Markku Varjosalo

The Mechanisms of Hedgehog Signal Transduction

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A C A D E M I C D I S S E R T A T I O N

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ABSTRACT

Studies in both vertebrates and invertebrates have identified proteins of the Hedgehog (Hh) family of secreted signaling molecules as key organizers of tissue patterning. Initially discovered in *Drosophila* in 1992, Hh family members have been discovered in animals with body plans as diverse as those of mammals, insects and echinoderms. In humans three related Hh genes have been identified: Sonic, Indian and Desert hedgehog (Shh, Ihh and Dhh). Transduction of the Hh signal to the cytoplasm utilizes an unusual mechanism involving consecutive repressive interactions between Hh and its receptor components, Patched (Ptc) and Smoothened (Smo). Several cytoplasmic proteins involved in Hh signal transduction are known in *Drosophila*, but mammalian homologs are known only for the Cubitus interruptus (Ci) transcription factor (GLI(1-3)) and for the Ci/GLI-associated protein, Suppressor of Fused (Su(fu)). In this study I analyzed the mechanisms of how the Hh receptor Ptc regulates the signal transducer Smo, and how Smo relays the Shh signal from the cell surface to the cytoplasm ultimately leading to the activation of GLI transcription factors. In *Drosophila*, the kinesin-like protein Costal2 (Cos2) is required for suppression of Hh target gene expression in the absence of ligand, and loss of Cos2 causes embryonic lethality. Cos2 acts by bridging Smo to the Ci. Another protein, Su(Fu) exerts a weak suppressive influence on Ci activity and loss of Su(Fu) causes subtle changes in *Drosophila* wing pattern. This study revealed that domains in Smo that are critical for Cos2 binding in *Drosophila* are dispensable for mammalian Smo function. Furthermore, by analyzing the function of Su(Fu) and the closest mouse homologs of Cos2 by protein overexpression and RNA interference I found that inhibition of the Hh response pathway in the absence of ligand does not require Cos2 activity, but instead critically depends on the activity of Su(Fu). These results indicate that a major change in the mechanism of action of a conserved signaling pathway occurred during evolution, probably through phenotypic drift made possible by the existence in some species of two parallel pathways acting between the Hh receptor and the Ci/GLI transcription factors. In a second approach to unravel Hh signaling we cloned > 90% of all human full-length protein kinase cDNAs and constructed the corresponding kinase-activity deficient mutants. Using this kinome resource as a screening tool, two kinases, MAP3K10 and DYRK2 were found to regulate Shh signaling. DYRK2 directly phosphorylated and induced the proteasome dependent degradation of the key Hh-pathway regulated transcription factor, GLI2. MAP3K10, in turn, affected GLI2 indirectly by modulating the activity of DYRK2.

Keywords: Hedgehog, signaling, patterning

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TIIVISTELMÄ

Hedgehog (Hh) -perheen kasvutekijät säätelevät alkion kehitystä ja kudosten erilaistumista niin selkärankaisissa kuin selkärangattomissakin eläinlajeissa. Hh-kasvutekijää koodaava geeni löydettiin vuonna 1992 banaanikärpäsestä (*Drosophila melanogaster*), jonka jälkeen tämän geeniperheen geenejä on löydetty useista muistakin eliölajeista –hyönteisistä nisäkkäisiin. Ihmisellä Hh geenejä on kolme: Sonic (Shh), Indian (Ihh) ja Desert (Dhh). Hedgehog kasvutekijän signaalinvälitykselle ovat tyypillisiä useat molekyylien väliset estävät vuorovaikutukset. Hh kasvutekijän sitoutuminen reseptoriproteiini Patchediin (Ptc) estää Ptc:n toiminnan ja vapauttaa signaalinvälittäjäproteiini Smoothenedin (Smo) Ptc:n estävästä vaikutuksesta. Useat Hh signaalinvälitykseen osallistuvat molekyylit on alunperin löydetty banaanikärpäsestä, ja nisäkkäiden Hh signaalinvälitykseen osallistuvista sytoplasmisista komponenteista on tunnistettu vain transkriptiotekijä Cubitus interruptusta (Ci) vastaavat GLI(1-3), ja Ci/GLI proteiineja sitova Suppressor of Fused (Su(Fu)). Tämän tutkimuksen tarkoituksena oli selvittää kuinka Shh-signaali välittyy solun pinnalta solun sisään johtaen lopulta GLI transkriptiotekijöiden aktivoitumiseen ja signaalinvälitysreitin kohdegeenien ilmentymiseen. Banaanikärpäsessä kinesiinin-kaltainen proteiini Costal2 (Cos2) säätelee keskeisesti signaalinvälitysreitin toimintaa ja Cos2 geenin puute johtaa kärpäsessä toukan kehityksen häiriintymiseen ja kuolemaan. Myös toinen proteiini, Su(Fu) osallistuu Ci-transkriptiotekijän aktiivisuuden hienosäätöön banaanikärpäsessä, mutta sen puutos aiheuttaa vain hyvin pieniä muutoksia siiven rakenteessa. Tutkimuksessamme havaitsimme yllättäen, että Hh-signaalinvälitysreitin toiminta eroaa merkittävästi nisäkkäiden ja kärpäsen välillä, alkaen heti solun pinnalla olevan Smo proteiinin rakenteesta. Eristettyämme Cos2 geenin nisäkäshomologit huomasimme myös, ettei niiden toimintaa tarvita nisäkkäissä. Nisäkkäissä signaalireitin aktiivisuutta sääteleekin keskeisesti Su(Fu). Tuloksemme osoittavat, että muuttumattomina pidetyt signaalinvälitysreitit voivat muuttua evoluution aikana. Löytääksemme uusia nisäkkäiden Hh-signaalinvälitysreitin toimintaa sääteleviä geenejä, eristimme myös yli 90% kaikista ihmisen proteiinikinaaseista ja analysoimme niiden merkitystä Shh-signaalireitin toiminnalle tehoseulontamenetelmin. Näin pystyimme löytämään kaksi uutta Shh-signaalireitin toimintaa säätelevää proteiinikinaasia, MAP3K10 ja DYRK2. Havaitsimme, että DYRK2 vaikuttaa suoraan GLI-transkriptiotekijöihin fosforyloimalla niitä ja johtaen näin niiden hajotukseen. MAP3K10 puolestaan vaikuttaa GLI-transkriptiotekijöihin epäsuorasti, säätelemällä muiden kinaasien, mm. DYRK2:n, toimintaa.

Avainsanat: Hedgehog, signaalinvälitys, kaavoittuminen

CONTENTS

ABBREVIATIONS

A	anterior
ABC	ATP-binding cassette
Arrb2	β-arrestin 2
ATP	adenosine 5'-triphosphate
BCC	basal cell carcinoma
BMP	bone morphogenetic protein
botv	brother of tout velu
cDNA	complementary DNA
Ci	Cubitus interruptus
CiA	155 kDa transcriptional activator form of Ci
CiR	75 kDa transcriptional repressor form of Ci
CKI	casein kinase I
CNS	central nervous system
col	collier
Cos2	Costal2
D	dorsal
Dhh	Desert hedgehog
Disp	Dispatched
DNA	deoxyribonucleic acid
dpp	decapentaplegic
Ehh	Echidna hedgehog
en	engrailed
EXT	Exostosin
FACS	fluorescent-activated cell sorting
Fu	Fused

GLI(1-3)	glioma-associated oncogenes 1-3
GLI3R	transcriptional repressor form of GLI3
GPI	glycosylphosphatidylinositol
GSK3	glycogen synthase kinase 3
GTP	guanosine-5'-triphosphate
Hh	Hedgehog
HhN	amino (N)-terminal Hh signaling domain
HhNp	dually lipidated HhN
HIP	Hedgehog interacting protein
HSPG	heparan sulfate proteglycans
Ihh	Indian hedgehog
P	posterior
PAGE	polyacrylamide gel electrophoresis
PKA	protein kinase A
PKC	protein kinase C
Ptc	Patched
Qhh	Qiqihar hedgehog
RND	resistance-nodulation-division
SANT	Smo antagonist
SDS	sodiumdodecylsulfate
Shh	Sonic hedgehog
sotv	sister of tout velu
Su(Fu)	Suppressor of Fused
TGFβ	transforming growth factor β
ttv	tout velu
V	ventral
wg	wingless

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to in the text by their Roman numerals:

I Varjosalo, M., Li, SP., Taipale, J. Divergence of hedgehog signal transduction mechanism between Drosophila and mammals. Developmental Cell 10, 177-186, 2006.

II De Rivoyre, M.*, Ruel, L.*, Varjosalo, M., Loubat, A., Bidet, M., Thérond, P., Mus-Veteau, I. Human receptors patched and smoothened partially transduce hedgehog signal when expressed in Drosophila cells. Journal of Biological Chemistry 281, 28584-28595, 2006.

III Varjosalo, M., Björklund, M., Cheng, F., Syvänen, H., Kivioja, T., Kilpinen, S., Sun, Z., Kallioniemi, O., Stunnenberg, H.G., He, W-W., Ojala, P., Taipale, J. Application of active and kinase-deficient kinome collection for identification of kinases regulating Hedgehog signaling. Cell, *In Press*, 2008.

*) These authors contributed equally to this work

1 INTRODUCTION

The Hedgehog (Hh) family of secreted signaling molecules regulates the morphogenesis of variety of tissues and organs during the development of organisms ranging from *Drosophila melanogaster* (fruit fly) to *Homo sapiens* (human) (McMahon et al., 2003). Hh signaling is also involved in homeostasis and stem cell proliferation in adult tissues. Furthermore, aberrant activation of the Hh pathway has been linked to the development of multiple human cancers (Taipale and Beachy, 2001). Mutations affecting the *Hh* gene were initially discovered by Nusslein-Volhard and Wieschaus (Nusslein-Volhard and Wieschaus, 1980) during their large-scale screen for mutations that would impair or change the development of the fruit fly larval body plan. They identified many genes for early *Drosophila* development including the segment polarity gene, *Hh*. The origin of the name Hedgehog derives from the short and "spiked" phenotype of the Hh mutant *Drosophila* larvae.

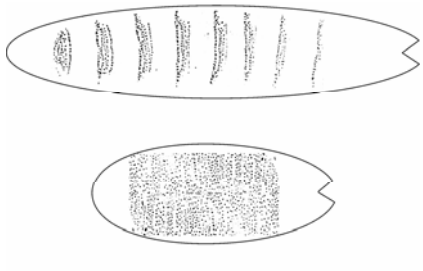

Figure 1. Wildtype (upper) and Hedgehog mutant (lower) larval cuticular phenotypes. The length of a Hedgehog mutant is approximately half of a wildtype larva, and exhibits a ventral lawn of denticles associated with the loss of naked cuticle from the ventral surface. The denticle pattern reflects a complete lack of segmentation (adapted from Mohler, 1988).

The molecular mechanisms of Hh signal transduction have been characterized mainly using elaborate *Drosophila* genetics and cell based assays. Currently, the mammalian Hh signaling pathway is incompletely understood and may harbor some differences and/or additional pathway components. The overall framework and most of the key components of the Hh signaling pathway are evidently conserved between *Drosophila* and vertebrates. In both cases the binding of Hh to its receptor Patched (Ptc) activates a signaling cascade that ultimately drives the expression of Hh target gene(s). The transcription factors that respond to the Hh signal are Cubitus interruptus (Ci) in *Drosophila* and the mammalian homologs GLI(1-3). It was long thought that the main difference between *Drosophila* and mammalian Hh signaling was the functional redundancy between proteins of the Hh subgroups and the three mammalian GLI(1-3) transcription factors. However, critical components of the *Drosophila* pathway, that relay the signal from Smoothened (Smo) to the Ci/GLI transcription factors, such as the protein kinase Fused (Fu) and the atypical kinesin Costal2 (Cos2), appear to be missing in vertebrates (publication I; Chen et al., 2005; Merchant et al., 2005; Svard et al., 2006).

