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Repressing the Hedgehog Signalling Pathway

*Functional Analysis of the Tumour Suppressors
Patched1 and Suppressor of Fused*

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ABSTRACT

The Hedgehog (Hh) signalling pathway is essential for metazoan development and aberrant activation of the pathway is found in tumours. Mutations in Hh pathway components are found in several tumour types, including basal cell carcinoma (BCC) and medulloblastoma (MB). The outline of the Hh pathway is evolutionary conserved, with Patched (Ptch) as the Hh receptor and Smoothed (Smo) as the signal transducer that relays the signal into the cytoplasm to activate the transcription factors Ci/Gli, which regulate gene transcription and cellular responses leading to cell proliferation and/or differentiation.

These studies focus on the function of Ptch1 and Suppressor of fused (Sufu), both negative regulators of Hh signalling. In paper I, we showed that genetic ablation of *Sufu* in mice results in embryonic lethality around embryonic day 9 with failure to close the neural tube. *Sufu*^{-/-} embryos showed high Gli activity that was not repressible at the level of Smo. *Sufu*^{+/-} mice develop jaw keratocysts and a skin phenotype with BCC-like lesions, alopecia, increased skin pigmentation, and aberrant sebaceous gland morphology. Our data show that in contrast to the situation in *Drosophila*, Sufu has a central role in mammalian Hh signalling, and its loss-of-function leads to ligand-independent activation of the Hh pathway.

In paper II, we have investigated the possibility that Sufu would modulate the phenotype of *Ptch1*^{+/-} mice or vice-versa. We found that the frequency of MB was not altered in *Sufu*^{+/-}*Ptch1*^{+/-} mice compared to *Ptch1*^{+/-} mice. All MB in *Ptch1*^{+/-} mice and all but one MB in *Sufu*^{+/-}*Ptch1*^{+/-} mice had lost expression of the *Ptch1* wt allele, indicating that this is the critical genetic change leading to MB formation in these mice. Skin from *Sufu*^{+/-}, *Ptch1*^{+/-} and *Sufu*^{+/-}*Ptch1*^{+/-} mice developed BCC-like epidermal lesions. The number of such lesions was increased in *Sufu*^{+/-}*Ptch1*^{+/-} mice compared to *Sufu*^{+/-} and *Ptch1*^{+/-} mice. Our data indicate a differential importance of Sufu and Ptch1 as tumour suppressors in MB versus the BCC-like lesions.

In paper III, we investigated the unique properties of PTCH1 isoforms generated by alternative first exon usage. All isoforms functioned as Hh receptors. However, the

isoforms induced by Hh signalling inhibited pathway activity to a higher extent than those not regulated by Hh signalling. In situ hybridization allowed the detection of the *Ptch1* isoforms in specific structures of mouse embryos. This study supports a role of splicing variation and/or promoter choice for Hh signalling regulation.

In paper IV, we created a conditional *Ptch1* allele in mice and deleted *Ptch1* by using the *ARR2PBi-Cre* transgenic line. *Ptch1* was deleted in prostate and seminal vesicles but we found no aberrant phenotype in these tissues up to the age of 12 months. In contrast, BCC-like epidermal lesions were initiated by Cre-mediated *Ptch1* recombination in solitary cells in interfollicular epidermis and hair follicles. Skin with BCC-like lesions showed upregulation of *Gli1*, an indicator of Hh pathway activity. The skin proliferations arose both from interfollicular epidermis and hair follicles. Our results indicate that loss of *Ptch1* in keratinocytes drives them into a hair follicle fate.

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LIST OF PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their roman numerals:

- I. **Svard J***, Heby-Henricson K*, Persson-Lek M, Rozell B, Lauth M, Bergstrom A, Ericson J, Toftgard R, Teglund S. Genetic elimination of Suppressor of fused reveals an essential repressor function in the mammalian Hedgehog signaling pathway. *Dev Cell*. 2006 Feb;10(2):187-97.
- II. **Svard J**, Rozell B, Toftgard R, Teglund S. Increased number of BCC-like skin lesions but not medulloblastoma in compound Patched1 and Suppressor of Fused heterozygous mice. Manuscript submitted.
- III. Shimokawa T, **Svard J***, Heby-Henricson K*, Teglund S, Toftgard R, Zaphiropoulos PG. Distinct roles of first exon variants of the tumor-suppressor Patched1 in Hedgehog signaling. *Oncogene*. 2007 Jul 26;26(34):4889-96.
- IV. **Svard J**, Rozell B, Toftgard R, Teglund S. BCC-like skin tumours induced by homozygous inactivation of Patched1 in mouse epidermal cells. Manuscript submitted.

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ABBREVIATIONS

BCC	Basal cell carcinoma
BMP	Bone morphogenic protein
C.elegans	Caenorhabditis elegans
Ci	Cubitus interruptus
Cos2	Costal 2
C-terminal	carboxyl terminal
Dhh	Desert hedgehog
Disp	Dispatched
Drosophila	Drosophila melanogaster
E	Embryonic day
Fu	Fused
GSK-3 β	Glycogen synthase kinase-3 β
Hh	Hedgehog
JAK	Janus-activated kinase
kDa	kilodalton
Ihh	Indian Hedgehog
LOH	Loss of heterozygosity
NBCCS	Nevoid basal cell carcinoma syndrome
N-terminal	amino terminal
Ptch	Patched
Shh	Sonic hedgehog
Smo	Smoothened
STAT	Signal transducers and activators of transcription
Sufu	Suppressor of fused
TGF	Transforming growth factor
UV	Ultraviolet
Wnt	Wingless/Int

INTRODUCTION

Cancer

Cancer is characterized by uncontrolled division of cells that invade nearby tissues or spread through the bloodstream and lymphatic system to other parts of the body in a process called metastasis. It has been proposed that most, if not all, types of human tumours share a number of capabilities. These capabilities are: self-sufficiency in growth signals; insensitivity to growth-inhibitory signals; evasion of programmed cell death; limitless replicative potential; sustained angiogenesis and tissue invasion and metastasis [1]. Many of the genes that control these cellular processes are proto-oncogenes and tumour suppressor genes. Mutations in these genes will result in the cell losing control over these cellular processes. Tumorigenesis is a multistep process, reflecting the need for several genetic alterations, that drives the progressive transformation of normal human cells into highly malignant derivatives [1]. Genetic alterations can occur at many levels. Entire chromosomes can be gained or lost or a single DNA nucleotide can be mutated. Alternatively, promoters can be methylated, resulting in transcriptional silencing. Carcinogens, such as tobacco smoke, radiation, chemicals, or infectious agents, may cause the genetic changes. They may also be acquired through errors in DNA replication or they may be inherited. In the latter case, the genetic change is present in all cells from birth.

Oncogenes

An oncogene is a gene that gives a cell one or more of the capabilities that characterize cancer cells (see above). A proto-oncogene is a normal gene that can become an oncogene due to mutation or increased expression. Mutations in oncogenes are dominant (mutation of only one gene copy is needed) as they involve gain-of-function mutations. Proto-oncogenes can be classified into five main groups: growth factors, growth factor receptors, signal transducers, transcription factors and regulators of cell death [2]. Examples of proto-oncogenes include *RAS* and *MYC*. The first oncogene *src*, was discovered by studying chicken retroviruses [3].

Tumour suppressor genes

Tumour suppressor genes are often inactivated in cancer cells, resulting in the loss of normal cellular functions, such as accurate DNA replication, control over the cell cycle, and orientation and adhesion within tissues. Mutation of tumour suppressor genes usually follows Knudson's two-hit hypothesis [4]. In this hypothesis, an inherited germ-line mutation in a tumour suppressor gene will only cause cancer if another mutation event that inactivates the other allele, occur later in life. Inactivation of the other allele is called loss of heterozygosity (LOH). Mutations of tumour suppressor genes that occur in germline increase the risk for cancer in subsequent generations. Members of these families have increased incidence and decreased latency of multiple tumours. The tumour types are typical for each tumour suppressor. Sometimes, inactivation of one allele of a tumour suppressor gene is sufficient to cause tumours. This phenomenon, called haploinsufficiency [5], has been demonstrated for p27 [6] and other tumour suppressors. Examples of tumour suppressors are p53, the retinoblastoma (*RB*) gene, Ptch1 and Suppressor of fused, of which the latter two are the main focus of this thesis.

