that Hh does not regulate the activity of any known kinase toward a generic substrate. Thus, the mechanism by which Hh signal is transduced appears not to depend on activation of pathway-specific kinases, but on regulation of access of substrates to relatively generic multifunctional kinases.

In conclusion, the mechanisms of mammalian Hh signaling have clearly diverged from those of *Drosophila*. This suggests that even signal-transduction mechanisms of conserved signaling pathways have not been "locked" early in evolution, and that they can be subject to evolutionary change. The divergence of the Hh pathway—arguably the last major signaling pathway to evolve—is also relevant to the evolution of multicomponent signaling pathways. Some pathways, such as the Notch pathway, where the same protein (Notch) functions as a receptor and a transcriptional coactivator are relatively simple and consist of a small number of pathway-specific components (Artavanis-Tsakonas et al. 1999; PiresdaSilva and Sommer 2003). Other pathways, such as the Hh signaling pathway in *Drosophila* are more complex. In addition to many multifunctional proteins, the Hh pathway consists of 11 known specific components: Hh, Skinny hedgehog (Ski), Dispatched, iHog/boi, Ptc, Smo, Cos2, Fu, Su(Fu), and Ci (Burke et al. 1999; Chamoun et al. 2001; Lum and Beachy 2004). The emergence of the Cos2–Fu system in invertebrates suggests that such multicomponent pathways may evolve by insertion of novel proteins between existing pathway components.

Regulation of GLI activity

In contrast to the differences in signaling between Smo and GLI, the activities of the GLI proteins themselves are regulated similarly to Ci—with the added complexity that the activator and repressor functions of Ci are divided in mammals to three GLI proteins, GLI1–3 (Jacob and Briscoe 2003; Ruiz i Altaba et al. 2007). GLI1 and GLI2 are responsible for most activator functions and have similar activities at protein level (Bai and Joyner 2001). Whereas loss of GLI2 is embryonic lethal (Mo et al. 1997; Ding et al. 1998; Matise et al. 1998), GLI1 is dispensable for normal development (Park et al. 2000). GLI1 expression is induced by Hh ligands, and its function appears to be primarily to provide positive feedback and to prolong cellular responses to Hh. GLI3, in turn, functions primarily as a repressor (B. Wang et al. 2000; Litingtung et al. 2002), and its loss or mutation leads to limb malformations in mice and humans (Vortkamp et al. 1991; Schimmang et al. 1992).

GLI activity appears to be regulated by Hh in a way that is very similar to that observed in *Drosophila*. In the absence of Hh, GLI3 is phosphorylated, recognized by β-TrCP, and proteolytically processed to a truncated repressor form (B. Wang et al. 2000; Pan et al. 2006). Whether similar processing of GLI2 results in complete degradation or generation of a truncated repressor form is

unclear (Pan et al. 2006; Wang and Li 2006). Addition of Shh leads to inhibition of processing and accumulation of full-length forms of both GLI2 and GLI3.

Dose-, time-, and context-dependent responses to Hh

The developmental processes that the *Drosophila* and vertebrate Hh signaling pathways regulate appear remarkably conserved (Ingham and McMahon 2001). At the cellular level, the effects of Hh range from growth and self-renewal to cell survival (Liu et al. 1998; Rowitch et al. 1999), differentiation, and/or migration. During embryogenesis, the Hh cascade is used repeatedly and in different tissues to induce a large number of developmental processes. The ability of a single morphogen to affect almost every part of the vertebrate body plan is made possible by the fact that cellular responses to Hh depend on the type of responding cell, the dose of Hh received, and the time the cell is exposed to Hh (see below). At the molecular level, the diverse cellular responses are effected by induction of different sets of target genes. Among the genes regulated tissue specifically by Hh signaling are those encoding other secreted signaling proteins, including *bone morphogenetic protein 4* (*BMP4*) (Astorga and Carlsson 2007), *fibroblast growth factor 4* (*FGF4*) (Laufer et al. 1994), and *vascular endothelial growth factor* (*VEGF*)-*A* (Pola et al. 2001), genes involved in cell growth and division (e.g., *N-Myc*) (Oliver et al. 2003), and many transcription factors that are essential for animal development, including members of the Myod/Myf, Pax, Nkx, Dbx, and Irx families (Pierani et al. 1999; Gustafsson et al. 2002; Jacob and Briscoe 2003; Vokes et al. 2007). The total number of genes that Hh regulates is only beginning to be discovered: A number of expression profiling studies have identified several novel target genes (for example, see Xu et al. 2006; Vokes et al. 2007), and our genome-wide in silico analyses identified 42 conserved enhancer modules with two or more GLI sites in the human genome (Hallikas et al. 2006).