This literature review focuses on current understanding of the different steps of Hh signaling: synthesis and processing of the Hh ligand; release and transport of Hh through tissues; Hh receptor binding and signal relay via intracellular processes; and activation of the Ci/GLI transcription factors and regulation of target genes. Also reviewed is the physiological significance of this key signaling pathway in embryonic development, the role of Hh in the pathogenesis of human disease and the possibilities of therapeutic intervention.

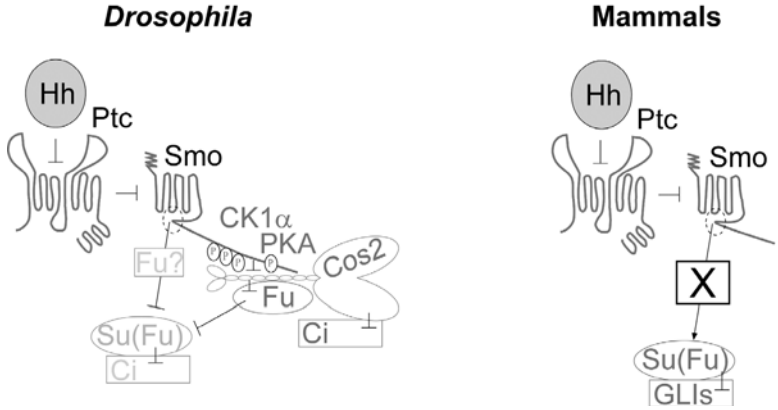

Figure 2. Overview of the Hedgehog signaling pathways in *Drosophila* and mammals.

2 REVIEW OF THE LITERATURE

2.1 Hedgehog and Hedgehog signaling in development

2.1.1 Cloning of the Hedgehog genes

Cloning of the *Drosophila* Hh gene in 1992 (Lee et al., 1992) was soon followed by the identification of *Mus musculus* (mouse), *Danio rerio* (zebrafish) and *Gallus gallus* (chicken) Hh genes (Echelard et al., 1993; Krauss et al., 1993; Riddle et al., 1993). Hh genes were then found in a range of other invertebrates including *Hirudo medicinalis* (leech) and *Diadema antillarum* (sea urchin) (Chang et al., 1994; Inoue et al., 2002; Shimeld, 1999). The *Rattus rattus* (rat) and human genes were cloned soon after (Marigo et al., 1995; Roelink et al., 1994). It is interesting to note that the model organism *Caenorhabditis elegans* (roundworm), has no Hh ortholog even though it has several homologous proteins to the Hh receptor Ptc (Kuwabara et al., 2000).

The vertebrate genome duplication (Wada and Makabe, 2006) has resulted in several Hh genes that can be categorized into three subgroups: Desert Hedgehog (Dhh); Indian Hedgehog (Ihh); and Sonic Hedgehog (Shh) (Echelard et al., 1993). Based on sequence similarity Dhh is the closest vertebrate homolog to *Drosophila* Hh. Shh and Ihh are more related to each other. Aves and mammals have one Hh gene in each subgroup, but due to a second whole-genome duplication (Jaillon et al., 2004), zebrafish has three extra Hh homologs, one in the Shh subgroup: tiggywinkle hedgehog (Twhh) (Ekker et al., 1995), and the other two in the Ihh group: echidna hedgehog (Ehh) (Currie and Ingham, 1996) and qiqihar (Qhh) (Ingham and McMahon, 2001) (Figure 2).

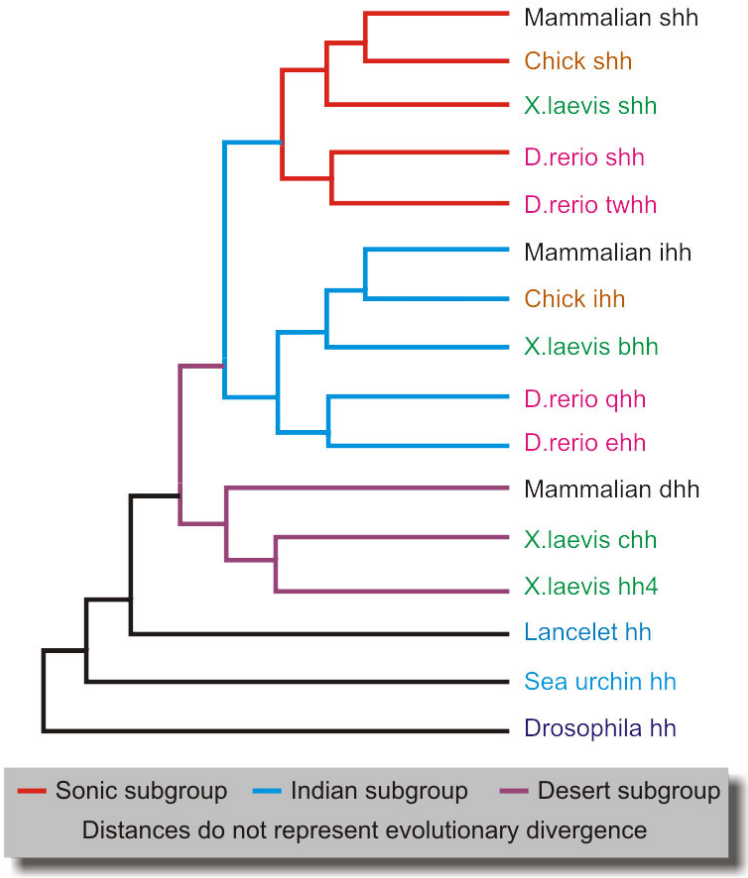

Figure 2. Phylogram of the evolution of Hedgehog proteins (illustrated by Peter Znamenskiy from Ingham and McMahon, 2001).

2.1.2 Hedgehog signaling during development

The developmental processes that the *Drosophila* and vertebrate Hh signaling pathways regulate appear remarkably conserved (Ingham and McMahon, 2001). During development, the Hh/Shh cascade is extensively used during embryogenesis to trigger a plethora of effects in a variety of tissues. These effects range from anterior-posterior or dorsal-ventral patterning, cell fate determination to tissue outgrowth, suggesting that Hh signaling acts in a context-dependent fashion. The Hh/Shh cascade induces the expression of transcription factors and signaling molecules that are essential for animal development.

2.1.3 Hedgehog signaling: *Drosophila* development

Hh is required throughout *Drosophila* embryogenesis and is responsible for the development of embryonic segments (Lee et al., 1992; Mohler, 1988; Nusslein-Volhard and Wieschaus, 1980), the patterning of the wings (Basler and Struhl, 1994; Mohler, 1988; Tabata and Kornberg, 1994), legs (Diaz-Benjumea et al., 1994) and eye discs (Dominguez, 1999; Heberlein et al., 1995). Other developmental events requiring Hh signaling are: the development of the optic lamina and gonads; migration of germ-cells; formation of abdomen, gut and tracheal system (Forbes et al., 1996; Glazer and Shilo, 2001; Huang and Kunes, 1996; Pankratz and Hoch, 1995; Struhl et al., 1997).

The best studied model of Hh signaling in *Drosophila* is the patterning of the wing. During the development of the *Drosophila* wing imaginal disc (Figure 3), posterior compartment cells express and secrete Hh protein. The secreted Hh then induces the expression of target genes in cells located in the anterior compartment. The Hh target genes include decapentaplegic (dpp), which encodes a member of the transforming growth factor β (TGFβ)/bone morphogenetic protein (BMP) family of secreted growth factors (Basler and Struhl, 1994; Tabata and Kornberg, 1994). Dpp then diffuses bidirectionally into both anterior (A) and posterior (P) compartments and functions as a long-range morphogen to control the growth and patterning of the entire wing. In addition, Hh can act as a short-range morphogen to direct cell patterning at the A/P boundary by activating ptc, collier (col) and engrailed (en). Low levels of Hh signal suffice to induce the expression of dpp whereas higher levels of Hh signaling are required to activate ptc and col. The induction of en, on the other hand, requires the maximal Hh signaling activity (Hooper, 2003).

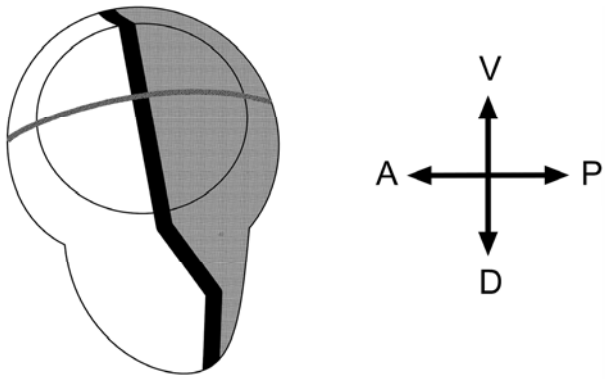

Figure 3. Establishment of signaling regions in *Drosophila* wing imaginal disc. Engrailed (en) is expressed in the posterior (P) compartment of the wing disc together with Hh (light gray). At the boundary of anterior (A) cells, Hh activates dpp (black), which is secreted into both compartments. Wingless (dark gray) is expressed at the dorsal-ventral (D-V) boundary.

2.1.4 Hedgehog signaling: vertebrate development

Vertebrate Hh signaling regulates patterning, cell fate, cell proliferation and cell survival. Hh can act in both short- and long-range signaling and also in a dose-dependent manner, similar to the signaling in *Drosophila*. Almost every part of the vertebrate body plan is influenced by Hh signaling. In some contexts, Ihh acts redundantly with Shh (Zhang et al., 2001). Ihh and Dhh are expressed in a very narrow tissue range; Ihh is expressed in gut (van den Brink, 2007), cartilage (Vortkamp, 2001) and in sertoli cells of the testis ((Bitgood et al., 1996). Shh is the best studied ligand of the vertebrate Hh signaling pathway.

During vertebrate embryogenesis, Shh is mainly expressed in the node, notochord, floor plate and in the zone of polarizing activity in the limb bud (Chang et al., 1994; Johnson et al., 1994; Marti et al., 1995; Riddle et al., 1993). Shh regulates ventral polarity in the neural tube and anterior-posterior polarity of the limbs (Riddle et al., 1993). Shh signaling has also been implicated in patterning the left right axis asymmetry (Pagan-Westphal and Tabin, 1998; Sampath et al., 1997; Schilling et al., 1999).

Throughout embryonic development, Shh is expressed in epithelial tissues including, tooth, hair follicles, lung epithelium (Bellusci et al., 1997; Bitgood and McMahon, 1995; Chuong et al., 2000; Dassule and McMahon, 1998; St-Jacques et al., 1998), gut (Roberts et al., 1995; van den Brink, 2007), pancreas (Apelqvist et al., 1997), kidney (Yu et al., 2002), bone and cartilage (Chiang et al., 1996; Ehlen et al., 2006), heart (Wagner and Siddiqui, 2007; Zhang et al., 2001), muscle (Duprez, 2002; Munsterberg et al., 1995), brain (Ruiz i Altaba et al., 2002; Wallace, 1999), spinal cord (Jacob and Briscoe, 2003), pituitary gland (Treier et al., 2001), in sensory organs like: eye (Jensen and Wallace, 1997; Neumann and Nuesslein-Volhard, 2000); olfactory systems (LaMantia et al., 2000); taste buds in the tongue (Miura et al., 2001), and also in ovary (Pangas, 2007; Wijgerde et al., 2005) and prostate (Podlasek et al., 1999; Shaw and Bushman, 2007) (Figure 4).