Metastasis

Cancer cells usually spread through the bloodstream and lymphatic system. The major cause of death from cancer is due to metastasis. For a tumour cell to metastasize it must leave the primary tumour, enter the lymphatic or blood circulation, survive within the circulation, overcome host defences, extravasate and grow as a vascularized metastatic colony. Tumours shed large numbers of tumour cells, but less than 0.01% of these cells develop into metastases [7].

Treatment

Cancer is usually treated with a combination of surgery, chemotherapy and radiotherapy. As research develops, treatments are becoming more specific for different varieties of cancer. There has been significant progress in the development of targeted drugs that act on specific molecular abnormalities in certain tumours and thereby minimizing damage to normal cells. Many of these agents, for example imatinib/Glivec, are inhibitors of receptor tyrosine kinases [8].

Cancer and development

During development from a fertilized egg, cells need to differentiate to perform different functions in order to form a mature adult organism. This process is highly regulated and depends on communication between cells to co-ordinate their proliferation and differentiation. Such communication depends on signals being transferred from the cell surface to the nucleus of the receiving cell to affect its gene transcription. There are a few evolutionarily conserved pathways that are used for signalling between cells during development. They include Wingless/Wnt, Notch, TGF/BMP, receptor tyrosine kinases, JAK/STAT, and Hedgehog (Hh) [9, 10]. Signals from these pathways are integrated in a cell to result in a cellular response.

A strong link exists between the biology of development and the biology of cancer. The signalling pathways that control proliferation and differentiation during normal development are often dysregulated in cancer. For example, tumours associated with Hh signalling typically arise from tissues in which the pathway normally operates. In tumours, mutations in Hh pathway components result in ligand-independent constitutive activity, leading to uncontrolled proliferation of tumour cells [11, 12], as will be thoroughly discussed later.

Hedgehog signalling in development

The Hh gene was discovered in *Drosophila melanogaster* by Nusslein-Volhard and Wieschaus in a genetic screen for mutations affecting the patterning of the *Drosophila* larval cuticle [13]. Hh was found to be one of several genes important for creating the differences between the anterior and posterior parts of individual body segments in *Drosophila*. The name "Hedgehog" was inspired from the phenotype of the mutant embryos, which instead of the normal segmented pattern of denticles are covered with a lawn of denticles resembling the spines of a hedgehog. The *Hh* gene encodes a secreted protein [14-16] that is essential for development in most metazoans. In *Drosophila*, Hh is important for development of wings, legs and eyes [17]. *Hh* genes have also been identified in several other species, including human, mouse, chick and zebrafish. In these different species the Hh proteins are important for growth, patterning and morphogenesis of many different tissues. [18-22]. In *C. elegans*, the

Hh signalling pathway has undergone considerable divergence. Obvious Hh homologues are absent, whereas homologues of the Hh receptor are present with functions in *C. elegans* development [23].

In mammals, there are three Hh homologues with largely non-overlapping functions [18]. Sonic hedgehog (Shh) is essential for patterning of several organs during development. It is expressed in the developing CNS, limb, lung, gut, teeth, and hair follicle [17, 22, 24-29]. Shh is for example needed for polarization of the anterior-posterior axis of the developing limb-bud and is the signal from the notochord involved in determining ventral cell fate of the neural tube. Indian hedgehog (Ihh) plays key functions in the development of bone and cartilage [30] and Desert hedgehog (Dhh) is involved in the development of testes and external genitalia [31]. All three Hh proteins bind the receptor Patched (Ptch) with equal affinities and use the same intracellular signalling pathway [32]. The unique expression patterns of the different Hh proteins partly explain their role in patterning of different organ systems.

In addition to its roles in cell fate and patterning, Hh proteins have also been implicated in regulating cell proliferation, cell migration, stem cell renewal, and tissue regeneration and repair [33-37].

Hedgehog signalling in disease

Disruption of Hh signalling during embryonic development leads to severe developmental defects. Holoprosencephaly, the failure of the embryonic prosencephalon to form cerebral hemispheres, is linked to mutations in *SHH*, *GLI2* and *PTCH1*, all components of Hh signalling [38]. Consumption of the Hh pathway inhibitor cyclopamine by pregnant mammals also results in holoprosencephaly [38]. Mutations in *GLI3* cause the Pallister-Hall syndrome and the Greig cephalopolysyndactyly syndrome, both of which manifest polydactyly [39]. Mutations in *PTCH1* cause the nevoid basal cell carcinoma syndrome (NBCCS) [40, 41], which is characterized by developmental defects and a predisposition to multiple basal cell carcinomas (BCCs) in the skin, medulloblastoma in the brain and rhabdomyoma in muscle [42, 43]. The NBCCS was the first link between Hh signalling and cancer.

Since then, aberrant Hh signalling has been associated with cancers in various organs, including brain, lung, prostate, and skin [44-51]. This will be discussed in detail later in this thesis.

Signal transduction of the Hedgehog pathway

The basic outline of the Hh pathway is relatively conserved from flies to humans (Fig. 2). Signalling starts with Hh being released from the producing cell. Hh then binds its receptor Ptch (Ptc in *Drosophila*) on the receiving cell, which leads to activation of the signal transducer Smoothed (Smo). Smo relays the Hh signal into the cytoplasm to activate the family of transcription factors Ci/Gli, which regulate transcription of target genes.

Production and secretion of Hedgehog

The Hh proteins need post-translational modifications to become fully active. First, Hh is autocatalytically cleaved to give an active N-terminal fragment (HhN) [14, 52, 53], which is modified by addition of a cholesterol moiety at its C-terminal [54]. Next, HhN is modified by the addition of a palmitoyl moiety at its N-terminal. The latter reaction is catalyzed by the acyltransferase Skinny hedgehog (Skn) [55-59]. The cholesterol and the palmitoyl moiety appears to be essential for long-range signalling [56, 60, 61].

In both *Drosophila* and mammals, release of Hh from producing cells requires the transmembrane protein Dispatched (Disp) [62-65]. *Disp* shares significant sequence similarity with *Ptch* [62]. Mice mutant for *Disp* have severe defects in the development of the ventral neural tube similar to *Smo* deficient mice [63, 64, 66].

Reception of Hedgehog

Without Hh, Ptch inhibits the activity of Smo, a seven-pass transmembrane protein, which is structurally similar to G-protein coupled receptors [17]. The mechanism for Ptch repression of Smo is still not known. Ptch was first thought to repress Smo activity by direct binding [67], but was later found to inhibit Smo at a ratio of 1:50,

suggesting that Ptch acts catalytically to inhibit Smo [68]. When Hh binds to Ptch the repression of Smo is relieved [67, 69-71].

Cdo and Boc belong to a newly recognized subfamily within the immunoglobulin superfamily of cell-surface molecules. They act as co-receptors for Hh, enhancing binding to Ptch [72, 73].

In vertebrates, the Hh-interacting protein (Hip) acts on the cell surface to sequester Hh [74]. Hip also regulates the amount of Hh available to bind Ptch [74]. Hip mutant mice die of respiratory failure shortly after birth [75].

Patched1

The *PTCH1/Ptch1* gene consists of 23 exons encoding a 1447/1434 amino acid glycoprotein [40, 41]. Human *PTCH1* maps to 9q22.3 and mouse *Ptch1* to chromosome 13. *Ptch1* is expressed in all major target tissues of Shh, such as the ventral neural tube, somites, and tissues surrounding the zone of polarizing activity of the limb bud [25, 76]. Ptch1 is predicted to have 12 transmembrane domains, two large extracellular domains where Hh binds, and a smaller intracellular domain [25, 77] (Figure 1).