The genes that are induced by Hh in many tissues, in turn, are generally involved in positive and negative feedback to the pathway itself and include Hib, GLI1, Ptc, and HIP (Fig. 1C). As Ci and the GLI proteins act as repressors in the absence of Hh and activators in its presence, many of the target genes also behave similarly, being repressed in the absence of Hh and induced in its presence.

Hh acts both directly and indirectly to pattern tissues

During the development of the *Drosophila* wing imaginal disc, posterior (P) compartment cells express and secrete the Hh protein (Fig. 5A). The secreted Hh then induces the expression of target genes in cells located in the anterior (A) compartment. Hh acts both directly at intermediate range to pattern the anterior wing tissues close to the A–P boundary and indirectly over long range by inducing the BMP family morphogen decapentaplegic (dpp) (Basler and Struhl 1994; Tabata and Kornberg 1994). Dpp diffuses bidirectionally into both A and P compartments and controls the growth and patterning of the entire wing. Dpp expression is normally repressed by CiR, and its activation only requires that this repression is lifted. Therefore, very low levels of Hh suffice to induce dpp expression (Methot and Basler 1999). The short and intermediate range effects of Hh require induction of target genes such as *collier* (*col*) and *engrailed* (*en*), whose expression require CiA function and higher levels of Hh (Methot and Basler 1999; Hooper 2003).

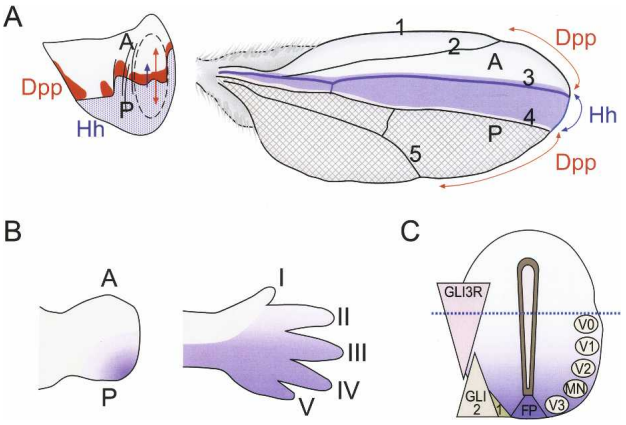

Figure 5. (A) Hh acts both directly and indirectly to pattern the *Drosophila* wing imaginal disc. (*Left*) Hh activates decapentaplegic (dpp; red) at the anterior side of the A–P boundary of the imaginal disc, which diffuses into and patterns both A and P compartments (red arrow). Hh (blue) also acts directly to pattern the anterior compartment close to the A–P boundary. (*Right*) Adult wing showing the regions derived from the anterior (A, *top*) and posterior compartment (P, *bottom*, shaded), and the regions patterned by Dpp (red arrows) and Hh (blue color, between wing veins 3 and 4). (*B*) Shh has a similar role in anterior-posterior patterning of the distal elements of vertebrate limbs and in specifying digit identity (roman numerals). (*C*) Time and dose-dependent action of Shh. The gradient of Shh (blue color) emanating from the notochord (not shown) and floor plate (FP) progressively defines five different neuronal subtypes in the ventral neural tube. The Shh protein gradient is converted to gradient of GLI activities shown on the *left*. GLI1 and GLI2 (*bottom*) act as transcriptional activators, whereas GLI3 functions as a repressor (GLI3R, *top*). (MN) Motoneuron; (V0–V3) interneurons. Dotted line indicates the dorsal limit of the domain patterned by the Shh gradient. Data adapted from Fuccillo et al. (2006).