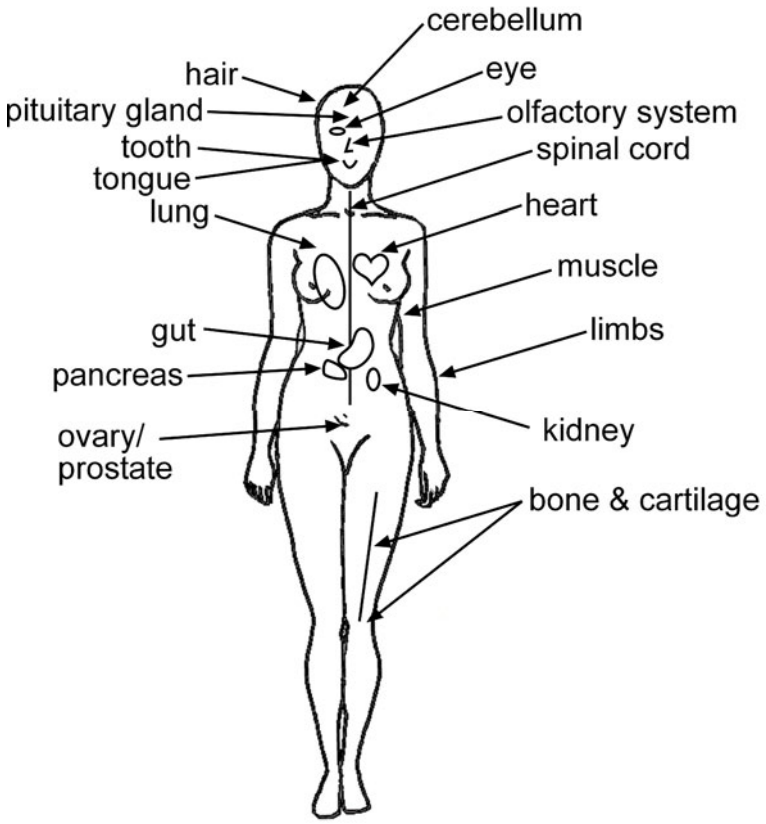

Figure 4. Shh controls development from an embryo to adult. In human development Shh affects the development of a wide range of organs/tissues.

The best characterized model of Shh activity is the neuronal specification of the ventral central nervous system (CNS) (Jessell, 2000; Marti and Bovolenta, 2002; Patten and Placzek, 2000). During neural tube development, Shh protein diffuses from the notochord and floor plate, creating a Shh concentration gradient across the ventral CNS (Figure 5). This concentration gradient drives distinct progenitor domains in the neural tube by regulating the expression of a set of homeodomain proteins that comprises members of the Pax, Nkx, Dbx and Irx transcription factor families (Jacob and Briscoe, 2003; Pierani et al., 1999). Induction or repression of these transcription factor families by the different Shh concentrations defines five neuronal subtypes at precise positions along the floor plate – roof plate axis (D/V axis) (Jacob and Briscoe, 2003).

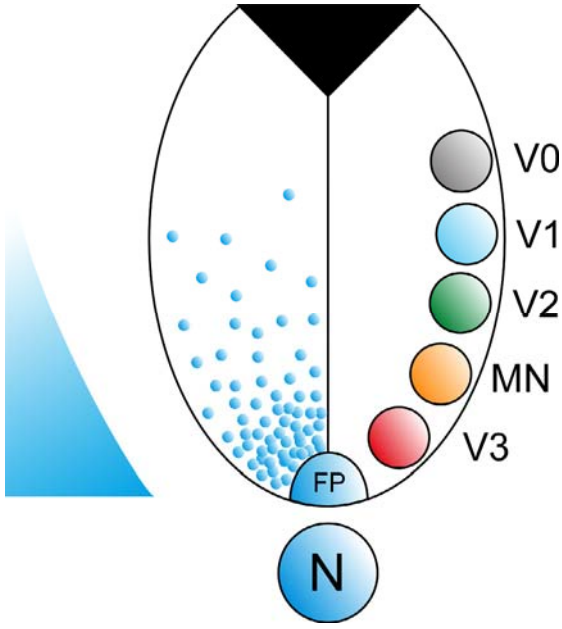

Figure 5. The gradient of Shh progressively defines the different neuronal subtypes; N notochord, FP floor plate, MN motoneuron, V0-V3 interneurons (adapted from Jacob and Briscoe, 2003).

2.2 Hedgehog pathway in developmental disorders and cancer

Loss of Hh signaling activity during vertebrate embryogenesis causes severe ventral CNS developmental disorders including holoprosencephaly, polydactyly, craniofacial defects and skeletal malformations (Hill et al., 2003; McMahon et al., 2003; Muenke and Beachy, 2000; Zhang et al., 2006a). 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), medulloblastomas (a childhood cancer with an invariably poor prognosis) (Berman et al., 2002; Goodrich et al., 1997) and rhabdomyosarcomas (Kappler et al., 2004) (Table 1). The Hh pathway in BCC and medulloblastoma is affected at the level of tumor-suppressor Ptc (Hahn et al., 1996; Johnson et al., 1996), or proto-oncogene Smo (Lam et al., 1999; Xie et al., 1998).

Shh signaling has also been suggested to play a role in other cancers such as breast (Kubo et al., 2004), esophageal (Berman et al., 2003; Watkins and Peacock, 2004), gastric (Berman et al., 2003), pancreatic (Thayer et al., 2003), prostate (Karhadkar et al., 2004; Sanchez et al., 2004) and small-cell lung carcinoma (Watkins et al., 2003). However, the exact mechanisms that lead to aberration of the Hh signaling are not known for these cancers.

The primary cilia has also been linked to regulation of 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 Bardet-Biedl syndrome, Kartagener syndrome, polycystic kidney disease and retinal degeneration (Pan et al., 2005). However, these results are largely preliminary and require further investigation.

Table 1. Cancers linked to aberrant Shh signaling. The most common Hedgehog related cancers are basal cell carcinoma and medulloblastoma (gray box). Hh signaling has also been linked to many other human cancers (white box).

Basal Cell Carcinoma (BCC):	(Hahn et al., 1996; Johnson et al., 1996)
Medulloblastoma:	(Berman et al., 2002)

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)
Rhabdomyosarcoma:	(Kappler 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)

2.3 Molecular mechanisms of the hedgehog signaling cascade

2.3.1 Hedgehog expression, processing and secretion

Hh proteins are involved in triggering an enormous array of physiological effects and accordingly, the expression of the different Hh isoforms is tightly regulated by highly complex and diverse transcriptional control mechanisms (McMahon et al., 2003; Sagai et al., 2005).

The mechanisms of Hh processing and secretion have been elucidated from *Drosophila* and are evidently evolutionally conserved, applying to all the mammalian Hh isoforms as well (Ingham and McMahon, 2001). Before being released from the secreting cell, the Hh protein undergoes multiple processing steps. The maturation of the newly synthesized ~45 kDa Hh precursor occurs in a four processing events, signal sequence cleavage, autoproteolytic-cleavage generating HhN, cholesterol addition and finally palmitoylation (Figure 6).

First, the signal sequence is cleaved. After the signal sequence is removed, the Hh molecule undergoes an intramolecular cleavage that occurs between the glycine-cysteine amino acid residues of the conserved glycine-cysteine-phenylalanine motif (Bumcrot et al., 1995; Lee et al., 1994). This reaction is catalyzed by the carboxyl (C)-terminus of the precursor Hh molecule (Lee et al., 1994; Porter et al., 1995) that has no known signaling activity. The C-terminal domain of the Hh polypeptide then catalyzes an intramolecular cholesterol transfer reaction. This results in the formation of an amino (N)-terminal Hh signaling domain (HhN) of ~19 kDa with a cholesterol-modified C-terminal. Already at this step of processing, the HhN molecule is able to activate the Hh cascade. The cholesterol modification results in the association of HhN with the plasma membrane, facilitating the final processing step of palmitoylation (Chamoun et al., 2001). The palmitic acid moiety (Pepinsky et al., 1998) is added to the N-terminus by the acyltransferase Skinny hedgehog (Ski) (Chamoun et al., 2001; Lee et al., 2001b). Palmitoylation increases the activity of HhN (Chamoun et al., 2001; Lee and Treisman, 2001) and therefore this last step generates a fully active, dually lipidated HhN (HhNp) signaling molecule.

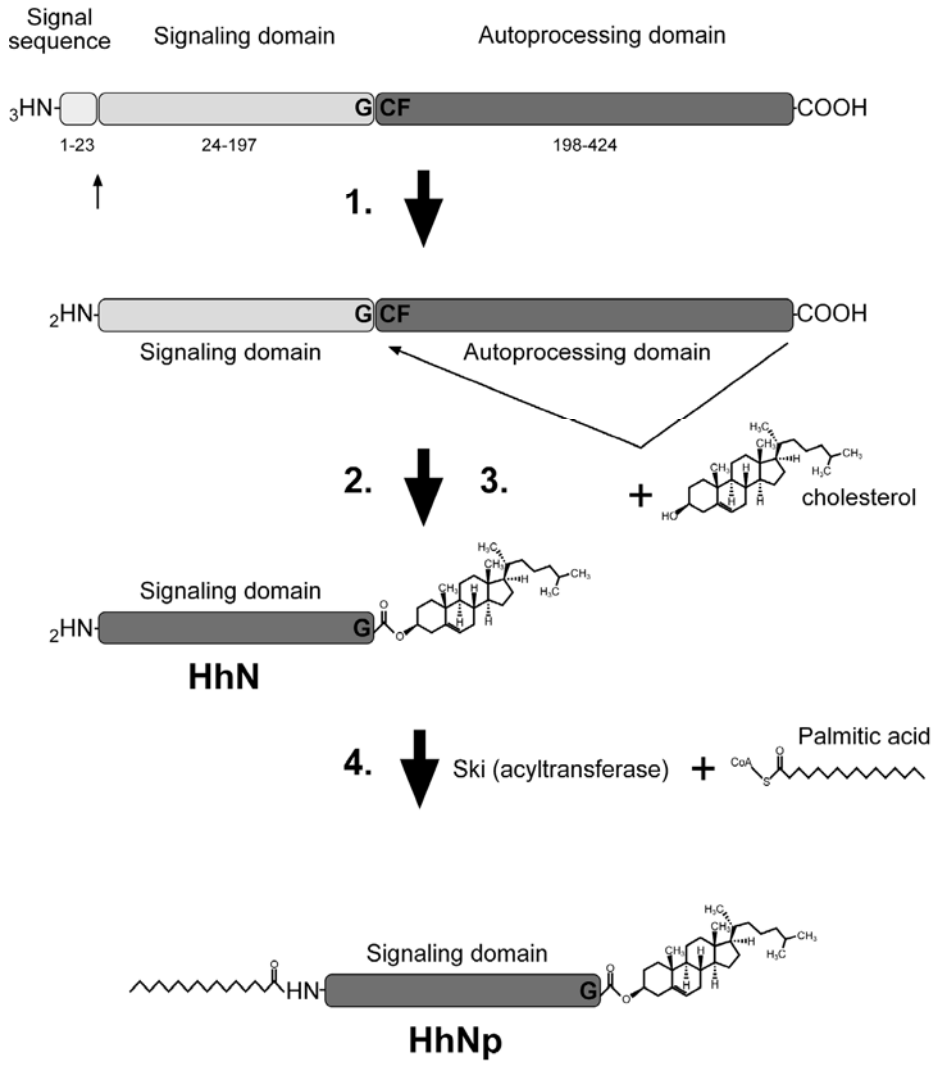

Figure 6. Hedgehog protein maturation. Hh protein undergoes multiple processing steps: 1) the signal sequence is cleaved; 2) the carboxyl (C)-terminal domain of the Hh polypeptide catalyzes an intramolecular cholesteroyl transfer reaction; 3) resulting in the formation of a C-terminal cholesterol-modified amino (N)-terminal Hh signaling domain (HhN); and 4) subsequent association of HhN with membranes, which facilitates the final modification step, the addition of a palmitic acid moiety to the N-terminus by the acyltransferase Skinny hedgehog, resulting in the formation of HhNp.

2.3.2 Release and transport of Hedgehog through tissues

Although HhNp is tightly associated with the plasma membrane, it is able to act directly over a long range (~50 µm in *Drosophila* wing imaginal disc and ~300 µm in vertebrate limb bud) in a time- and concentration-dependent manner (Stamataki et al., 2005). The cholesterol modification of HhNp also influences the range of Hh action by affecting Hh palmitoylation, stability, diffusion and/or transport (Callejo et al., 2006; Gallet et al., 2003; Lewis et al., 2001). In both *Drosophila* and vertebrates, the secretion of HhNp from the cell requires the activity of the 12-span transmembrane protein, Dispatched (Disp). Disp, like Ptc, belongs to the bacterial resistance-nodulation-division (RND) family of transport proteins (Burke et al., 1999; Ma et al., 2002).