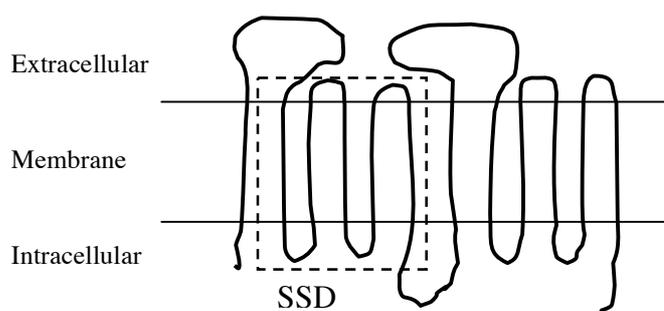


Figure 1. Topology of Patched1. Patched1 is predicted to have 12 transmembrane domains, two large extracellular domains, and a smaller intracellular domain. Five of the membrane-spanning domains show homology with sterol sensing domains (SSD).

Transmembrane domains 2-6 show homology with sterol sensing domains (SSD) found in several proteins involved in cholesterol homeostasis [78]. These include the protein responsible for the Niemann-Pick C disease [79, 80], and HMG-CoA reductase [78]. The role of the SSD of Ptch1 is unknown. It has been proposed that the response of target tissues to Hh is dependent on the detection of intracellular sterol levels by the SSD [81].

Ptch binds all Hh proteins with low nanomolar affinity [67]. Ptch is structurally similar to the resistance-nodulation-division (RND) family of prokaryotic permeases [68, 82] that transport small molecules across membranes. Mutations in conserved residues in the RND-like structure impair Ptch function [68, 83, 84]. Ptch has therefore been proposed to function as a transporter of a small molecule that acts as a Smo agonist or antagonist [68]. Several candidates for this small molecule have been proposed, including pro-vitamin D3 [85] and oxysterols [86].

Ptch1 is essential during development. In humans, germline *PTCH1* mutations cause NBCCS [42] and in mice, *Ptch1*^{-/-} embryos die in midgestation with an open neural tube [87, 88]. *PTCH1* is also a tumour suppressor. Mutations in *PTCH1* have been identified in basal cell carcinoma, medulloblastoma, meningioma, breast carcinoma, oesophageal carcinoma, squamous cell carcinoma, and trichoepithelioma [89], PTCH Mutation Database: www.cybergene.se/PTCH/.

Patched1 variants

PTCH1 is reported to have at least four first exons, 1, 1A, 1B and 1C [40, 41, 90-92] and undergoes exon skipping [92, 93] or alternative exon insertion events [94]. The alternative promoters for exons 1B and 1C have GLI1 consensus binding sites and HH signalling results in upregulation of both PTCH1-1B and -1C [91]. These isoforms may thus work as mediators of the negative feedback loop, which characterizes HH signalling. The *PTCH1* alternative promoters and first exons influence the expression pattern and the stability of the proteins as well as their capacity to act as inhibitors of HH signalling [95].

Patched2

Patched2 (Ptch2), found in vertebrates, has an unclear role in Hh signalling [96-98]. Human *PTCH2* is localized to chromosome 1, consists of 22 exons and encodes a 1203 amino acid protein [98]. *PTCH1* and *PTCH2* have a high amino acid sequence identity (57%) [96, 99] and bind SHH, DHH, and IHH with similar affinities [96]. *PTCH2* is like *PTCH1* a target gene of Hh signalling [97, 100]. It is expressed preferentially in skin and testis [96]. During epidermal development, *PTCH1* and

PTCH2 are expressed differentially suggesting specific roles for each protein. *PTCH1* is expressed in mesenchymal cells, while *PTCH2* and *SHH* are co-expressed in epithelial cells [97].

Rare mutations in *PTCH2* have been found in BCC and MB [98]. Mice with a targeted mutation of *Ptch2* develop alopecia and epidermal hyperplasia [101]. Loss of *Ptch2* also affects tumour formation in combination with *Ptch1* haploinsufficiency. *Ptch1*^{+/-}*Ptch2*^{-/-} and *Ptch1*^{+/-}*Ptch2*^{+/-} mice showed a higher incidence of MB and rhabdomyosarcoma than *Ptch1*^{+/-} [102]. *PTCH2* may act as a Hh receptor that fine tunes the signalling in various cellular environments.

Signalling from Smo to Ci/Gli

Smo transduces the Hh signal across the plasma membrane. In *Drosophila*, Hh induces multiple phosphorylations in the Smo C-terminal cytoplasmic tail, leading to its cell surface accumulation and activation [103-106]. Phosphorylation activates Smo by inducing a conformational change and dimerization of Smo cytoplasmic tails, which is essential for pathway activation [107]. Smo transmits the signal to a cytoplasmic complex [108-111] composed of the kinase Fused (Fu), the kinesin-like protein Costal 2 (Cos2), the novel protein Suppressor of Fused (Sufu), and the transcription factor Cubitus interruptus (Ci). Interaction between this complex and Smo, presumably via a direct association with Cos2, is necessary for transducing the signal from the membrane [112-115]. This complex controls the processing, activity, and subcellular distribution of the Ci transcription factor responsible for Hh target gene activation. *Smo* and *Shh/Ihh* compound mutants have identical phenotypes: embryos fail to turn, arresting at somite stages with a small, linear heart tube, an open gut and cyclopia [116].

Drosophila and mammalian Hh signalling have diverged between Smo and Ci/Gli. Based on functional analysis and sequence conservation of putative Cos2 orthologues, Sufu, Smo, and Ci/GLI it was found that major Cos2-like activities are absent in mammalian cells and that the inhibition of the Hh pathway in the absence of ligand depends on Sufu [117].

The function of Fu is also different. In *Drosophila*, Hh-induced Smo accumulation is inhibited in *fu* mutant clones [118]. Mice deficient in *Fu* do not exhibit phenotypes indicative of defective Hh signalling during embryonic development [119, 120]. Postnatally, they show growth defects and lethality due to progressive hydrocephalus (HC). HC is also found in *Ptch1* heterozygous mice [121].