Shh has an analogous role in controlling vertebrate limb patterning. Shh expressed by the ZPA located at the posterior margin of developing limb buds diffuses to adjacent tissues, forming a temporal and spatial gradient that specifies the anterior–posterior pattern of the limbs (Fig. 5B).

Time and dose dependency of the Hh response

The effect of Hh dose on target tissue responses is best characterized in the specification of cell identities in the ventral neural tube (Jessell 2000; Patten and Placzek 2000; Marti and Bovolenta 2002). During neural tube de-

velopment, Shh protein diffuses from the notochord and floor plate, creating a concentration gradient across the ventral neural tube (Fig. 5C). Different doses of Shh within this gradient specify five neuronal subtypes at precise positions along the floor plate–roof plate axis. Initially, Shh induces Class II homeodomain (e.g., Nkx2.2, Nkx6.1) (Pierani et al. 1999; Jacob and Briscoe 2003) and represses Class I homeodomain (Pax6, Pax7, Irx3, and Dbx1/2) transcription factors. Cross-repressive interactions between these factors then act to sharpen the expression boundaries and to subsequently direct cells to differentiate into specific lineages (Briscoe and Ericson 2001).

The activity of Shh as a morphogen was initially thought to be due to concentration-dependent responses, but the duration of Shh signal seems also to critically affect cellular responses. Both during neural tube and limb development, the pattern of cellular differentiation is controlled not only by the amount but also by the time of Shh exposure (Briscoe and Ericson 2001; Ahn and Joyner 2004; Harfe et al. 2004). The changing of the concentration or duration of Shh seem to have an equivalent effect on intracellular signaling.

Chick neural cells convert different concentrations of Shh into time-limited periods of signal transduction, such that signal duration is proportional to Shh concentration (Dessaud et al. 2007). This depends on the gradual desensitization of cells to Shh caused by up-regulation of patched (Ptc) (Taipale et al. 2002). Thus, in addition to its role in shaping the Shh gradient (Chen and Struhl 1996; Briscoe et al. 2001; Jeong and McMahon 2005), Ptc participates cell-autonomously in gradient sensing. This mechanism integrates Shh signal strength over time, allowing cells to more accurately determine their distance from the Hh source—resulting in more robust patterning of the nervous system.

Role of Hh signaling in young and adult mammals

The multiple roles of Hh signaling in embryonic patterning are discussed above and reviewed in more detail in McMahon et al. (2003). Much less is known about the roles played by Hh in pupal development and in maintaining homeostasis of tissues during adult life.

During maturation of mouse pups, Ihh signaling is important for bone growth. Permanent deletion of *Ihh* in chondrocytes of mice carrying conditional and inducible null alleles of *Ihh* results in permanent defects in bone growth, inhibiting proliferation and promoting differentiation of chondrocytes, leading to dramatic expansion of the hypertrophic zone (Razzaque et al. 2005; Maeda et al. 2007) and truncation of long bones. Interestingly, similar phenotype was observed with treatment of young mice with Smo antagonist for just 48 h (Kimura et al. 2008). In adults, Hh pathway controls bone homeostasis; activation of the Hh pathway in osteoblasts leads to bone resorption, and conversely, Hh inhibition protects aging mice against bone loss (Mak et al. 2008; Ohba et al. 2008). Adult mice seem to tolerate Hh antagonists well, suggesting that short-term Hh pathway inhibition might not interfere with the possible role of Hh as a stem cell factor (Berman et al. 2002; Kimura et al. 2008).

The best-characterized role for Hh signaling in adults is in the reproductive system, and Hh proteins are expressed and required for maturation of the germ cells in multiple species. In *Drosophila* ovary, Hh acts as a somatic stem cell factor, directly controlling the proliferation and maintenance of ovarian somatic stem cells (Zhang and Kalderon 2001). In mammals, Ihh and Dhh produced by granulosa cells act as paracrine factors to induce target gene expression in the developing theca cell compartment. This suggests that hedgehog signaling regulates the theca cell development in growing follicles (Wijgerde et al. 2005). Dhh also has a role in the regulating the development and function of the somatic cells of the testis (Bitgood et al. 1996; Yao et al. 2002).