Formation of the Hh concentration gradient emanating from the secreting cells is facilitated by multiple macromolecules, which control release, transport and sequestration of Hh. How HhNp moves over a long distance is still not clear, but soluble Shh multimers that contain lipids and that have strong signaling potency have been described (Zeng et al., 2001).

The large extracellular heparan sulfate proteglycans (HSPG) macromolecules seem to be important for Hh signaling (and other morphogens, like wg and dpp). Loss of 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; Bornemann et al., 2004; Han et al., 2004a) impairs HhNp distribution throughout the extracellular matrix and its signaling activity. In addition, the glycosylphosphatidylinositol (GPI)-linked HSPG (also called glypicans) Dally and Dally-like (substrates of ttv), also affect Hh signaling by facilitating HhNp to cell surfaces (Han et al., 2004b; Lum et al., 2003a; Nakato et al., 1995). The mammalian orthologs for ttv and sotv,(EXT1 and EXT2) are also linked to Hh distribution and signaling mediation by HSPGs (Koziel et al., 2004; Lin et al., 2000), supporting the view that that HSPGs also control the range of Hh signaling in vertebrates.

In addition to the Hh receptor Ptc, which sequesters and shapes the HhNp gradient in all species, several species-pecific proteins that influence HhNp transport have been described (Chen and Struhl, 1996). Vertebrates have an additional

transmembrane protein, Hedgehog interacting protein (HIP), which binds to HhNp and reduces the range of movement (Chuang and McMahon, 1999). *Drosophila* have a secreted Hh interacting protein Shifted (Shf) (Gorfinkiel et al., 2005) that is critically required for HhNp spreading in *Drosophila* wing imaginal discs (Glise et al., 2005; Gorfinkiel et al., 2005).

2.3.3 Receiving the Hedgehog signal

A hallmark of the Hh signaling cascade is the apparent squealching of the Hh signal by a negative feedback loop. In response to the Hh signal, Ptc expression is increased which restricts the spreading of the Hh signal through tissues. The binding of HhNp to Ptc, internalizes HhNp through Dynamin-dependent endocytosis and targets Hh to the lysosomes for degradation (Gallet and Therond, 2005; Incardona et al., 2000).

In addition to the glypican-family cell surface proteoglycans, the binding of HhNp to cells is further facilitated by the transmembrane proteins ihog and boi (Cdo and Boc in vertebrates) (Tenzen et al., 2006; Yao et al., 2006). The expression of these two related cell-surface proteins are down regulated in response to Hh signaling in mammals, but they synergistically facilitate the binding of HhNp to Ptc (Tenzen et al., 2006; Yao et al., 2006). Both Cdo and Boc and their *Drosophila* homologs, ihog and boi, bind to HhNp via conserved fibronectin repeats (Yao et al., 2006).

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 HhNp to Ptc results in repression of Ptc activity. Subsequently, Ptc relieves its inhibition of Smo which then transduces the Hh signal to the cytoplasm (Stone et al., 1996; Taipale et al., 2002). The relationship between Ptc and Smo is evolutionary conserved. In both flies and vertebrates, Ptc binds the Hh ligand and relieves its repression on Smo (Marigo et al., 1996). Genetic analyses show Smo acting downstream of Ptc (Alcedo and Noll, 1997). How Ptc represses Smo activity is currently not known. The inhibition of Smo by Ptc apparently is catalytic, as Ptc is still able to inhibit Smo even at a stoichiometry 1:50 (Taipale et al., 2002). Vertebrates have two Ptc genes, Ptc1 and Ptc2. Ptc2 is dispensable for embryonic development, but a deficiency in Ptc2, in conjunction with Ptc1 heterozygosity,

increases the incidence of several types of tumors in adult life (Lee et al., 2006b). 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., 2002a; Frank-Kamenetsky et al., 2002) and possibly endogenous metabolites such as lipids (Taipale et al., 2002), oxysterols (Corcoran and Scott, 2006) and vitamin D3 derivatives (Bijlsma et al., 2006).

2.3.4 Divergence of the Hedgehog pathway at the level of Smoothened

Smo localization is differently regulated in *Drosophila* and mammals. *Drosophila* Smo accumulates to the cell-surface after activation of the Hh pathway (Denef et al., 2000). Mammalian Smo however, is internalized after pathway activation (Incardona et al., 2002). Experimental data reveals that oncogenically activated mammalian Smo proteins localize to the endoplasmic reticulum (Chen et al., 2002a). Furthermore, Smo phosphorylation appears to be differently regulated between insects and mammals. *Drosophila* Smo activation is coupled to the hyperphosphorylation of 26 serine/threonine residues located within the 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). None of these PKA or CKI phosphorylation sites are conserved in vertebrate Smo, although most of them are within or near the evolutionarily conserved region (Figure 7). Evidently, the trafficking and activity of vertebrate Smo is not regulated by these two kinases.

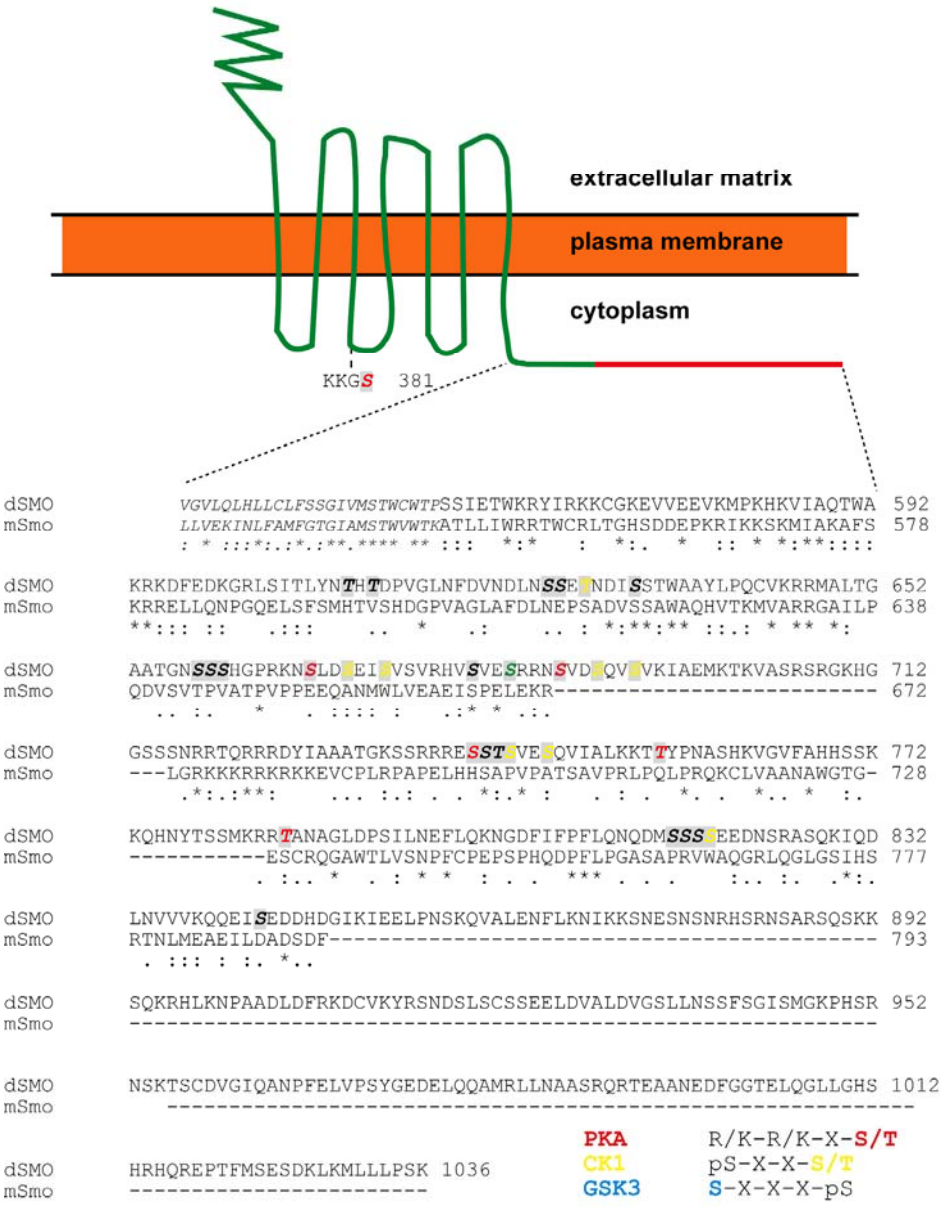

Figure 7. The PKA and CK1 phosphorylation sites required for *Drosophila* Smo hyperphosphorylation and activity are missing from the mouse Smo sequence (adapted from (Varjosalo et al., 2006; Zhang et al., 2004).

However, multiple kinases have been proposed to phosporylate vertebrate Smo. Some evidence suggests that mammalian Smo could be phosphorylated by GRK2, which then would promote the Smo association with β-arrestin 2 (Arrb2) and the consequent endocytosis of Smo via chlathrin-coated pits (Chen et al., 2004). Mutant mice lacking Arrb2 are viable and do not show even a mildly abrogated Hh pathway phenotype (Kohout et al., 2001). Based on the similarity of structure of Smo to heterotrimeric GTP-binding regulatory proteins (G-protein) -coupled receptors, it has been suggested that Smo couples to heterotrimeric (α,β,γ) G-proteins and activate G(i)-family members (Riobo et al., 2006c). Similarly, it has also been reported, that phorbol esters, acting through protein kinase C-δ (PKCδ) and mitogen-activated protein/extracellular signal-regulated kinase-1 (MEK-1) can transduce the Smo signal to the GLI proteins. Furthermore, cytoplasmic phosphoinositide 3-kinase (PI3-kinase)-dependent Akt activation is essential for Shh signaling (Riobo et al., 2006a; Riobo et al., 2006b). However, the mechanisms of how mammalian Smo signals downstream to the GLI transcription factors remain to be convincingly demonstrated.

2.3.5 Relaying the Hedgehog signal from cell surface to the nucleus

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 the endocytosis and/or recycling is blocked. This stabilized and activated Smo then accumulates on the cell surface.

Drosophila Smo transduces the Hh signal by direct recruitment of Cos2 to the Smo C-terminus (Jia et al., 2003; Lum et al., 2003b; Ogden et al., 2003; Ruel et al., 2003). Cos2, in turn, tightly associates with Fu (Therond et al., 1996) and the full-length transcriptional activator form of Ci, CiA (150 kDa) (Robbins et al., 1997). Cos2 also recruits the kinases PKA, GSK3β, CKIα and CKIε, that (hyper)phosphorylate the C-terminal domain of CiA. The hyperphosphorylation promotes CiA truncation by procuring the activity of Slimb (vertebrate homolog β-TrCP) (Jiang and Struhl, 1998), which ultimately leads to the formation of a truncated (75 kDa) transcriptional repressor form of Ci, CiR. (Chen et al., 1999b; Jia et al., 2002; Jia et al., 2005; Price and Kalderon, 1999; Price and Kalderon, 2002;

Wang et al., 1999). Slimb and β-TrCP are subunits of SCF (Skp1, Cul1 (a.k.a cdc53) and F-box), a ubiquitin ligase complex that targets phosphorylated substrates for ubiquitinylation, followed by proteosomal degradation (Maniatis, 1999). In addition to promoting CiR formation, Cos2 also regulates Ci by tethering it in the cytoplasm preventing nuclear translocation (Wang et al., 2000b).

The sequestering of Cos2 by Smo to the plasma membrane prevents CiA being converted to CiR. However, this is not enough for full pathway activation as some CiA is still retained in the cytoplasm by another protein, Suppressor of Fused (Su(Fu)) (Pham et al., 1995) (Methot and Basler, 2000). In *Drosophila,* Fu inactivates Su(Fu), possibly through direct phosphorylation (Ohlmeyer and Kalderon, 1998), which results in the release of the remaining CiA. Upon entering the nucleus, CiA binds to specific Ci/GLI DNA elements in promotor and enhancer regions and thereby controls transcription of Hh target gene(s). The inactivation of Cos2 by Smo and Su(Fu) by Fu results in full activation of the pathway (Lefers et al., 2001; Lum et al., 2003b). Thus, Ci activity is not regulated only by phosphorylation and proteolytic processing, but by other mechanisms, such as cytoplasmic retention and protein-protein interactions.