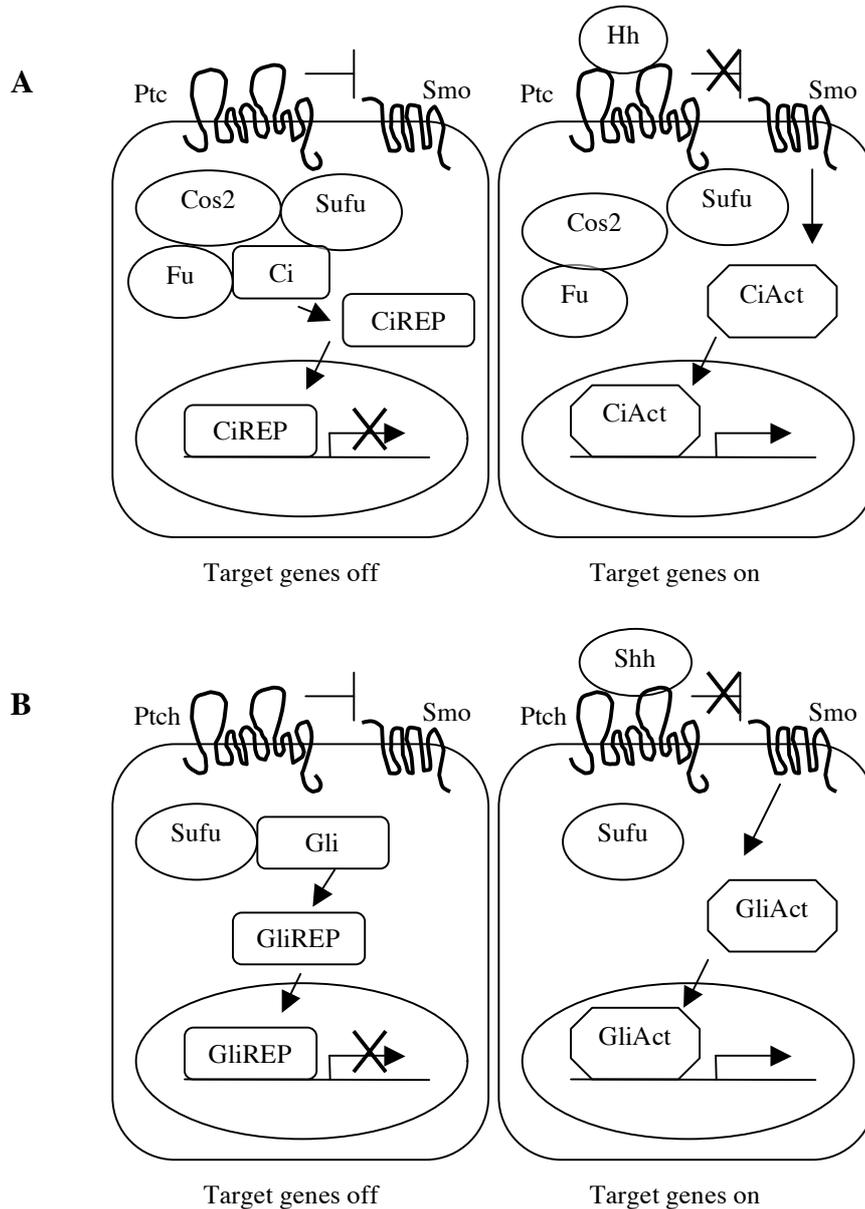


Figure 2. Schematic representation of the Hedgehog signalling pathway in *Drosophila* and in mammals. Shown are the core components. However, there are several other proteins known to modulate the pathway that are not depicted. In the absence of Hedgehog (Hh) induction, Patched (Ptc) inhibits the activity of Smoothened (Smo). **(A)** In *Drosophila*, the complex with Suppressor of fused (Sufu), Costal 2 (Cos2) and Fused (Fu) allows processing of Ci into a repressor, CiREP, which inhibits transcription of target genes. In the presence of Hh (right), the inhibition of Smo is relieved and Ci is converted into an activator, CiAct, which induces transcription of target genes. **(B)** In mammals, Sufu can sequester Gli in the cytoplasm and inhibit transcription of target genes in concert with the Gli repressor form (GliREP). In the presence of Shh (right), the inhibition of Smo is relieved and GliAct is produced, leading to activation of target-gene transcription. In mammals, obvious Cos2 and Fu homologues are missing.

Suppressor of fused

Sufu was identified in *Drosophila*, where loss of *Sufu* suppresses the fused wing vein phenotype of *Fu* mutants [122]. Flies lacking *Sufu* are viable, fertile, and display only subtle developmental defects [122, 123]. Human *SUFU* is 37% identical with *Drosophila Sufu* and 97% identical with mouse *Sufu*. Two alternately spliced protein isoforms include a 433 amino acid 48 kDa form and a 484 amino acid 54 kDa form [124]. Human *SUFU* contains a PEST domain [124] but no other known motifs. The mouse *Sufu* and human *SUFU* genes contain 12 exons [125] and are found on chromosome 10 and 19 respectively.

Sufu, like Ptch, is a negative regulator of Hh signalling [123, 126, 127]. *Drosophila Sufu* appears to inhibit Ci by blocking nuclear accumulation of full-length Ci [123, 126, 128]. *SUFU* interacts with GLI1, GLI2 and GLI3 [124, 129]. Over-expression of mouse *Sufu* or human *SUFU*, in cell-lines, inhibits Gli-dependent transcription and promotes cytoplasmic localization of Gli1. Sufu has a nuclear export signal (NES) and is thought to sequester Gli1 in the cytoplasm by restricting its nuclear localization [127, 130-132]. Furthermore, Sufu can recruit the histone deacetylation (HDAC) machinery to Gli1 through interaction with SAP18, a component of the mSin3A protein complex [133]. Sufu has also been shown to be a negative regulator of β -catenin [134, 135].

Mouse *Sufu* is expressed in various tissues throughout the developing embryo. It is particularly expressed in the nervous system, ectoderm, and limbs. Whole-mount *in situ* hybridization analysis detected expression throughout mid gestation in the neural tube and, later, in the brain, overlapping with expression of other components of the Hh signalling pathway [125, 136].

Mutations in human *SUFU* have been found in MB, prostate cancer and rhabdomyosarcoma, implicating it as a tumour suppressor [134, 137, 138]. Germline deletion of *SUFU* was found in a patient with some features of NBCCS [134]. *Sufu* heterozygous mice develop MB in combination with loss of p53 [139].

The Ci/Gli transcription factors

In *Drosophila*, the zinc finger transcription factor Ci is responsible for activation and repression of Hh target genes [140]. The activity of Ci is mainly controlled at the post-translational level [141]. In cells not exposed to Hh, full-length Ci is found in the cytoplasmic complex with Cos2, Fu, and Sufu. In the absence of Hh, complex-bound full-length Ci is targeted for processing [142-144]. This removes the transcriptional activation domain generating a truncated Ci (CiREP) that retains the zinc finger DNA binding domain and an N-terminal repressor domain. CiREP translocates to the nucleus and represses expression of target genes [141, 145]. In the presence of Hh, processing of Ci is inhibited and the cytoplasmic complex dissociates, Ci relocates to the nucleus and target genes are activated. This activation is thought to result from a combination of lifting of repression by CiREP [141], nuclear import of full-length Ci [142], and maturation of full-length Ci into a labile transcriptional activator (CiACT) [123]. Loss of CiREP alone may be sufficient for expression of some target genes, whereas the expression of other Hh targets requires CiACT.

In vertebrates, the Ci homologues: Gli1, Gli2 and Gli3 are responsible for activation and repression of Hh target genes. Gli1 was first identified as being upregulated in glioblastoma due to gene amplification [146]. Although Ci and the Gli proteins are highly similar, there are important differences in their regulation. Unlike Ci, Gli1 is a canonical target gene activated in response to the Hh signal [147, 148]. Gli1 lacks an apparent N-terminal repressor domain and seems to only function as an activator [148-150]. Like Ci, Gli2 and Gli3, act as bipotential transcription factors possessing both activation and repression domains [147, 149, 150]. Proteolytical processing, by the proteasome, converts them into repressors [141, 151-153]. It has been proposed that Gli2 and Gli3 are the primary mediators of Hh signalling [149], and when converted to their active forms they activate Gli1 at the transcriptional level [147, 149]. In mammalian cells, Gli1 and Gli2 serve mainly as positive transcriptional regulators, whereas Gli3 mainly acts as a repressor [17]. Activation of Hh target genes is thought to result from the synergistic action of Gli1, 2 and 3. The expression and post-translational stabilization of the various Gli family members creates a distinct combination of transcriptional activators and repressors that results in a specific biological readout [154]. Several proteins, for example PKA [151, 155] and GSK-3 β

[156], can modulate the ratio of Gli activator to Gli repressor and thereby determine the level of Hh signalling perceived by the cell [154].

Downstream targets of Hedgehog signalling

The Ci/Gli transcription factors regulate gene expression by association with a consensus binding site (5'-GACCACCCA-3') in the promoter region of target genes. Activator and repressor forms bind the same consensus sequence, but it appears that the sensitivity of particular promoters to regulation by either activators or repressors is determined by additional cis-regulatory elements [157].

In *Drosophila*, targets of Hh signalling include *wingless* (homologous to the vertebrate Wnt genes), *decapentaplegic* (a member of the TGF/BMP superfamily) as well as *ptc* [158, 159].

Downstream targets of vertebrate Hh signalling include Bcl2, CyclinD, FoxM1, FoxE1, Hip, Ptch1, Ptch2 and Gli1 [74, 100, 160-165]. Since many of these genes are directly involved in cell cycle regulation (cyclins) and cell survival (Bcl2), activation of these genes is one mechanism contributing to proliferation and tumour development. Upregulation of Ptch1/2 and Gli1 provide negative and positive feedback regulation of Hh signalling, respectively.

