Hedgehog signaling

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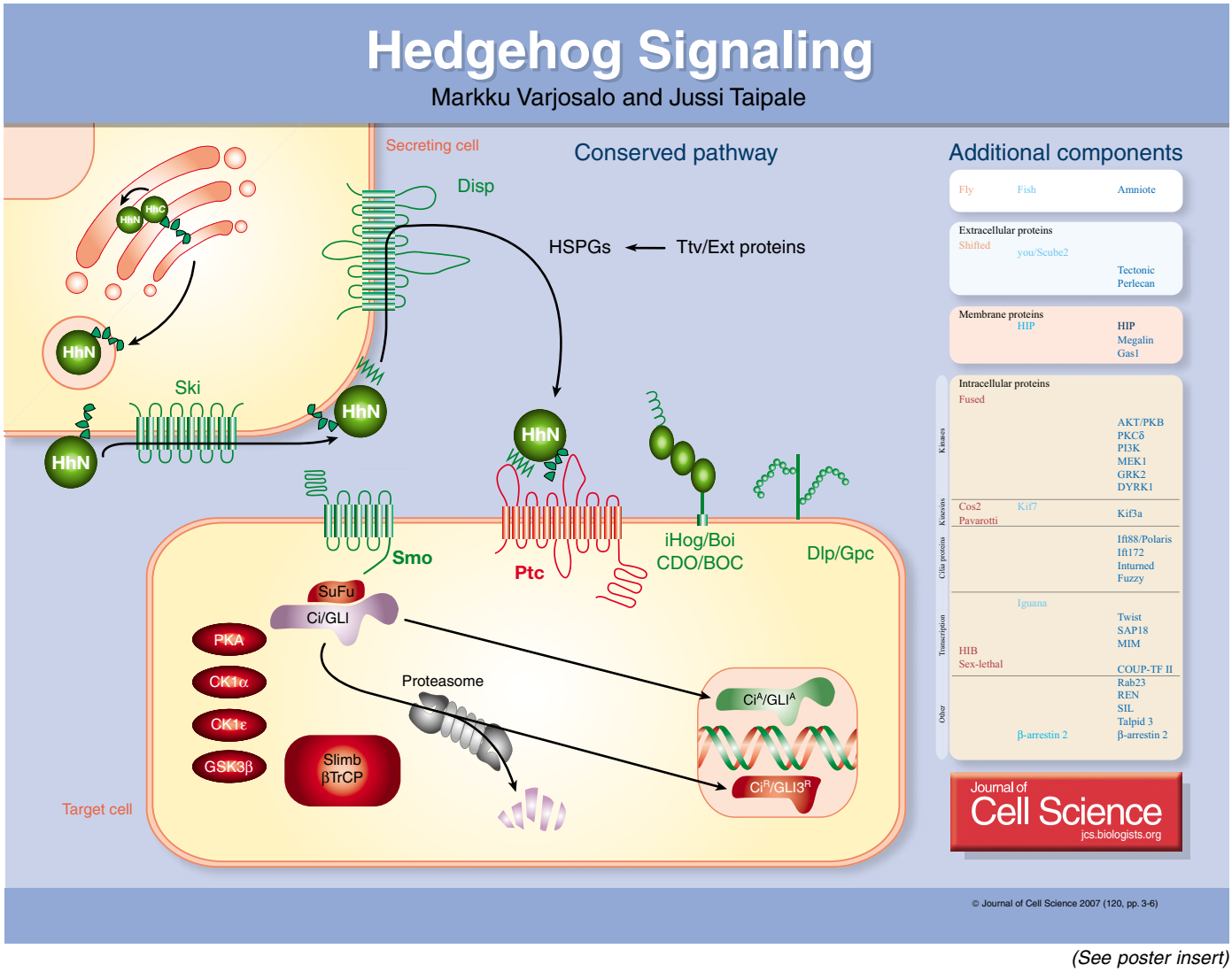
The Hedgehog (Hh) family of secreted signaling proteins plays a crucial role in development of diverse animal phyla, from *Drosophila* to humans, regulating morphogenesis of a variety of tissues and organs (McMahon et al., 2003). Hh signaling is also involved in control of stem cell proliferation in adult tissues and aberrant activation of the Hh pathway has been linked to multiple types of human cancer (Taipale and Beachy, 2001). A lot has happened since the initial discovery and the molecular cloning of *Drosophila* Hh (Lee et al., 1992; Nusslein-Volhard and Wieschaus, 1980). Many components involved in Hh signal transduction in *Drosophila* have since been identified and characterized. However, the vertebrate Hh signaling pathway may yet offer some surprises. Here we present an overview of Hh signaling in the light of recent data, which has revealed an unexpected level of divergence of the mechanism of Hh signaling in flies, fish and amniotes.