In contrast, the mechanisms by which mammalian Smo signals to the cytoplasm and the GLI transcription factors is still unclear. Evidence suggests that the cytoplasmic components and the mechanism of Hh signal transduction diversified between *Drosophila* and mammals. Mouse Smo is not stabilized after Shh addition, and mouse Smo does not recruit either Kif27 or Kif7, the two mammalian orthologs of Cos2 (publication I). Moreover, neither overexpression nor RNAi mediated knockdown of Kif27 or Kif7 has any effect on Shh pathway activity or GLI2 transcriptional activity (publication I). Consistent with the divergence of the intracellular Hh signaling pathway between fly and mammals, the Fu knock-out mouse had no phenotypes of impaired Hh signaling during embryogenesis (Chen et al., 2005; Merchant et al., 2005). Su(Fu)-knockout mice embryos essentially have the same phenotype as Ptc-knockout mice resulting from complete activation of the Hh pathway (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).

2.3.6 Regulation of the Ci/GLI transcription factors appears to be conserved

Our current understanding of the mechanisms by which Hh signals are relayed through the cytoplasm to the nucleus indicate that the *Drosophila* and vertebrate pathways have diverged. Interestingly though, within the nucleus, transcriptional regulation by the Ci/GLI transcription factors, yet again share conserved components. Vertebrates have three GLI proteins, GLI(1-3). GLI2 and GLI3 are the primary mediators of Hh signaling (Bai et al., 2004; Dai et al., 1999; Ding et al., 1998; Lee et al., 1997; Matise et al., 1998). GLI1 is a transcription activator and responds to the Hh signal only at the transcriptional level. The activation of GLI1 expression in response to increased pathway activity creates a positive-feedback loop and enhances the specificity of the Hh signal. GLI2 functions as transcriptional activator, whereas GLI3 is a repressor (Jacob and Briscoe, 2003; Ruiz i Altaba et al., 2007). In some developmental contexts GLI2 can act as a transcriptional repressor and GLI3 as an activator (Bai et al., 2004; McDermott et al., 2005; Motoyama et al., 2003; Tyurina et al., 2005).

GLI2 and GLI3 are proteolytically processed to a truncated form in a fashion similar to Ci which is also inhibited by the Hh/Shh signal. GLI2 and GLI3 processing initially depends on the phosphorylation of four PKA sites (Pan et al., 2006; Wang et al., 2000a). Subsequently, adjacent CK1 and GSK site are also phosphorylated. This hyperphosphorylation is similar to that of Ci (Jia et al., 2005; Price and Kalderon, 2002). Following the recognition of GLI2 and GLI3 by β-TrCP (Pan et al., 2006; Wang and Li, 2006), the ubiquitinylation and degradation steps seem to be conserved between species.

Another mechanism of Ci/GLI processing and degradation has been reported to use the Broad Complex, Tramtrack, and Bric a Brac (BTB) protein hib/SPOP (Hh induced MATH and BTB protein) and Cul3 E3 ubiquitin ligase. hib/SPOP fine-tunes the Hh signaling responses. Expression of hib/SPOP increases in response to the Hh signal. This in turn reduces GLI2 and GLI3 transcriptional activity (Kent et al., 2006; Zhang et al., 2006b). The two E3 ubiquitin ligase systems have different effects on Ci/GLI processing. Hib/SPOP-mediated ubiquitinylation leads to complete degradation of Ci/GLI (Zhang et al., 2006a). Slimb/ β-TrCP-mediated ubiquitinylation leads to partial Ci/GLI degradation. Furthermore, Su(fu) and hib

compete to bind to the same region of CiA (Ci155). Su(fu) binding to Ci inhibits hib recoginition and degradation of Ci (Croker et al., 2006; Huntzicker et al., 2006; Zhang et al., 2006a). The HECT family E3 ligase, Numb, promotes Itch-dependent ubiquitination and degradation of GLI1 (Di Marcotullio et al., 2006). In addition, disruption of the BTB family gene *REN* has been linked to medulloblastomas, suggesting that REN inhibits Shh pathway activity (Di Marcotullio et al., 2004).

2.3.7 Possible vertebrate-specific components of Hedgehog signaling

A number of genes and their encoded proteins have been reported to be involved in the regulation of the vertebrate Shh signaling pathway. The best studied examples are the genes involved in the primary cilia function. Increasing evidence suggest that the primary cilium may be critical for mammalian Hh signaling. Mutations of several proteins required for the formation of primary cilia, including Kif3a, Ift88 and Ift172 result in embryonic phenotypes characteristic of the loss of Shh signaling (Huangfu et al., 2003) and biochemical studies have linked these proteins to the processing of GLI transcription factors (Haycraft et al., 2005). The role of cilia in Hh signaling seems to be specific to mammals (Nybakken et al., 2005; Sun et al., 2004). In addition, a number of vertebrate-specific Hh regulators have been reported which either have no *Drosophila* ortholog, or whose orthologs appear not to affect Hh signaling in *Drosophila* (Table 2). However, more compelling evidence is required to link them to the regulation of vertebrate Hedgehog signaling.

Table 2. Vertebrate specific components of Hedgehog signaling pathway. Listed is gene name, protein function, activy on mammalian Hh signaling (positive activity ↑, negative activity ↓) and reference to the original publication.

GENE	PROTEIN FUNCTION	ACTIVITY	REFERENCE
TECTONIC	SECRETED AND TRANSMEMBRANE PROTEIN	↑	(REITER AND SKARNES, 2006)
MEGALIN	ENDOCYTIC RECEPTOR FOR SHH	↓	(MCCARTHY ET AL., 2002)
GAS1	GPI-LINKED MEMBRANE GLYCOPROTEIN	↓	(LEE ET AL., 2001A)
INTURNED	PLANAR CELL POLARITY EFFECTOR	↑	(PARK ET AL., 2006)
FUZZY	PLANAR CELL POLARITY EFFECTOR	↑	(PARK ET AL., 2006)
TWIST	NUCLEAR BASIC HELIX-LOOP-HELIX TRANSCRIPTION FACTOR	↑	(VILLAVICENCIO ET AL., 2002)
SAP18	COMPONENT OF MSIN3- HDAC COREPRESSOR COMPLEX	↓	(CHENG AND BISHOP, 2002)
SIN3	COMPONENT OF MSIN3- HDAC COREPRESSOR COMPLEX	↓	(CHENG AND BISHOP, 2002)
MIM	ACTIN-BINDING PROTEIN	↑	(CALLAHAN ET AL., 2004)
COUP-TF II	ORPHAN NUCLEAR RECEPTOR	↑	(LEE ET AL., 2006A)
RAB23	VESICLE TRANSPORT PROTEIN	↓	(EGGENSCHWILER ET AL., 2001)
SIL	CYTOPLASMIC PROTEIN	↑	(IZRAELI ET AL., 2001)
TALPID3	CYTOPLASMIC PROTEIN ?	↑↓	(DAVEY ET AL., 2006)
YOU/SCUBE2	ENDOCYTIC RECEPTOR	↑	(HOLLWAY ET AL., 2006)
KIF7	KINESIN	↓	(TAY ET AL., 2005)
DZIP1	ZINC-FINGER PROTEIN	↑↓	(SEKIMIZU ET AL., 2004)
DYRK1A	DUAL-SPECIFICITY TYROSINE-(Y)-PHOSPHORYLATION REGULATED KINASE	↑	(MAO ET AL., 2002)

2.4 Hedgehog pathway target genes

The presence of the different forms of the Ci/GLI proteins leads to the activation (CiA, GLI1-2A) or repression (CiR, GLI3R) of many different types of genes including other growth and transcription factors. In *Drosophila* the Hh pathway activates the expression of dpp, wg, ptc, col and en (see 2.1.3 and (Methot and Basler, 1999). The expression of these genes requires different levels of Hh activity. The expression of dpp is activated even when the repressor activity of CiR is lost. The expression of en on the other hand requires the maximal pathway activity for induction of expression (Hooper, 2003).

Vertebrates apparently have more Hh target genes than *Drosophila*. Interestingly, the list of target genes also include components of the vertebrate Hh signaling pathway itself, GLI1, Ptc and HIP. Among the other genes regulated by Hh signaling are other secreted signaling proteins including BMP, fibroblast growth factor (Laufer et al., 1994), N-Myc (Oliver et al., 2003), cyclins D1-2 (Duman-Scheel et al., 2002; Oliver et al., 2003), vascular endothelial growth factor-1, angiopoietins-1 and -2 (Pola et al., 2001), insulin-like growth factor 2, platelet derived growth factor receptor-α, Bcl2 (Cayuso et al., 2006), Bmi1 (Leung et al., 2004), Wnt (Mullor et al., 2001), Hes1 (Ishibashi, 2004), SPOP (Zhang et al., 2006b). *In vivo* Shh signaling in the developing vertebrate neural tube, induces dose-dependent gene expression activation of Nkx6.1 and Nkx2.2, or repression of Pax6, Irx3 and Dbx2 homeodomain transcription factors (Briscoe and Ericson, 2001).

The total number of genes that Hh-GLI signaling regulates could be dramatically larger, as a genome-wide prediction of mammalian enhancers idenfied 42 genes to have Shh enhancer modules with two or more GLI-binding sites (Hallikas et al., 2006).

2.5 Targeting Hedgehog related cancers with small molecules

Aberrant activation of the Hh pathway has been associated with numerous malignancies including BCC, medulloblastoma, prostate, pancreatic and breast cancers. As the Hh pathway regulates both cell proliferation and differentiation, clearly, a dysregulated Hh pathway can contribute to the onset of cancer and accelerate its progression (see 2.2).

As the Hh pathway in BCC and medulloblastoma is affected at the level of Ptc or Smo, small molecule antagonists should act at/or downstream of these components. It seems that Smo is an attractive drug target of the Hh signaling pathway. *In vitro* and *in vivo* evidence indicates that antagonizing of excessive Hh signaling through Smo could provide a way to intervene with tumorigenesis and tumor progression. The most used antagonist of the Hh pathway is a plant alkaloid derived from corn lilies, cyclopamine (Taipale et al., 2000). Cell based high-throughput screening has revealed a few distinct classes of antagonist, which, like cyclopamine, bind to Smo; SANT(1-4) (Chen et al., 2002b), KAAD-cyclopamine (Taipale et al., 2000), jervine, compound-5 and compound-Z (Borzillo and Lippa, 2005) and Cur-61414 (Frank-Kamenetsky et al., 2002).

Excessive Hh pathway activity has also been reported to cause other tumors (see Table 1). It has been proposed that Hh ligand overexpression can promote certain cancers (Dahmane et al., 1997). Heghehog specific antibody (5E1) can block the growth of the first identified tumor overexpressing Hh, small-cell lung carcinoma (Watkins et al., 2003). Evidently the specific blocking of Hh signaling may offer an effective treatment for various cancers originating from abnormal Hh pathway activation.

3 AIMS OF THE STUDY

The main goal of this work was to determine how the Shh signal is transduced from the Ptc receptor to the cytoplasm. The mechanisms by which the seven transmembrane protein Smo transduces the Hh signal to the cytoplasm and effects activation of the GLI transcription factors are unclear. Furthermore, despite, the critical role in *Drosophila,* no mammalian ortholog for Cos2 had been identified. In this study I aimed to identify the mammalian Cos2 orthologs and test their role in mammalian Hh signal regulation. The hypothesis was that transduction of the Shh signal from the receptor to cytoplasm would involve novel components and mechanisms that are different from *Drosophila* Hh pathway.

After the initial discovery that Cos2-like activity is not required for regulation of mammalian Hh signaling, and the finding that Shh pathway regulation heavily depends on the activity of Su(Fu), we concentrated our efforts to identify novel Hh pathway components. Our efforts were further directed to protein kinases by two reports on the Fused knockout animal showing no Hh-like phenotype. The reasoning was that novel Shh pathway specific kinase(s) would be required for pathway regulation, especially phosphorylating Su(Fu) and thus regulating its activity.