Cilia in mammalian Hedgehog signalling

Genetic studies in mice revealed that a number of components of the intraflagellar transport (IFT) machinery are required for mammalian Hh signalling [166, 167]. IFT proteins are essential for the assembly and maintenance of cilia and flagella [168] and several mammalian Hh signalling components including Ptch1, Smo, Sufu, and all three Gli proteins have been found to localize to cilia [167, 169]. In IFT mutants the localization of Smo to cilia is disrupted [170]. Cilia localization of Smo is stimulated by Hh and blocked by a Hh pathway inhibitor [171]. Ptch1 inhibit Smo by preventing its accumulation within cilia. When Shh binds to Ptch1, Ptch1 leaves the cilia, leading to accumulation of Smo and activation of signalling [169]. In the absence of Hh, cilia localization of Gli3 could be essential for its processing to a repressor, as Gli3

processing is compromised in IFT mutants [166, 167, 172, 173]. It has been suggested that cilia may function as a signalling centre for Hh signal transduction, concentrating Hh pathway components to allow more efficient protein-protein interactions.

In *Drosophila*, IFT mutants are viable and do not exhibit any phenotype associated with defective Hh signalling. In zebrafish, morpholino knockdown (antisense oligos) experiments and mutagenesis studies have failed to reveal any role for IFT proteins in Hh signalling. Thus, involvement of cilia in Hh signalling seem to have evolved in mammals [167].

Dysregulated Hedgehog signalling in tumours

Nevoid basal cell carcinoma syndrome

Nevoid basal cell carcinoma syndrome (NBCCS), also called Gorlin syndrome, was briefly mentioned earlier. It is an autosomal dominant disorder with high penetrance and variable expression [42, 43]. The incidence of NBCCS is estimated to be approximately 1 in 56,000 [174]. NBCCS is characterized by developmental defects and a predisposition to tumour formation with multiple organs being affected. It occurs with equal frequency in both sexes. Mean height is increased and the head is sometimes enlarged with frontal bossing. Approximately 80% of the patients are affected by odontogenic keratocysts (jaw cysts). In skin, multiple and early onset, basal cell carcinomas (BCC) are characteristic. The BCCs occur mostly on the face and upper trunk. Other skin features include; palmar and plantar pits, which are holes, formed in areas with defective keratinisation, benign dermal cysts, palmar and plantar keratosis. Skeletal defects manifested as splayed, cervical, or bifid ribs and calcification of the falx cerebri are common. Polydactyly are found in about 5% of NBCCS patients. Other tumours than BCC found in NBCCS patients are; medulloblastomas, astrocytomas, meningioma cardiac and ovarian fibromas, and others with low frequency.

The NBCCS gene was localized to 9q22-31 by linkage analysis [175]. The underlying genetic event was found to be inactivation of the *PTCH1* gene, resulting in activation

of the Hh signalling pathway [40, 41]. NBCCS patients have germline mutations in one *PTCH1* allele. Subsequent loss of the remaining allele results in cancer formation.

Basal cell carcinoma

BCC of the skin is the most common cancer type in humans. It has the potential for local invasion and destruction but rarely metastasizes. BCCs develop mostly on hair-bearing skin, most commonly on sun-exposed areas. Ultraviolet (UV) exposure, race, age, gender, and decreased DNA repair capacity are known risk factors [176].

Mutations in *PTCH1* play a major role in BCC development, with frequent LOH of the *PTCH1* locus both in sporadic BCCs and in hereditary BCCs in NBCCS [177-179]. *SMO* activating mutations [180] and *PTCH2* [98] mutations are also found in BCCs. More than 70% of sporadic BCCs have detectable genetic mutations in components of the Hh signalling pathway that lead to elevated levels of the GLI1 transcription factor [181, 182]. Additional evidence that the Hh signalling pathway is involved in BCC formation comes from transgenic mouse models. Over-expression of *Shh*, *Smo*, *Gli1*, or *Gli2* in keratinocytes leads to spontaneous development of BCC-like lesions [183-187]. *Ptch1*^{+/-} mice develop multiple BCCs after exposure to UV or ionizing radiation [177, 188].

Another gene frequently inactivated in BCC is *p53*, a tumour suppressor involved in genome surveillance through the regulation of cell proliferation and death [189-192]. Aberrant Notch signalling can cause BCC and other skin tumours. The absence of Notch activity allows Wnt and Shh signalling to persist in a tissue where they are normally repressed [193].

The origin of BCC

The continuous loss of cells from stratified epidermis and the cyclic growth and degeneration of hair follicles require a balance between cell proliferation, differentiation and cell loss. Maintenance of the epidermis and its appendages is ensured by stem cells [194-196] that have been shown to reside in the basal layer of the epidermis and in the bulge region of hair follicles. Under physiological conditions, bulge stem cells are used for the cyclic regeneration of the hair follicle, while they can

also regenerate the sebaceous gland and the epidermis after injury [196, 197]. Whether Hh signalling is inappropriately activated in stem cells or in committed cells is unclear [198]. According to the cancer stem cell hypothesis, deregulation of signalling pathways in cancer stem cells that control self-renewal is the principle cause of tumorigenesis [199]. Cancer stem cells can either be derived from normal tissue stem cells that have lost proper growth control or differentiated cells that acquire the stem cell property of self-renewal [200].

BCCs are poorly differentiated tumours that resemble in some aspects undifferentiated hair follicle-like structures, suggesting that BCCs could be hair follicle derived tumours or aberrant formation of hair follicles. Expression analysis of Hh pathway components in normal hair follicles showed that Hh responsive genes, including *PTCH*, *GLI1* and *GLI2* as well as the *GLI* targets *FOXE1* and *BCL2*, are expressed in the outer root sheath (ORS), while *SHH* is restricted to the matrix region [161, 162, 201-203]. It is therefore possible that BCCs derive from the ORS, where ligand-independent Hh signalling (caused by inactivating *PTCH1* mutations or *SMO* activating mutations) may cause increased proliferation at the expense of differentiation. BCC can also originate from the basal layer of interfollicular epidermis, maybe even from cells that have entered the differentiation pathway, since expression of *GLI2* in human epidermal cells in vitro is able to oppose differentiation signals and induce re-entry into the cell cycle [204].

Medulloblastoma

Medulloblastoma (MB) is the most common malignant brain tumour in children. It grows aggressively and has a tendency to metastasize via cerebrospinal fluid [51]. The tumour arises in the cerebellum, presumably from granule neuron precursors (GNP) proliferating in the external granular layer (EGL) of the cerebellum during late embryogenesis and early postnatal period [205, 206]. Shh, secreted from Purkinje cells in the cerebellum, drives this proliferation of GNP in the EGL [207, 208] by inducing the expression of, for example, the proto-oncogene *N-myc* [209] and the G1-S phase promoter, *cyclin D* [210, 211]. MBs arise both sporadically and in familial tumour syndromes including NBCCS [212]. Several studies have shown that deregulated Hh signalling is critical to the genesis and survival of sporadic MB [134,

213-216]. Genetic alterations associated with MB include activating mutations of *SMO* and inactivating mutations of *PTCH1* and *SUFU* [134, 214, 217]. Transgenic mice with an overactive Hh signalling pathway are predisposed to MB. These include *Ptch1* heterozygous mice and *Smo* overexpressing mice [87, 88, 218]. Loss of *Sufu* promotes MB development on a p53 null background [139]. Thus, Hh signalling regulates normal cerebellar development, and deregulation of the pathway plays a critical role in the formation of MB.

Activation of the phosphatidylinositol 3-kinase (PI3K) signalling pathway by insulin-like growth factor-II, inactivation of *p53*, loss of DNA damage repair mechanisms, and ectopic expression of Myc oncoproteins cooperate with Hh signalling to enhance MB formation in mice [219].

Prostate cancer

Prostate is another organ where Hh signalling is important for both organogenesis and cancerogenesis [220]. Hh signalling is not required for the initial formation of the prostate, but for subsequent growth and ductal patterning [221, 222]. Prostate regeneration also requires Hh pathway activity [223]. In prostate cancer, there is a correlation with increased invasiveness and metastatic potential and elevated Hh signalling [223]. Furthermore, cultured primary prostate carcinoma cells are dependent on Hh signalling for growth and survival [224].