Hh expression, secretion and processing

Hh is involved in the patterning of a wide variety of tissues in many species (McMahon et al., 2003). Accordingly, the expression of the different Hh isoforms is tightly controlled by highly complex and divergent transcriptional enhancers (see Sagai et al., 2005 and references therein). The mechanisms for subsequent Hh processing and secretion appear to be conserved in evolution and are likely to apply to all Hh isoforms, including the mammalian Hh proteins Sonic hedgehog (Shh), Desert hedgehog (Dhh) and Indian Hedgehog (Ihh) (Ingham and McMahon, 2001).

The Hh proteins undergo multiple processing steps. First, the signal sequence is cleaved. Then the C-terminal domain of the Hh polypeptide catalyzes an intramolecular cholesteroyl transfer reaction resulting in formation of a C-terminally cholesterol-modified N-terminal Hh signaling domain (HhN) (Porter et al., 1996). The cholesterol



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modification results in the association of Hh with membranes, facilitating the final processing step in which a palmitoyl moiety (Pepinsky et al., 1998) is added to the N-terminus of HhN by the transmembrane acyltransferase Skinny hedgehog (Ski) (Chamoun et al., 2001; Lee et al., 2001). This generates the fully active, dually lipid-modified HhN.

Release and transport of Hh through tissues

Despite its tight membrane association, Hh is able to affect patterning of distal tissues, acting directly over a long range in a time- and concentration-dependent manner (Stamataki et al., 2005). The formation of the gradient of Hh activity emanating from the secreting cells is facilitated by multiple macromolecules, which control release, transport and sequestration of Hh. Hh is released from the secreting cell by Dispatched (Disp), a conserved protein that shares sequence similarity with transmembrane transporters (Burke et al., 1999; Zhu and Scott, 2004). Subsequent transport of Hh through tissues requires heparan sulfate, as indicated by the failure of Hh transport in embryos lacking heparan-sulfate-synthesizing enzymes of the EXT/tout velu (ttv) family (The et al., 1999; Zhu and Scott, 2004). The cholesterol modification of Hh also affects the range of Hh action by affecting its palmitoylation, stability, diffusion and/or transport (Callejo et al., 2006; Dawber et al., 2005; Gallet et al., 2006; Lewis et al., 2001; Li et al., 2006; Mann and Beachy, 2004).

Several other proteins that affect Hh transport and/or shape the Hh gradient have been described in different species. For example, in addition to the Hh receptor Patched (Ptc), which sequesters Hh and restricts its range of action in all species analyzed (Chen and Struhl, 1996; Zhu and Scott, 2004), vertebrates have an additional transmembrane protein, Hh-interacting protein (Hip), which binds to Hh proteins and reduces their range of movement (Chuang and McMahon, 1999; Zhu and Scott, 2004).

Receiving the Hh signal

The binding of Hh to cells is facilitated by two classes of accessory receptor: the glypican-family of cell surface proteoglycans (e.g. dally-like in *Drosophila*) (Lum et al., 2003a) and the transmembrane proteins iHog and Boi (CDO and BOC in vertebrates) (Tenzen et al., 2006; Yao et al., 2006). iHog and Boi also increase the binding affinity of Hh for the signaling receptor Ptc, a 12-span transmembrane protein related to bacterial transmembrane transporters of the resistance-nodulation-division (RND) family. In the absence of Hh, Ptc catalytically inhibits the activity of the seven-transmembrane-span receptor-like protein Smoothened (Smo) (Taipale et al., 2002), potentially by affecting localization and/or concentration of a small molecule. Smo activity can be modulated by many synthetic small molecules (Chen et al., 2002b). Of endogenous metabolites, oxysterol derivatives (Corcoran and Scott, 2006) and vitamin D3 derivatives (Bijlsma et al., 2006) have been suggested to mediate the effects of Ptc on Smo.

Binding of Hh to Ptc results in loss of Ptc activity, and consequent activation of Smo, which transduces the Hh signal to the cytoplasm (Stone et al., 1996; Taipale et al., 2002), ultimately leading to the activation of the Ci/GLI family of transcription factors (Lum and Beachy, 2004; Matise and Joyner, 1999; Methot and Basler, 2001).

Divergence of the mechanism of intracellular Hh signaling

The components and mechanisms involved in Hh signaling from secretion to reception of signal thus appear largely conserved. However, a major divergence of mechanism appears to have taken place between the Smo signal transducer and the Ci/GLI transcription factors.

In *Drosophila*, Smo accumulates at the cell surface after Hh stimulation (Denef et al., 2000). By contrast, oncogenically activated mammalian Smo proteins localize to the endoplasmic reticulum (Chen et al., 2002a), and mammalian Smo has been reported to internalize after pathway activation (Incardona et al., 2002). Thus, it seems that Smo localization is differentially regulated in vertebrates and invertebrates.