4 MATERIALS AND METHODS

More detailed descriptions of the materials and methods used can be found from the original publications (I-III) as indicated in Table 3 below.

Table 3. Methods used in this study**.**

Method	Publication
Data analysis	I, II, III
Drosophila cell culture	I, II
Eukaryotic cell transfection	I, III
Fluorescent-activated cell sorting	I, II
Gel electrophoresis	I, II, III
Generation of transgenic mouse embryos	I
Immunoblotting	I, II, III
Immunoprecipitation	I, II, III
Immunostaining of tissues and cultured cells	I, II, III
In vitro kinase assay	III
In vitro transcription	II
Mammalian cell culture	I, III
Mass spectrometry	III
Microscopy (including confocal and high content)	I, II, III
Northern blotting	I
Plasmid construction and DNA manipulation	I, II, III
Protein microarray	III
Recombinant protein production in bacteria	I, II, III
Recombinant protein production in mammalian cells	III
RNAi	I, II, III
Reporter assays	I, III
SDS-PAGE	I, II, III

5 RESULTS AND DISCUSSION

5.1 Patched receptor function is conserved between *Drosophila* and mammals (I, II)

Signaling mechanisms involving the Hh receptor Ptc and the signal transducer Smo are less well characterized in mammals than in fruit fly. To analyze the conservation of Hh signal transduction between *Drosophila* and mammals, we performed a functional comparison of Ptc and the effect on signal transducer Smo in these two distinct organisms.

5.1.1 Human Patched can rescue the *Drosophila* hedgehog pathway activity in Schneider S2 cells depleted of endogenous Patched

Using RNAi to knock-down *Drosophila* Ptc (dPtc), we observed a constitutive activation of the Hh pathway in Schneider S2 (S2) cells, which was consistent with previous observations (Lum et al., 2003a; Ruel et al., 2003). To study the conservation of *Drosophila* and mammalian Hh signaling components Ptc and Smo, we investigated whether human Ptc (hPtc) could rescue the function of dPtc and inhibit endogenous *Drosophila* Smo (dSmo) in S2 cells. S2 cells contain a functional and Hh responsive Hh signaling pathway, from Ptc to the Fu-Cos2 complex. However, S2 cells lack the transcriptional response due to the lack of Ci expression (Chen et al., 1999a). In response to Hh, dSmo becomes stabilized and accumulates to the plasma membrane (publication II and Denef et al., 2000; Jia et al., 2004; Zhang et al., 2004). Consequently, we detected that, based on their mobility shift in SDS-PAGE, Fu and Cos2 became phosphorylated. By knocking down the endogenous dPtc from S2 cells stably expressing hPtc, we observed that hPtc could rescue the repression of the pathway activity in the absence of Hh. Consistently, Fu and Cos2 were also phosphorylated and the pathway was activated when Hh was added. This demonstrated that hPtc can repress dSmo activation in the absence of Hh. Futhermore, dHh is able to bind to hPtc, repressing the activity of hPtc on dSmo, resulting in the activation of Hh signal transduction. These observations indicate that hPtc is able to replace at least partially dPtc functions in the *Drosophila* Hh pathway.

These results were suprising as the amino acid sequence identity between hPtc and dPtc is relatively low at only 36%. However, several motifs of 5–15 amino acids are totally conserved between the two species. Two of these conserved motifs are localized in the extracellular domains 1 and 2, and are present in all Ptc sequences, suggesting that these conserved motifs are involved in Hh protein interaction. Our results indicate that dHh is able to interact with hPtc. Interestingly, two other conserved motifs are localized in extracellular domains 1 and 2, and three other evolutionary conserved motifs are found in the transmembrane segments (TMS) 3, 4, 9, and 10. In addition to being conserved in all Ptc sequences, these motifs are conserved in various ATP-binding cassette (ABC) transporters. Mutation in one of these motifs in Ptc (glycine 477 to arginine in TMS 3) has been shown to abolish Smo repression without compromising the ability of Ptc to bind and internalize HhNp through Dynamin-dependent endocytosis (Strutt et al., 2001). This suggests that, apart from interacting with Hh and endocytosing it, a key and conserved function of Ptc could be the transport of a molecule involved in inhibition or activation of Smo (publication II and Bijlsma et al., 2006; Chen et al., 2002b; Corcoran and Scott, 2006). The observation that hPtc is able to inhibit dSmo in the absence of Hh and that Hh binding releases this inhibition suggests that the same, or similar, molecule is transported by *Drosophila* and human Ptc.

5.2 Divergence of Smoothened (I, II)

We have established that the mechanisms of function of the Hh receptor, Ptc, are conserved between *Drosophila* and mammals (II). Subsequently, we tested whether the conservation of these signaling pathways, would extent to the Hh pathway co-receptor and signal transducer Smo. Due to the well-established and important role of Ptc, Smo and Cos2 in *Drosophila* Hh signaling, we decided to analyze the structural and functional similarities of mammalian and *Drosophila* Smo. Furthermore, we investigated whether mammalian Smo acts through Cos2-like proteins. To elucidate the role of Smo and Cos2 for mammalian Shh signaling, we decided to analyze whether the C-terminal cytoplasmic domains would be required for mouse Smo (mSmo) function. It was known that in *Drosophila*, upon Hh induction, Cos2 interacts with the Smo C-terminus and the consequent events relay the signal to the cytoplasm and lead to the activation of the Ci transcription factor (Jia et al., 2003; Lum et al., 2003b).

We generated a Smo deficient mouse fibroblast cell line (Smo-/-) derived from 8.5 days old Smo null embryos (E8.5) (Ma et al., 2002). The measurement of mammalian Shh signaling activity was performed using a GLI-luciferase reporter construct, that is sensitive to both activator and repressor forms of GLI (Figure 9). The activity of the GLI-luciferase reporter can be induced by GLI2 expression or Shh treatment, and inhibited by GLI3 expression or with forskolin (induces the formation of endogenous GLI3 repressor) (Taipale et al., 2000; Taipale et al., 2002).

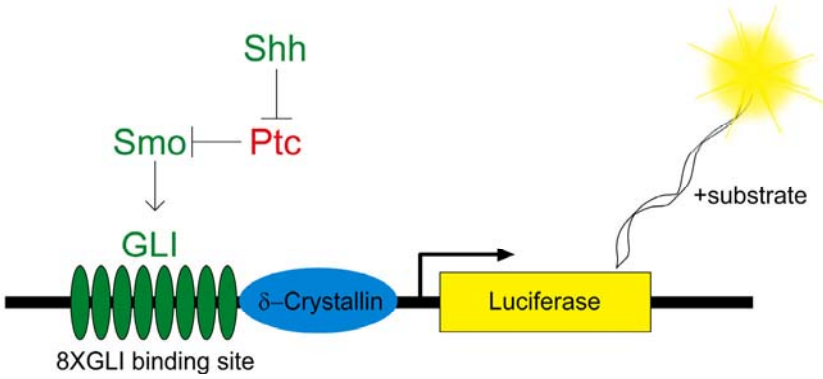

Figure 9. GLI-Luciferase reporter. The binding of Shh to Ptc activates the Shh signaling cascade which ultimately leads to activation of the GLI transcription factors. Subsequent binding of GLI to specific GLI binding sites (8xGLI) drives the expression of luciferase.

5.2.1 The requirement of the cytoplasmic tail region of mouse Smoothened for Sonic hedgehog signaling

Using the mouse fibroblast Smo-/- cells together with the GLI-reporter, we wanted to establish whether mSmo lacking the corresponding regions of dSmo's Cos2 binding regions would still be active. In dSmo, there are two domains that are required for activity and Cos2 binding. These domains are located between amino acids 652-686 (Lum et al., 2003b) and 730-1035 (Jia et al., 2003) (see also Figure 7). dSmo lacking both of these domains is inactive and does not bind to Cos2 (Lum et al., 2003b; Nakano et al., 2004). The corresponding mSmo construct (mSmo637ΔC), however, had reduced, but still strong activity. Furthermore, the activity of this mSmo mutant could be enhanced by increasing its expression level, further showing that the deleted domains were not critical for mSmo function. Maximal pathway activity could not be observed even with the highest expression

levels of mSmo637ΔC, so we constructed another mSmo mutant in which we had deleted amino acids 638-656. This construct had activity similar to wild-type mSmo. In contrast, in *Drosophila* a construct corresponding to mSmo714ΔC (dSmo724ΔC) had very weak activity and failed to rescue the loss of endogenous dSmo. For maximal dSmo function, both Cos2 binding domains are required (Nakano et al., 2004). These results show that the two corresponding domains required for Cos2 binding and Smo activity in *Drosophila*, are not needed for mammalian Smo function and for the regulation of pathway activity.

5.2.2 Alanine scanning mutagenesis identifies eight mouse Smoothened inactivating mutations

To identify the critical residues for mSmo downstream signaling, we mutated all conserved residues, between flies and mammals, on the cytoplasmic side of mSmo to alanine. This alanine scan identified two residues in the third intracellular loop, and six residues between amino acids 570 and 580 in the C-terminal cytoplasmic tail. All mutations resulted in significant loss of mSmo activity. The predicted secondary structure for this C-terminal region (aa 570-580) is an α helix, in which these residues reside on the same side, raising the possibility that, together with the third Smo intracellular loop, this region may form an interaction surface involved in the inactivation of Su(Fu) or activation of Ci/GLI. All the inactive mutants were normally expressed and folded, as indicated by their ability to bind to the Smo antagonist, cyclopamine (Chen et al., 2002b). Only one of the identified mutants (isoleucine 573 to alanine; I573A) has been described before. The corresponding mutation (I586A) in fruit fly results in an unstable dSmo protein (Lum et al., 2003b). The instability was interpreted to cause the lack of activity of this mutant. As the dSmo function is coupled to dSmo stabilization (Alcedo et al., 2000), it is more likely that the I586A mutant is degraded due to its failure to become activated. This is in contrast to the corresponding I573A mutant in mammals, probably because activation of the Shh pathway is not coupled to mSmo stabilization. In mammals, Smo is not stabilized after the addition of ShhNp in Shh-responsive Smo-/- cells stably expressing a myc-tagged version of mSmo. This corresponds well with the fact that dSmo stabilization requires the activity of Cos2 (Lum et al., 2003b) and is consistent with the lack of mSmo stabilization and requirement for Cos2 binding domains in mammals.

5.2.3 Overexpression of myristoylated Smoothened carboxyl-terminus does not activate mammalian Sonic hedgehog pathway

In *Drosophila*, overexpression of myristoylated-SmoC, a Smo C-terminal cytoplasmic domain that is anchored to the internal leaflet of the plasma membrane by a myristoyl moiety (Hooper, 2003; Jia et al., 2003). This anchoring inhibits processing of Ci to a repressor form by inducing a loss of Cos2 activity. In contrast, the expression of mouse myr-SmoC did not induce the Hh signaling cascade in NIH-3T3 or Smo-/- cells. It is possible that subtle induction of the pathway, such as that induced by a loss of GLI3 processing to a repressor form, would not be detected in our cultured cell assay. Therefore, we made transgenic mouse embryos expressing myr-SmoC, and analyzed the phenotype at E16. A relatively minor induction of the Hh pathway would be readily visible as polydactyly at this stage, however, the mouse embryos displayed wildtype limb patterning. These results suggest that, in contrast to the *Drosophila* Smo C terminus, the mouse Smo C-terminal domain does not bind to and inactivate proteins, such as Cos2.

5.2.4 The PKA and CK1 phosphorylation sites required for *Drosophila* Smoothened activation are not conserved in mouse Smoothened

We tested the ability of the dSmo C-terminal domain to function in the context of mSmo. The mSmo C-terminus (from amino acids 638-end) was replaced with the corresponding dSmo C-terminus. This substitution resulted in formation of a chimeric Smo protein. The chimeric Smo could rescue Hh pathway activity in mSmo-/- cells. Furthermore, the inclusion of the multiple phosphorylation site mutation (Zhang et al., 2004) that renders dSmo inactive (serines to alanines) or hyperactive (serines to glutamic acids), did not affect the activity of the chimeric Smo. This indicated that the mechanisms that activate mSmo are divergent to that of dSmo.