Other tumours with aberrant Hedgehog signalling

Activation of the Hh pathway via ligand-over-expression has been demonstrated in small-cell lung cancer (SCLC) [44], as well as in upper gastrointestinal malignancies arising from pancreas, oesophagus and stomach [45, 47], and prostate cancer [223, 225]. These tumours lack mutations in *PTCH*, identifying a mechanism of pathway activation distinct from the NBCCS syndrome. The mechanism by which Hh acts in ligand-overexpressing cancers is still unclear. One model suggests that these tumours rely on autocrine/paracrine signalling, where Hh ligand produced by tumour cells stimulate their growth and/or survival.

Treatment of tumours with aberrant Hedgehog signalling

Cancer treatment is often associated with major side effects. Therefore, new non-toxic therapies, targeting specific molecular defects need to be developed. Inhibition of the Hh pathway provides a potential novel therapy for the many tumour types with activated Hh signalling. Today, there are no clinically available drugs that specifically targets the Hh signalling pathway but cyclopamine and other Smo-targeting compounds (Curis Inc., Cambridge, MA), is currently in phase I trials.

Of all the components of the Hh pathway, Smo appears to be an especially susceptible target of small molecules [226] since several compounds have been described that inhibit Hh signalling at the level of Smo [215, 227, 228]. Inhibition of medulloblastoma growth in cell culture and in mouse models was possible using cyclopamine [215]. Another Smo antagonist, HhAntag, blocked the function of Smo in mice with medulloblastoma. This resulted in inhibition of cell proliferation, increase in cell death and, at the highest dose, complete eradication of tumours. Long-term treatment with HhAntag prolonged medulloblastoma-free survival [229]. Animals receiving even the highest drug doses showed no toxic side effects.

Targeting Hh signalling at the level of Smo would not inhibit activity in tumours with *SUFU* mutations, which have been found in medulloblastoma [134], prostate cancer [137], and rhabdomyosarcoma [138]. It would also not inhibit activity in tumours with GLI gene amplifications [230] or GLI2 protein stabilization [231]. Therefore it seems preferable to target Hh signalling at the level of the Gli transcription factors.

Recently, a screen for small-molecule antagonists of GLI-mediated transcription revealed two molecules, GANT61 and GANT58, which were able to selectively inhibit GLI-mediated gene transactivation. Both compounds efficiently inhibited *in vitro* tumour cell proliferation and blocked cell growth in an *in vivo* xenograft model using human prostate cancer cells with downstream activation of the Hh pathway [232].

Hopefully, such GLI-inhibiting compounds will have favourable pharmacokinetic properties and low toxicity in humans that would allow the use of them in cancer patients.

AIMS OF THIS THESIS

The general aim of this thesis was to get a better understanding of the role of *Ptch1* and *Sufu* in embryogenesis and tumour formation.

Specifically, the aims were to:

1. Study the function of *Sufu* by developing and characterizing *Sufu* knock-out mice.
2. Test the hypothesis that compound *Sufu* and *Ptch1* heterozygous mice would have an increased incidence or a different tumour spectrum than either *Ptch1* or *Sufu* heterozygous mice.
3. Investigate the function *PTCH1* isoforms.
4. Study the role of *Ptch1* loss in the skin and prostate by tissue-specific deletion of *Ptch1* using conditional knock-out mice.

RESULTS AND DISCUSSION

Role of Sufu in embryo development and tumour formation (Paper I)

Sufu was originally identified in *Drosophila* by its ability to suppress overactive *Fu* mutations. Sufu is highly conserved but the lack of known functional domains makes predicting how it acts difficult. To find out more about the function of Sufu in mammals we generated *Sufu* mutant mice by homologous recombination in embryonic stem cells. We found that in contrast to the mild phenotype seen in *Drosophila sufu* mutants, loss of *Sufu* in mice results in embryonic lethality around embryonic day 9.5 (E9.5). *Sufu*^{-/-} embryos appeared phenotypically very similar to *Ptch1*^{-/-} embryos with an open neural tube.

We analyzed the expression of Hh pathway components by whole mount *in situ* hybridization in E9.5 embryos and found that the expression domains of *Gli1* and *Ptch1* were much broader in *Sufu*^{-/-} embryos compared to wild-type (Wt) embryos. This shows that loss of *Sufu* results in constitutive expression of Hh target genes in *Sufu*^{-/-} embryos.

Hh signalling has an important role in patterning of the dorso-ventral aspects of the developing neural tube where Shh secreted from the notochord and floor plate controls cell fate and position of the different neuronal subtypes of the ventral neural tube. In *Ptch1*^{-/-} embryos, the neural tube is ventralized. To investigate how loss of *Sufu* affects patterning of the E9.5 neural tube, we analyzed expression of markers for different neuronal cell types by immunofluorescence. We found that FoxA2, normally expressed in the most ventral part of the neural tube, was expressed along the entire dorso-ventral axis. In contrast, Pax6 expression, normally found in the dorsal neural tube, was essentially lost in both *Sufu*^{-/-} and *Ptch1*^{-/-} neural tubes. These data demonstrate that the *Sufu*^{-/-} neural tube shows a strong ventralization similar to *Ptch1*^{-/-} embryos.

Next, we analyzed the activity of the Gli transcription factors in mouse embryonic fibroblasts (MEF) using a luciferase reporter assay. *Sufu*^{-/-} MEFs showed 12-15-fold

increase in reporter activity relative to Wt MEFs. This increased activity is dependent on intact Gli binding sites. Transient transfection of human *SUFU* repressed the Gli activity to Wt levels, demonstrating that the observed phenotype is Sufu-dependent and not due to other genetic alterations in the ES cells or MEF cells. Furthermore, it shows that human *SUFU* can functionally substitute for mouse *Sufu*.

To test if the Gli-mediated Hh signalling activity in the *Sufu*^{-/-} MEFs could be further stimulated, we incubated the *Sufu*^{-/-} MEFs with Smo agonist, but no further increase in Gli reporter activity was observed. Conversely, we tested whether cyclopamine, an inhibitor of the Hh pathway acting on Smo, could inhibit Gli reporter activity. However, again no effect was observed indicating that neither stimulation nor inhibition of the Hh pathway at the level of Smo has any significant effect on Gli activity in the absence of Sufu. These results indicate that *Sufu*^{-/-} MEFs display maximal Hh pathway activation, supporting a central role for Sufu in Hh pathway repression.

Protein kinase A (PKA) is known to have a negative effect on Hh signalling. To test what effect PKA has in cells lacking *Sufu*, we treated *Sufu*^{-/-} MEF cells with forskolin, an activator of PKA, and could inhibit the Gli-mediated response by approximately 50%. The same result was obtained by expressing a constitutively active form of the catalytic subunit of PKA. In contrast, an almost complete suppression was observed in the *Ptch1*^{-/-} MEFs, as has been shown before. This suggests that the full repressive effect of PKA on the Hh pathway depends on more than one mechanism.

Previous reports have demonstrated that Sufu binds and sequesters Gli1 in the cytoplasm. Therefore we wanted to determine the subcellular localization of GLI1 in the absence of Sufu. We transiently transfected the MEFs with GLI1 fused to EGFP (EGFP::GLI1). EGFP::GLI1 localized predominantly in the cytoplasm of *Sufu*^{-/-} MEFs. No significant difference to Wt and *Ptch1*^{-/-} MEFs was observed. The cytoplasmic localization of GLI1 in the *Sufu*^{-/-} MEFs is dependent on active nuclear export as leptomycin B treatment of EGFP::GLI1-transfected MEFs resulted in strong nuclear accumulation of the EGFP::GLI1 fusion protein. This argues against a role for

Sufu in subcellular localization of Gli1. The primary function of Sufu may instead be to repress Gli1 targets in the nucleus by recruiting the HDAC machinery by interaction with SAP18.

Sufu^{+/-} mice were born at the expected Mendelian ratio, appeared normal at birth, show normal growth, and were fully fertile. However, they developed a skin phenotype with 100% penetrance, characterized by ventral alopecia, increased pigmentation, aberrant sebaceous gland morphology and papules and nodules on the paws and tail, macroscopically visible in mice older than 18 months and becoming more severe as they age. The earliest microscopic alterations were seen as small basaloid evaginations arising from basal epidermal cells on the palmar aspect of the paws at about six months of age. Immunohistochemical staining for the proliferation marker Ki67 revealed a relatively low number of positive cells, consistent with the observed slow growth of these changes that were most similar to basaloid follicular hamartomas. Furthermore, we frequently observed mandibular keratocysts in *Sufu*^{+/-} mice, which is a typical finding in NBCCS patients.