Aberrant Hh signaling in disease

Loss of Hh signaling activity during vertebrate embryogenesis causes severe developmental disorders including holoprosencephaly, polydactyly, craniofacial defects, and skeletal malformations (Muenke and Beachy 2000; Hill et al. 2003; McMahon et al. 2003; L. Zhang et al. 2006). It is now also becoming evident that components required for the function of primary cilia are required in mammalian Shh signaling (Huangfu et al. 2003). It is therefore possible that Hh signaling may also be altered in human syndromes caused by defects in cilia, including Meckel, Bardet-Biedl and Kartagener syndromes, polycystic kidney disease, and retinal degeneration (Pan et al. 2005; Kyttala et al. 2006).

On the other hand, aberrant activation of Hh signaling can cause basal cell carcinoma (BCC, the most common type of skin cancer) (Hahn et al. 1996; Johnson et al. 1996), medulloblastoma (a childhood cancer with an invariably poor prognosis) (Goodrich et al. 1997; Berman et al. 2002), and rhabdomyosarcoma (Table 1; Kappler et al. 2004). These tumor types occur at an increased rate in patients or mice with germline mutations in Ptc, and sporadic cases are often associated with mutations in the Hh pathway components Ptc, Smo, or Su(Fu), or more rarely, the amplification of GLI1.

Aberrantly activated Shh signaling has also been suggested to play a role in other cancers, such as glioma, breast, esophageal, gastric, pancreatic, prostate, and small-cell lung carcinoma (see Table 1 for references). With the exception of rare GLI1 amplifications found in gliomas (Kinzler et al. 1987), the mutational basis of Hh pathway activation in these types of cancer has not been ascertained.

Multiple lines of evidence suggest that Hh acts to promote cancer by directly regulating cellular growth and/or survival. Loss of one ptc allele causes larger body size in mice (Goodrich et al. 1997) and in humans (Gorlin 1987). Several common human single nucleotide polymorphisms affecting body height map close to Hh pathway components, including Ihh, Ptc, and Hip (Lettre et al. 2008; Weedon et al. 2008), suggesting that individual variation in height is determined in part by the strength

Table 1. *Cancers linked to aberrant Shh signaling*

Cancer type	References
Basal cell carcinoma (BCC)	(Hahn et al. 1996; Johnson et al. 1996)
Medulloblastoma	(Goodrich et al. 1997; Berman et al. 2002)
Rhabdomyosarcoma	(Hahn et al. 1996; Kappler et al. 2004)
Glioma	(Kinzler et al. 1987)
Breast cancer	(Kubo et al. 2004)
Esophageal cancer	(Berman et al. 2003; Watkins and Peacock 2004)
Gastric cancer	(Berman et al. 2003)
Pancreatic cancer	(Thayer et al. 2003)
Prostate cancer	(Karhadkar et al. 2004; Sanchez et al. 2004)
Small-cell lung cancer	(Watkins et al. 2003)
Biliary tract cancer	(Berman et al. 2003)
Bladder cancer	(Hamed et al. 2004)
Oral cancer	(Nishimaki et al. 2004)

Mutations in Hh pathway components have been identified in BCC, medulloblastoma, rhabdomyosarcoma, and glioma (top).

of negative feedback loops that fine-tune Ihh signaling during bone growth. Hh pathway controls growth also during embryonic development—transgenic mice that overexpress *ptc* are consistently smaller than control mice, but remarkably well proportioned, illustrating that Hh signaling controls growth in many tissues. However, whether this growth effect is direct or indirectly caused by altered placental or vascular development is unclear.

In development of midbrain and forebrain, Shh is crucial in driving the rapid, extensive expansion of the early brain vesicles. The action of Shh is mediated through coordination of cell proliferation and survival (Britto et al. 2002). In addition, Shh has been implicated in regulating cell proliferation and survival in a number of other cell types, including retinal precursor cells (Jensen and Wallace 1997), myoblasts (Duprez et al. 1998), optic nerve astrocytes (Wallace and Raff 1999), cerebellar granule cells (Dahmane and Ruiz i Altaba 1999), and neural crest cells (Ahlgren and Bronner-Fraser 1999).