5.2.5 Mouse Smoothened can be rendered Costal2 sensitive by replacing the carboxyl-terminus with *Drosophila* carboxyl-terminal sequence

We next tested the effect of co-expression of *Drosophila* Cos2 along with wild-type mSmo or chimeric Smo proteins in mSmo-/- cells. Expression of *Drosophila* Cos2 had no effect on wild-type mSmo. To our surprise, the expression of *Drosophila*

Cos2 completely abolished the activity of the Smo chimera, regardless of whether the multiple phosphorylation sites were mutated to be active or inactive (see 5.2.4). These results clearly demonstrate that *Drosophila* Cos2 is expressed in a functional form in mouse cells, but has no effect because mSmo lacks the corresponding interaction amino acid sequences. This further demonstrates that the C-terminal domains of dSmo cause it to be Cos2 sensitive. Consistent with these results, Cos2 did not affect the transcriptional activity of mammalian GLI2 in S2 cells, although GLI2 is active in *Drosophila, in vivo* (Aza-Blanc et al., 2000). In contrast, even a low level of Cos2 expression was able to completely inhibit Ci activity. Furthermore, mutant I586A dSmo did not affect binding to *Drosophila* Cos2. This revealed, that the conserved aspect of the Smo mechanism that is inactivated by the isoleucine to alanine mutation, does not involve the regulation of Cos2-binding.

5.2.6 Human Smoothened is expressed and correctly folded in Schneider S2 cells, but does not rescue the loss of endogenous Smoothened

To study the conservation of Smo structure and function in the fruit fly system, we tested if hSmo could rescue RNAi-mediated depletion dSmo in S2 cells. After dSmo depletion, Fu and Cos2 phosphorylation was not induced by Hh. Expression of hSmo could be detected in stably transfected S2 cells at a similar protein level with or without treatment by Hh. When these cells were treated with dsRNAi directed against dSmo, no phosphorylation Fu and Cos2 in response to Hh was detected. This result suggests that hSmo is not able to compensate for the absence of dSmo.

We next investigated if hSmo was expressed in a functional state and why hSmo does not transduce the Hh signal in S2 cells. The steroidal alkaloid cyclopamine has been shown to specifically bind to the mammalian Smo heptahelical domain, but interestingly it does not bind to dSmo. The cyclopamine binding is very sensitive to the conformational state of mammalian Smo, as it binds with 10 times less affinity to the oncogenic Smo mutant, and recognizes only the intact binding site (Chen et al., 2002a; Taipale et al., 2000) (Figure 10).

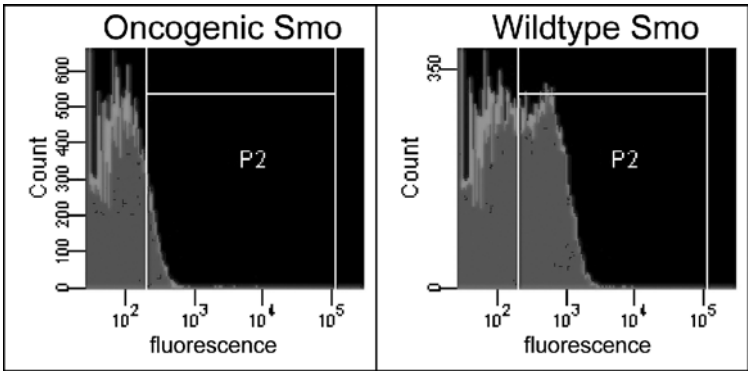

Figure 10. Cyclopamine binding assay. Oncogenic Smo-mutant and wildtype Smo expressing mammalian cells were incubated with fluorescence bodiby-cyclopamine and the binding was analyzed by FACS.

To test the conformational state of hSmo expressed in S2 cells, the cells were treated with fluorescence bodiby-cyclopamine. The binding efficiency of bodiby-cyclopamine to hSmo was measured using fluorescent-activated cell sorting (FACS) flow cytometry. The results indicated that hSmo was able to bind cyclopamine and suggested that the hSmo expressed in the S2 stable cell line was in a native conformational state. Using fluorescence confocal microscopy and FACS we demonstrated, that hSmo was enriched on the S2 cell surface in response to Hh. All these results suggest that hSmo is able to respond to Hh when expressed in S2 cells. Even though hSmo localizes on the S2 plasma membrane, it could not compensate for the absence of dSmo activity, most likely because hSmo fails to bind to Cos2.

The localization of Smo correlates with subsequent downstream signaling. In mammalian cells transfected with Smo, it is internalized immediately after Hh signal (Incardona et al., 2002). This is in contrast to dSmo which accumulates on the cell surface after Hh signaling. Mammalian Smo internalization can also be enhanced by Hh agonists and inhibited by the Hh antagonist cyclopamine (Chen et al., 2004). When expressed in *Drosophila* cells, hSmo is not internalized in response to Hh but is enriched on the plasma cell surface like dSmo. These results further promote the fact that Smo localization is differently regulated in fruit fly and mammals. The plasma membrane localization of hSmo in S2 cell somewhat resembles recent results showing Smo to be enriched and localized to primary cilia in response to Hh (Corbit et al., 2005). In addition to Smo, Su(Fu) and full length GLI proteins are also reported to localize to cilia after pathway activation (Haycraft et al., 2005). It is thus possible that this signal-dependent localization of Smo, and other Hh signaling pathway components to cilia, could be the mammalian functional equivalent to

Cos2. The critical role of Cos2 for the fly Hh signaling could result from the lack of cilia in *Drosophila* cells, except in sensory neurons and spermatids (Avidor-Reiss et al., 2004).

5.3 Divergence of Costal2 and Suppressor of Fused functions between mammalian and *Drosophila* Hedgehog signaling (I, II)

To futher rule-out the role of Cos2-like activity on mammalian Hh signaling, we cloned the two closest homologs to *Drosophila* Cos2, the kinesin-like proteins Kif27 and Kif7. The kinesin family members are a class of motor proteins that can move along microtubules powered by the hydrolysis of ATP. Several cellular functions including mitosis, meiosis and transport of cargo proteins require the support from the active movement of kinesins. Kinesins consists of a globular motor domain that is connected via a short, flexible linker neck to the stalk, a long central coiled-coil region, ending in a tail region. Two kinesin molecules form the functional kinesin dimer, where the cargo proteins bind to the tail while each head has two separate binding sites, one for the microtubule and the other for ATP. ATP binding, hydrolysis, and subsequent ADP release changes the conformation of the microtubule-binding domains, resulting in the motion of the kinesin. Extensive amino acid sequence analysis showed that the total domain similarity of Kif27 and Kif7 to Cos2 was 37.9% and 37.1%, respectively. The comparison of domain similarity of the closest orthologs to Cos2 is shown in Figure 11.

Mouse GENE	DOMAIN SIMILARITY TO *Drosophila* COS2 (%)			
	All	**Cargo**	**Stalk**	**Motor**
Kif27	37.9	33.7	42.4	38.9
Kif7	37.1	24.1	43.7	37.5
KIF4	36.2	29.6	40.5	36.9
KIF17	34.9	29.5	36.7	35.5
KIF5B	34.6	24.5	39.5	33.5
Kif21A	31.3	19.3	37.5	34.9

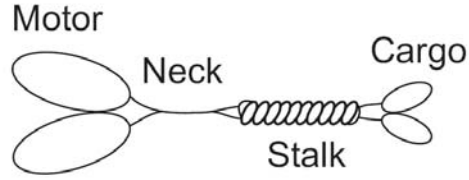

Figure 11. Comparison of the domain similarity of kinesin family members with the *Drosophila* Cos2.

Comparison of the amino acid sequences of kinesin family members with Cos2, showed that the *Drosophila* and mosquito (*Anopheles gambiae*) Cos2 proteins have significantly diverged from the other kinesins. They lack the conserved residues in the nucleotide binding and switch motifs in the motor domains, suggesting that they may be able to bind to microtubles, but are not able to actively move along them. In contrast vertebrate Kif27 and Kif7 have all the sequence characteristics for microtubule-dependent molecular motors, indicating that they have not been subjected to selective pressure similarly than the insect Cos2 proteins.

5.3.1 Overexpression of Kif27 and Kif7 has no effect on Sonic hedgehog pathway activity

Northern Blot analyses revealed that Kif27 mRNA was found to be expressed strongly in mouse testis, and weakly in other tissues, with the expression starting from E11. Kif7 was expressed in mouse kidney and in testis as an alternatively spliced-form. The expression of Kif27 or Kif7 had little or no effect in Hh-responsive mouse fibroblast cells (NIH-3T3). In addition, our study showed that Kif27 or Kif7 had no effect on transcriptional activity of co-expressed GLI1-3. This is in contrast to *Drosophila*, in which Cos2 inhibited the response of S2 cells to Hh, and co-expression of Cos2 with Ci, strongly suppressed Ci transcriptional activity.

5.3.2 Kif27 and Kif7 fail to associate with mammalian Smoothened and do not change the subcellular localization of the GLI proteins

We investigated the biochemical interactions between the Kif proteins and mSmo by co-immunoprecipitation. *Drosophila* Cos2 binds to dSmo through its cargo domain (Jia et al., 2003). In contrast, mammalian Kif27 or Kif7 did not immunoprecipate with mSmo. To rule out all requirements of Cos2-like activity on mammalian Shh signaling, we tested whether Kif27 or Kif7 would affect to the localization of the GLI proteins. *Drosophila* Cos2 binds to Ci and prevents Ci nuclear localization. However, the co-expression of Kif27 or Kif7 did not affect the nuclear localization of GLI2-3. In contrast, as shown previously for GLI1 (Kogerman et al., 1999), co-expression of Su(Fu) completely blocked the nuclear localization of GLI2-3.

5.3.3 Human Smoothened does not interact with Costal2 in Schneider S2 cells

Co-immunoprecipitation with antibodies directed against Cos2, co-precipitated dSmo and Fu, and Hh-treatment further enriched this association of dSmo with Cos2 and Fu. In hSmo-expressing S2 cells, we observed that although dSmo co-immunoprecipitated with phosphorylated Cos2 and Fu in the presence of Hh, hSmo did not. Similarly, the use of antibodies directed against a hemagglutinin epitope tag at the C-terminal end of hSmo revealed that neither Cos2 nor Fu co-immunoprecipitated with hSmo. These experiments indicate that in the hSmo-expressing S2 cells, Cos2 interacts with dSmo but not with hSmo. To avoid competition between hSmo and dSmo, we performed an immunoprecipitation of hSmo in dSmo RNAi-treated cells. Although the amount of endogenous dSmo was dramatically decreased, no interaction was observed between hSmo and the Fu-Cos2 complex. It has been previously shown that Cos2 interacts with the cytoplasmic tail of dSmo (publication I and Jia et al., 2003; Lum et al., 2003b). The Smo C-terminal sequences are not well conserved between *Drosophila* and vertebrates (publication I and Zhang et al., 2004)). To further determine if the cytoplasmic tail is responsible for the absence of association between Cos2 and hSmo, we analyzed the interaction between Cos2 and chimeric Smo where the N-terminal part of mouse Smo was fused with the cytoplasmic tail of *Drosophila*. We demonstrated that Cos2 and Fu do co-immunoprecipitate with the chimera, strongly suggesting that the cytoplasmic tail of dSmo is necessary to provide interaction with Cos2, and the lack of interaction of hSmo is due to the abscence of specific Cos2 binding sites.