To test if the Hh pathway was activated in the skin of *Sufu*^{+/-} mice, we measured the levels of *Gli1* expression by quantitative real-time PCR. Skin from ~2-year-old *Sufu*^{+/-} mice showed a 10-16-fold increase in *Gli1* mRNA levels compared to Wt control. In ~1-year old mice with less advanced proliferations, smaller but still significant increases (4-10-fold) were found. Thus, the increase in Gli1 expression levels correlate with extent of the epidermal skin changes.

Our data demonstrate that Sufu is an essential repressor of Hh signalling in mammals with loss of *Sufu* leading to ligand-independent activation of the pathway. Mammalian Sufu may have assumed new functions, perhaps as a result of the absence of a functional mammalian Cos2 homologue, an important repressor in the *Drosophila* Hh signalling pathway. It is still unclear how Sufu inhibits Gli activity. Future studies examining the phosphorylation, processing, and activity of the Gli proteins in *Sufu* mutants will give important information about the function of Sufu.

Tumour spectrum in compound *Sufu* and *Ptch1* heterozygous mice (Paper II)

It has been suggested that haploinsufficiency of *Ptch1* and *Sufu* together can result in tumour formation. We decided to investigate if *Sufu* could modulate the phenotype of *Ptch1*^{+/-} mice or vice-versa. Since *Ptch1*^{+/-} mice are larger than Wt mice we measured the weight of *Sufu*^{+/-}*Ptch1*^{+/-}, *Ptch1*^{+/-}, *Sufu*^{+/-} and Wt mice from 4-20 weeks age and found that both *Sufu*^{+/-}*Ptch1*^{+/-} and *Ptch1*^{+/-} are larger than Wt and *Sufu*^{+/-} mice, but not different from each other. *Sufu*^{+/-} mice are not larger than Wt mice indicating that the larger body size is connected to the *Ptch1* allele.

The 1-year survival rate was similar in *Sufu*^{+/-}*Ptch1*^{+/-} and *Ptch1*^{+/-} mice. The main causes of death in *Sufu*^{+/-}*Ptch1*^{+/-} and *Ptch1*^{+/-} mice are medulloblastoma (MB) and hydrocephalus. 48% of *Sufu*^{+/-}*Ptch1*^{+/-} and 37% of *Ptch1*^{+/-} mice developed MB to the age of 1 year. This difference was not statistically significant.

We found that MB from *Sufu*^{+/-}*Ptch1*^{+/-} and *Ptch1*^{+/-} mice had a similar histology. There were also no differences in growth and apoptosis rates in MB from the different genotypes as measured by immunohistochemical staining with anti-phospho histone H3 and anti-cleaved caspase 3, respectively. CyclinD1 and PDGFR- α , markers known to be expressed in MB, were expressed at similar levels in MB from *Sufu*^{+/-}*Ptch1*^{+/-} and *Ptch1*^{+/-} mice.

Quantitative real-time RT-PCR showed increased *Gli1* and *Gli2* levels in all MB from *Sufu*^{+/-}*Ptch1*^{+/-} and *Ptch1*^{+/-} mice compared to normal cerebellum. All MB in *Ptch1*^{+/-} mice and all but one MB found in *Sufu*^{+/-}*Ptch1*^{+/-} mice had lost expression of the *Ptch1* wt allele, indicating that this is the critical genetic change leading to MB formation in *Ptch1*^{+/-} and *Sufu*^{+/-}*Ptch1*^{+/-} mice. The cause of tumour formation in the MB that retained the Wt *Ptch1* allele may be a mutation of *Ptch1* or *Sufu*. *Sufu* expression was upregulated in MB from *Ptch1*^{+/-} and *Sufu*^{+/-}*Ptch1*^{+/-} mice indicating that *Sufu* expression is regulated by Hh activity. This may constitute another negative feedback loop in the Hh signalling pathway. Also, this shows that expression of *Sufu* was not lost in the MB from the *Sufu*^{+/-}*Ptch1*^{+/-} retaining the Wt

Ptch1 allele. However, the high levels of Sufu were not able to inhibit pathway activity.

Ptch1^{+/-} mice developed rhabdomyosarcoma (RMS) with a frequency of 2-9 % depending on genetic background. In this study, we found that the frequency of RMS was similar in *Ptch1*^{+/-} and *Sufu*^{+/-}*Ptch1*^{+/-} (6 and 7 %, respectively). No RMS was detected in *Sufu*^{+/-} or wt mice.

A few *Ptch1*^{+/-} (1/49) and *Sufu*^{+/-}*Ptch1*^{+/-} (4/56) but no *Sufu*^{+/-} or wt mice developed cystic changes of the pancreas. The cystic epithelium was positive for PAS and Alcian blue, which stains intestinal-type mucins. PAS- or Alcian blue-positive epithelium is not found in normal pancreatic parenchyma but is a feature of mucinous cystic tumors as well as adenocarcinoma of the pancreas. We also noted a few diverticular hamartomatous lesions in the intestine and stomach of *Ptch1*^{+/-} mice and *Sufu*^{+/-}*Ptch1*^{+/-} mice.

Skin from *Sufu*^{+/-}, *Ptch1*^{+/-} and *Sufu*^{+/-}*Ptch1*^{+/-} mice developed BCC-like epidermal lesions. When counting the number of lesions on the plantar surface of the hind foot, we found that they were more frequent in *Sufu*^{+/-}*Ptch1*^{+/-} mice compared to *Sufu*^{+/-} and *Ptch1*^{+/-} mice. The number of lesions was also higher in *Sufu*^{+/-} mice than in *Ptch1*^{+/-} mice.

Quantitative real-time RT-PCR showed increased *Gli1* levels in skin from *Sufu*^{+/-} (2.7 times), *Ptch1*^{+/-} (4.6 times) and *Sufu*^{+/-}*Ptch1*^{+/-} mice (4.0 times) compared to Wt skin. The number of lesions did not, however, seem to correlate with *Gli1* levels.

It may be that in skin, Sufu is haploinsufficient while Ptch1 is haplosufficient and LOH of *Ptch1* is needed for these basal cell lesions to occur. Our results indicate a differential importance of Sufu and Ptch1 as tumour suppressors in MB versus the BCC-like lesions.

Expression and function *PTCH1* first-exon variants (Paper III)

The regulation of SMO by *PTCH1* is the core control system of the HH pathway. *PTCH1* has several first exons and undergoes exon-skipping events. The alternative promoters for exons 1B and 1C have GLI1 binding sites. Consequently HH signalling results in transcriptional upregulation of the *PTCH1*-1B and -1C isoforms.

In this study, we screened a human cDNA panel by PCR. The primers were in the different first exons and in exon 3. We found tissue-specific expression of two novel alternative variants initiating at exon 1C. One, found in liver, had skipped exon 2 (*PTCH1*-1C Δ E2), another, found in kidney, used an alternative 5' splice site within exon 1C (*PTCH1*-1Ckid). A third variant had joined the alternative 5' splice site within exon 1C directly to exon 3 (*PTCH1*-1Ckid Δ E2). The open reading frames (ORF) of *PTCH1*-1C Δ E2 and *PTCH1*-1Ckid Δ E2 were the same as the ORFs of the previously characterized variants *PTCH1*-1 and *PTCH1*-1A. On the other hand, *PTCH1*-1Ckid had a fusion ORF, resulting from the joining of an upstream ORF to the *PTCH1*-1C ORF and consequently coded for a *PTCH1* variant with a unique N-terminal tail, Figure 3.