The molecular mechanisms by which Shh controls growth are beginning to be unraveled. In vitro studies have shown that the Shh protein up-regulates N-myc expression in cerebellar granule neuron progenitor (CGNP) cultures and that N-myc overexpression promotes CGNP proliferation even in the absence of Shh (Kenney et al. 2003). N-myc is thought to promote proliferation of CGNPs synergistically with cyclins D and E (Knoepfler et al. 2002), whose expression is also regulated by Shh (Duman-Scheel et al. 2002).

Direct evidence for the role of N-myc in pathway-associated cancer comes from a study of Shh pathway-induced medulloblastoma formation in mice, where it was shown that the disruption of N-myc, but not c-myc, inhibits cellular proliferative responses to Shh (Hatton et al. 2006). This provides in vivo evidence that N-myc plays a central role in Shh-mediated proliferation in CGNPs and in medulloblastoma cells, which are thought to be derived from CGNPs (Hatton et al. 2006).

Potential for therapeutic intervention

As the Hh pathway in BCC and medulloblastoma is often affected at the level of Ptc or Smo, small molecule antagonists should act at/or downstream from these components (Taipale et al. 2000). Furthermore, several studies have shown that Smo can be targeted by small molecule drugs, and that antagonizing Smo could provide a way to interfere with tumorigenesis and tumor progression. The most commonly used antagonist of the Hh pathway is the plant alkaloid cyclopamine (Taipale et al. 2000). Cell-based high-throughput screening has revealed several distinct classes of antagonists, which, like cyclopamine, bind to Smo. These include SANTs 1–4 (Chen et al. 2002b); KAAD-cyclopamine (Taipale et al. 2000), compound-5 and compound-Z (Borzillo and Lippa 2005), and Cur-61414 (Frank-Kamenetsky et al. 2002). Although one phase I clinical trial has already reported promising results of Hh pathway antagonist in advanced BCC (Garber 2008), further clinical studies are needed to establish which of these antagonists are suitable for therapeutic use. As it has been proposed that autocrine Shh expression is required for growth of some cancers (Dahmane et al. 1997; Karhadkar et al. 2004), and stromal cell-derived Shh can also activate the Hh pathway in tumors (Becher et al. 2008), it might also be possible to treat tumors with Shh-specific monoclonal antibodies. In fact, one such antibody, 5E1, has been shown to block the growth of some tumors, including small-cell lung carcinoma (Watkins et al. 2003). In addition to targeting tumors that themselves have hyperactive Hh pathways, antagonists of the Hh pathway could also affect growth of tumors that use Hh ligands to induce angiogenesis (Pola et al. 2001; Nagase et al. 2008) or recruit other types of stromal cells supporting tumor growth. Further studies are needed to characterize the role that Shh plays in such tumor–host interactions.

Because adults can tolerate inhibition of the Hh pathway (Berman et al. 2002; Kimura et al. 2008), specifically blocking Hh signaling offers an effective treatment for the various cancers originating from aberrant Hh pathway activation. However, systemic treatment of pediatric tumors such as medulloblastoma may not be feasible due to the severe effects that transient inhibition of the Hh pathway has on bone growth (Kimura et al. 2008).

Perspective

The Hh signaling pathway was first identified in *Drosophila* 16 yr ago. Subsequently, it has taken its rightful place among the major signaling pathways controlling animal development, being found to regulate the morphogenesis of a variety of tissues and organs during the development of organisms ranging from *Drosophila* to human (McMahon et al. 2003). In addition, the Hh pathway has been linked to multiple forms of human cancer,

and the possibilities for therapeutic intervention are being actively pursued.

The synthesis and processing of the Hh ligand, its release and transport through tissues, and mechanisms of signal transduction in the receiving cells have been studied extensively. However, many aspects of Hh signaling remain incompletely understood. Further research is needed in multiple areas, including the study of Hh target gene responses, which is required to understand in detail how the graded Hh signals are converted to discrete cell-fate decisions, and to decipher the molecular mechanisms that underlie the exquisite specificity of cellular responses to Hh. In addition, the therapeutic potential of Hh pathway agonists and antagonists in human degenerative diseases and cancer should be further investigated.

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