5.3.4 Patched and Suppressor of Fused functions are required for normal mammalian pathway activity, whereas Kif27 or Kif7 are not involved in pathway regulation

The co-expression of Su(Fu) together with GLI(2-3), very efficiently inhibits the nuclear localization of GLI proteins (see 5.3.2). However, no Su(Fu) loss-of-function experiments had been performed. We designed short hairpin RNA (shRNA) constructs against the two known positively acting components of Shh signaling pathway, Smo and GLI2. All shRNAs against Smo and GLI2 significantly suppressed the pathway response to ShhNp in NIH-3T3 cells. We then designed shRNAs against other known or possible Shh pathway components; Ptc, Su(Fu), Kif27 and Kif7. RNAi against Kif27 or Kif7 had no effect, whereas, the knock-down of Ptc and Su(Fu) significantly elevated pathway activity. The effect of Su(Fu) knock-down was partial, probably

because Su(Fu) is in abundance in cells, and in excess to the GLIs (Paces-Fessy et al., 2004). We sensitized the assay by expressing low levels of GLI2 protein, and even under these conditions, the knock-down of Kif27 or Kif7 had no effect. Su(Fu) knock-down, instead, resulted in complete activation of the Shh pathway. These results indicated that endogenous Kif27 or Kif7 are not regulating GLI2 activity, and that Su(Fu) is the critical regulator of GLI2 activity. These results are in contrast to *Drosophila*, where Cos2 has a central role and Su(Fu) a minor role in pathway regulation. The results, are consistent with the studies of Cooper et al. (Cooper et al., 2005) and Svärd et al. (Svard et al., 2006), who revealed that loss of Su(Fu) in mouse embryos results in complete activation of the Hh pathway, similarly to the loss of Ptc. Together, these results also clearly show that mouse cells and embryos (Svard et al., 2006) lack a Cos2-like activity that, in *Drosophila*, can completely suppress the Hh pathway in the absence of Su(Fu).

Taken together, our study indicates that a significant divergence in the mechanism of signal transduction has occurred between vertebrates and invertebrates Hh signaling cascades. We show that the Hh receptor Ptc, and the mechanisms by which it inhibits the signal transducer Smo, are most likely conserved. However, the mechanism of Smo activation and the localization in response to the pathway activation is different. Our study also revealed that Cos2-like activities are absent in mouse cells based on three observations: (1) domains in Smo that are required in *Drosophila* to bind to Cos2 are not required for mSmo function; (2) mouse Shh signaling is insensitive to expression of *Drosophila* Cos2, but can be rendered Cos2 sensitive by replacing the mSmo C-terminal domain with the dSmo C-terminal domain; (3) overexpression or RNAi-mediated suppression of mouse Cos2 homologs has no effect on Hh signaling, even under sensitized conditions. These results are also consistent with divergence of the sequence of domains involved in Cos2 binding in Ci/GLI proteins and Smo between fly and mammals.

5.4 Identification of kinases that affect the activity of Sonic hedgehog signaling (III)

The lack of Hh pathway related phenotype with the Fu knock-out mouse (Chen et al., 2005; Merchant et al., 2005) and the drastic phenotype of Su(Fu)- knock-out mouse (Svard et al., 2006), suggests that there are unidentified components of the mammalian cytoplasmic signaling of Hh. Due to the lack of Fu in mammals, another kinase might regulate the activy of Su(Fu). To identify potential kinases regulating

Su(Fu) activity, we performed a screen in Hh-responsive mouse fibroblast cells (NIH-3T3) using the GLI-luciferase reporter (see Figure 9 and Taipale et al., 2002). To be able to perform the screen, we constructed a kinome-wide expression cDNA library.

5.4.1 Generation of the kinome-wide expression-ready cDNA collection

The initial set of full-length human protein kinase cDNAs were isolated from ~800 000 individual sequenced cDNA clones derived from 20 different tissue sources. Subsequently, cDNAs for those kinases that were not identified by the sequencing method were isolated by PCR screening of pooled and plated libraries resulting in 568 full-length kinome cDNA constructs. To increase the completeness of the collection, we used direct PCR to attempt to clone all of the remaining kinases from five separate sources of cDNA, representing mRNAs from 48 different human tissues and 34 unique cell lines. Using this approach, 16 more kinases were cloned, bringing the total to 93% of all kinases identified by the *in silico* analyses of the human genome (Manning et al., 2002).

5.4.2 Generation of the catalytically inactive kinase cDNA collection

Kinases with active site mutations are commonly used to dissect the specificity of the kinase activity in a cellular context and to identify functions that are independent of catalytic activity. However, no large collection of catalytically inactive kinases was available. To facilitate the downstream analysis of the kinases identified in various screens, we used high-throughput site-directed mutagenesis to change the important catalytic lysine residue of the valine-alanine-isoleucine-lysine motif to methionine. The resulting inactive kinome library contains 425 kinase cDNAs, representing 351 unique inactive kinases. Of these, 390 (316 unique kinases) (Manning et al., 2002) were inactive (Carrera et al., 1993; Ebina et al., 1987; Manning et al., 2002). Almost all (95%) of the kinases showing robust activity in wild-type form were inactivated by the lysine to methionine mutation.

5.4.3 Kinome expression screening to identify kinases regulating Sonic hedgehog signaling

We used the kinome-library to identify novel kinases affecting Shh signaling in mammalian cells. The Hh pathway reporter screen (see Figure 9) identified two novel kinases that affect mammalian Shh pathway, MAP3K10 and DYRK2. Both of these kinases affected Shh signaling *in vivo*, and loss of MAP3K10 or DYRK2 function resulted in failure of Shh signaling. In contrast to other signaling pathways where activation of pathway-specific kinases is central to signal transduction, addition of Shh to responsive cells did not appear to regulate the activity of expressed DYRK2 or MAP3K10. This is consistent with the lack of effect of Hh on activities of CK1α, CK1ε, GSK3β and PKA, known kinases whose activity is required for Hh signaling in both *Drosophila* and mammals. The mechanism by which the Shh signal is transduced thus appears to depend on multiple relatively generic kinases, with activity of the pathway likely controlled by access of these kinases to pathway-specific substrate(s).

In contrast to the nuclear kinase DYRK1, which has been reported to activate GLI1 (Mao et al., 2002), our study shows that DYRK2 localizes to the cytoplasm and inhibits Shh signaling by decreasing GLI activity. DYRK2 directly phosphorylated GLI2 sequences and resulted in the loss of co-expressed GLI proteins, indicating that DYRK2 acts by inducing the phosphorylation and degradation of GLI proteins via the ubiquitin/proteasome pathway. The other kinase we identified, MAP3K10, acts positively on the Shh pathway by increasing the transcriptional activator-activity of GLI2. MAP3K10 has previously been linked to JNK signaling (Gallo and Johnson, 2002) and to the regulation of trafficking of clathrin-coated vesicles (Akbarzadeh et al., 2002). The effect of MAP3K10 on GLI2 appears not to be direct. Instead, MAP3K10 binds to multiple kinases regulating Shh pathway, including CK1α, CK1ε, GSK3β and DYRK2. In addition, MAP3K10 has been shown to associate with Kif3a (Nagata et al., 1998), a component of cytoplasmic Kinesin II, which is required for primary cilia function and Shh pathway regulation in mice (Huangfu et al., 2003). Our protein microarray experiments confirmed this finding and further indicated that Kif3a is also a substrate of MAP3K10. Although both MAP3K10 and DYRK2 act on GLI2 but with opposing effects, MAP3K10 action cannot be explained solely by its effect on DYRK2, as these kinases have different activity on GLI2-VP16 transcriptional activity. Many connections of MAP3K10 with different pathway components suggest that MAP3K10 action on GLI2 is likely to be complex and require further study.

6 CONCLUSIONS

The conclusions of this study can be summarized as follows:

1. *Drosophila* and mammalian Hh signaling mechanisms have diverged, and in mouse cells, the Costal2 –like activities are absent. Furthermore, the inhibition of the Hedgehog pathway in the absence of ligand critically depends on Suppressor of Fused in mammalian cells.

2. The upstream part of the Hedgehog pathway involving Hedgehog interaction with Patched, regulation of Smoothened by Patched, and Smoothened enrichment at the plasma membrane is highly conserved between *Drosophila* and mammals –in contrast, signaling downstream of Smoothened is not.

3. Kinome expression screening is a highly effective way to identify physiological signaling pathway components, and revealed the role of MAP3K10 and DYRK2 in Sonic hedgehog signal regulation.

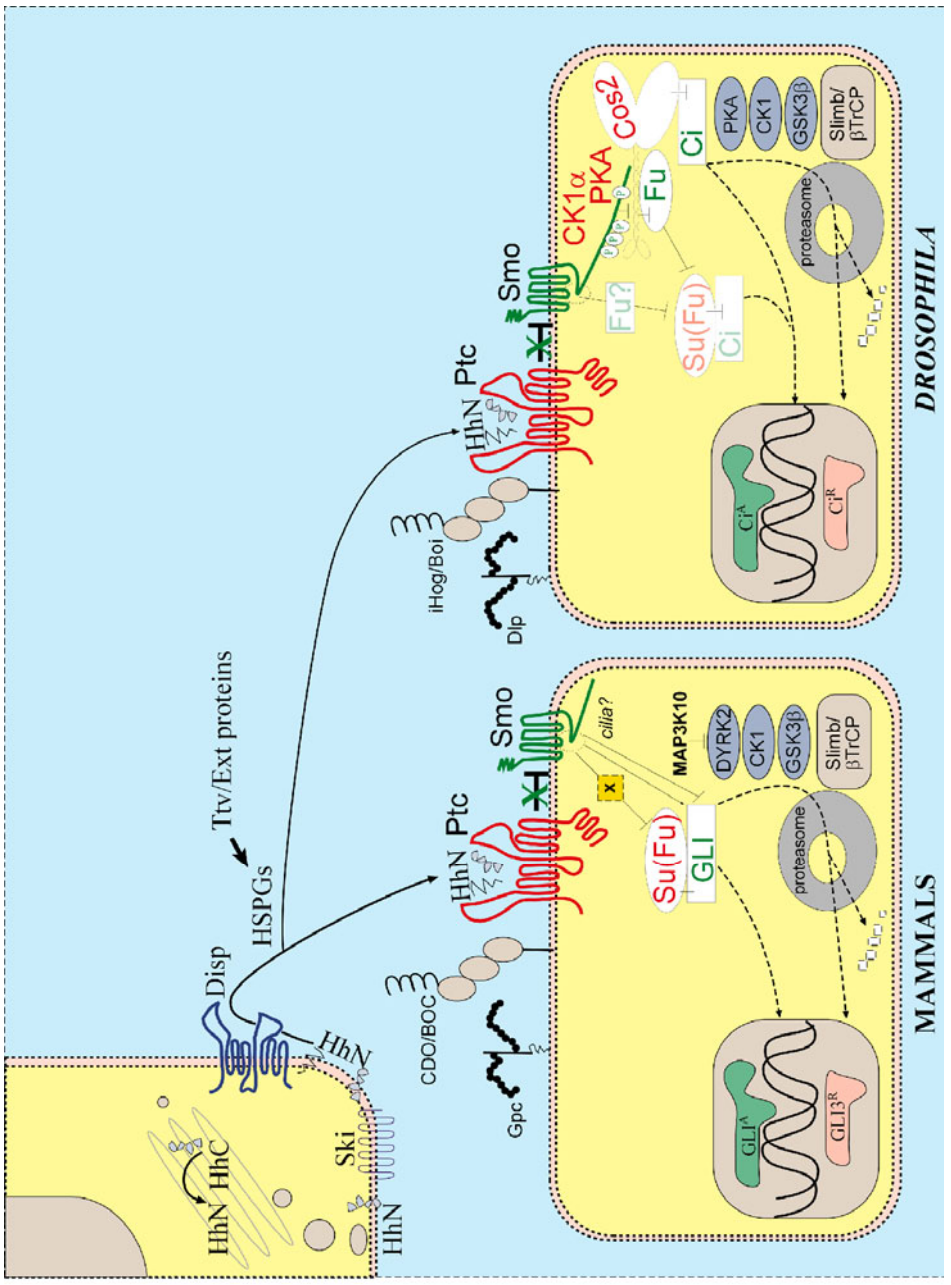

Figure 12. The mammalian and *Drosophila* Hh signaling pathways.

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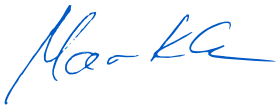

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