Furthermore, phosphorylation of Smo is also differentially regulated in *Drosophila* and in mammals. In *Drosophila*, Smo activation is coupled to the hyperphosphorylation of 26 serine/threonine residues of its C-terminal cytoplasmic tail by protein kinase A (PKA) and casein kinase I (CKI) (Apionishev et al., 2005; Jia et al., 2004; Zhang et al., 2004). However, none of these phosphorylation sites is conserved in mammals, although many of them are located within or at the border of the evolutionarily conserved region of Smo (Lum et al., 2003b; Varjosalo et al., 2006).

Further evidence of divergence comes from analysis of the atypical kinesin Costal2 (Cos2), which is a key negative regulator of the Hh pathway downstream of Smo in *Drosophila* (Hooper and Scott, 2005). Cos2 forms a tight protein complex with Fused (Fu), a protein kinase that acts positively on the Hh pathway. Cos2 also bridges Smo to the Ci transcription factor by associating directly with both of these proteins (Jia et al., 2003; Lum et al., 2003b; Ruel et al., 2003). In the absence of Hh, full-length Ci is retained in the cytoplasm by Cos2 and another protein: Suppressor of Fused [Su(Fu)]. Cos2 also promotes phosphorylation of Ci by PKA, GSK3β and CKI. Phosphorylated Ci associates with the Slimb/βTrCP E3 ubiquitin ligase, and is processed by the proteasome to a repressor form (Ci^R). In the presence of Hh, Smo is stabilized and activated, leading to increased association of Cos2 to Smo. Cos2 bound to active Smo is not able to facilitate Ci processing, and Ci enters the nucleus as a transcriptional activator (Ci^A) (Lum and Beachy, 2004).

By contrast, mouse Smo is not stabilized after Shh addition, and mouse Smo does not bind to either Kif27 or to Kif7, the two mouse orthologs of Cos2. Furthermore, neither overexpression or RNAi-mediated knockdown of the these two Cos2 orthologs has any effect on Shh pathway activity or on Gli transcriptional activity (Varjosalo et al., 2006). Thus, it appears that mammals lack a functional equivalent of Cos2. Mammalian Fu also appears not to act on the Hh pathway: Fu-knockout mice fail to show any indications of disturbance of Hh signaling during embryogenesis (Chen et al., 2005; Merchant et al., 2005). Thus, although mammalian GLI proteins are regulated by

phosphorylation and proteolytic processing analogously to Ci (Pan et al., 2006; Wang et al., 2000), coupling of Smo to the regulation of GLI activation and processing appears not to require Cos2 or Fu orthologs.

If Cos2 is not needed to suppress the mammalian Hh pathway in the absence of ligand, can Su(Fu) alone suppress the pathway? This seems to be the case because loss of Su(Fu) results in complete activation of the Hh pathway in mouse embryos, essentially phenocopying the effects of loss of Ptc function (Svard et al., 2006). This is in striking contrast to *Drosophila*, in which the Su(Fu)-null mutant phenotype is so mild that it was initially not reported and only later identified by a detailed study of Su(Fu)-null fly wings (Ohlmeyer and Kalderon, 1998).

Vertebrate-specific components in Hh signaling

A number of vertebrate-specific Hh regulators either have no known *Drosophila* ortholog or have orthologs that appear not to affect Hh signaling in *Drosophila*. For example, mutations in several components required for formation of primary cilia, including Kif3a, Ift88 and Ift172, result in embryonic phenotypes characteristic of loss of Shh signalling (Huangfu et al., 2003). Subsequent biochemical studies have linked these proteins to processing of GLI transcription factors (Liu et al., 2005). It has also been reported that Smo, Su(Fu) and unprocessed Gli proteins are localized to the primary cilium (Corbit et al., 2005; Haycraft et al., 2005). However, the role of these cilia proteins in Hh signaling seems to be specific to mammals, because loss of their orthologs appears not to affect Hh signaling in *Drosophila* or Zebrafish (Nybakken et al., 2005; Sun et al., 2004).

Future directions

Although many players in the Hh signaling cascade have been identified, we know surprisingly little about the precise mechanisms by which they act. Open questions in the Hh signaling field concern, for example, how Ptc controls Smo activity. How is Smo activity coupled to phosphorylation of Ci/GLI? What is the role of cilia in mammalian Hh signaling, how is Su(Fu) activity and Ci/GLI nuclear localization regulated, and which target genes mediate the effects of Hh in different tissues? Owing to the many unconventional components in this pathway, the established signaling paradigms do not seem to apply. Thus, new thinking and innovative experimental strategies are needed to reveal the mechanisms that lie beneath this key signaling pathway.

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