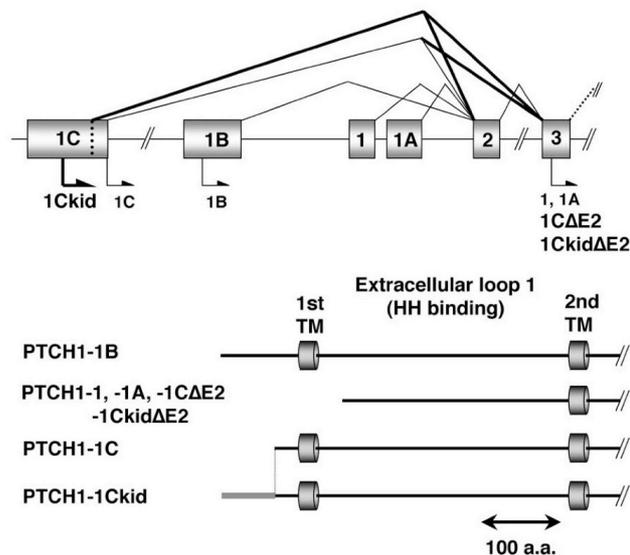


Figure 3. First-exon *PTCH1* variants. Upper panel: Schematic representation of the 5' end of the *PTCH1* gene. The individual exons are shown and the splicing events joining the first exons to exons 2 or 3 are indicated. Arrows mark the positions of the initiator methionine codons. Lower panel: Schematic representation of the N-terminal region of the *PTCH1* variants. The extracellular loop 1 involved in HH binding and the first two transmembrane domains (TM) are indicated. The unique amino terminal sequence of *PTCH1*-1Ckid is shown by a thicker line.

In a luciferase reporter assay in NIH3T3 cells, we compared the ability of the different isoforms to inhibit activation by a constitutively active form of SMO. *PTCH1*-1Ckid inhibited SMO activation similarly to *PTCH1*-1C but not to the same

extent as PTCH1-1B. To examine if the PTCH1 variants could act as receptors of HH proteins, we co-transfected *SHH* with the *PTCH1* isoforms into NIH3T3 cells. PTCH1-1B partially repressed this activation, but the other PTCH1 isoforms could not inhibit the activity of exogenous SHH. We also co-transfected the isoforms into *Ptch1*^{-/-} MEF cells where the HH signalling pathway is constitutively active. In these cells, all PTCH1 isoforms inhibited pathway activity but PTCH1-1B had the strongest inhibitory effect.

All PTCH1 isoforms strongly inhibited GLI1. PTCH1-1B and -1C completely repressed full length GLI2, but only partially repressed the active form of GLI2 that lacks the amino terminal region. PTCH1-1 and PTCH1-1Ckid partially inhibited both the full length and the active form of GLI2. This suggests that the primary target genes of GLI2 may still be expressed under the partial PTCH1 inhibition.

We examined the protein levels of the different PTCH1 variants in presence or absence of SHH. The amount of PTCH1 protein was remarkably different depending on which alternative first exon was present in the transfected construct. PTCH1-1B levels were the highest of all variants and remained high even in the presence of SHH. On the other hand, PTCH1-1C levels were dramatically down-regulated by SHH. Cycloheximide treatment revealed that the rate of degradation of PTCH1-1C was higher than that of PTCH1-1B. Thus, it is apparent that the unique N-terminal sequence of PTCH1-1B stabilizes the protein.

To elucidate the distribution of the *Ptch1* variants in embryogenesis, we analyzed mouse embryos at 8.5 and 9.5 dpc by whole mount *in situ* hybridization. Riboprobe E2-11, detecting all variants, showed strong expression in the neural tube and forelimb and weak, broader expression around these regions. *Ptch1-1B* showed a similar expression pattern as riboprobe E2-11. *Ptch1-1* also revealed a similar expression pattern but this was more widely and broadly distributed. By contrast, *Ptch1-1A* was slightly detected in the blood vessels. *Ptch1-1C* showed a strong signal in the yolk sac and broad expression all over the embryo. *Ptch1-1* and *-1C* were expressed in the otic placode.

In conclusion, we found that the PTCH1 alternative promoters and first exons influenced the expression pattern and the stability of the proteins as well as their capacity to act as inhibitors of HH signalling. This study supports a role of splicing variation and/or promoter choice for Hh signalling regulation.

A model based on the results from this and earlier studies was proposed. Without HH, the *PTCH1-1* isoform is expressed. Since this variant has sufficient capacity to inhibit endogenous SMO, transcription of *PTCH1-1B*, *-1C* and *GLII* are repressed. Ligand availability allows PTCH1-1 to bind HH, resulting in signalling activation and upregulation of *PTCH1-1B*, *PTCH1-1C* and *GLII*. However, the presence of HH keeps suppressing the PTCH1-1B and -1C activity, and sustains pathway activation. During this period, the cell accumulates mRNAs for *PTCH1-1B* and *-1C*. Termination of ligand availability activates the PTCH1-1B and -1C proteins, and these inhibit signal transduction. This model allows for an explanation of the strict switching between activation and inactivation depending on HH protein levels and the effective negative feedback loop that regulates HH signal transduction.

Homozygous deletion of Patched1 in prostate and skin (Paper IV)

In this study we created a conditional *Ptch1* allele with exon 2 flanked by loxP sites in the mouse. Recombination of the conditional allele by the Cre recombinase results in a null allele. Mice carrying the conditional allele were crossed with *ARR2PBi-Cre* transgenic mice. Recombination of *Ptch1* were confirmed in prostate and seminal vesicles but we found no aberrant phenotype in these tissues up to the age of 12 months. However, we observed BCC-like epidermal lesions in all skin locations. We showed that the *ARR2PBi-Cre* transgenic line expressed Cre in solitary cells in interfollicular epidermis and hair follicles as visualized by X-gal staining in *R26R;ARR2PBi-Cre* mice. Expression of Cre in skin resulted in recombination of the conditional *Ptch1* allele. Skin where *Ptch1* had been deleted showed upregulation of Gli1, indicating abnormal Hh pathway activity. The skin lesions arose both from interfollicular epidermis and hair follicles. Also, *de novo* rudimentary hair follicles forming hair shafts appeared.

The epidermal lesions expressed Keratin 5, normally found in the basal cells of interfollicular epidermis (IFE) and the outer root sheath (ORS) of hair follicles. They also expressed Keratin 17, normally expressed in the ORS of hair follicles and in the embryonal placode, which will give rise to appendages such as hair, glands, and teeth during skin development. Keratin 17 is also strongly expressed in human BCCs.

Cyclin D1 is more frequently expressed in mouse BCCs compared to basaloid follicular hamartomas. In the follicular hamartomas that develops in *Sufu*^{+/-} mice relatively few cyclin D1 positive cells were found. Immunostaining for cyclin D1 in the skin of *Ptch1* conditional mutant mice revealed many strongly positive cells in the outer rim of the proliferations, which support a closer relationship with BCCs than with hamartomas.

Our results support the idea that BCC development represents aberrant hair follicle formation.

FUTURE PERSPECTIVES

The Hh pathway is activated in many types of cancer. This raises questions about the normal role of the pathway in those tissues. Recent studies indicate a role for Hh signalling in the renewal and maintenance of adult tissues through effects on tissue stem cells. The connection between Hh and cancer may be related to the normal function of this pathway in regulating stem cell activity. A tumour may be viewed as an aberrant organ formed by a cancer cell that acquired the capacity for indefinite proliferation. This suggests that tumorigenic cancer stem cells undergo processes that are analogous to the self-renewal and differentiation of normal stem cells. In most tissues, stem cells are rare and a question is whether Hh signalling is abnormally activated in stem cells or if committed cells can become stem cell-like after activation of the Hh pathway.

BCC has been suggested to be aberrant formation of hair follicles after activation of Hh signalling. The tumours originate both from interfollicular epidermis and from hair follicles. Can activation of Hh signalling in different cell types drive them into the same path to form hair follicles? In the mouse models of BCC used in these studies, hair follicle-like structures form after loss of *Ptch1* or *Sufu* in the skin. Better characterization of the cells in which the tumours are initiated and the normal function of Hh signalling in these cells will give us more information on how activation of Hh signalling promotes tumour formation.

In future experiments, the *Ptch1* conditional mouse model developed in this study can be used to delete *Ptch1* function in many different cell types to better define which cell types can contribute to tumour formation. For example, *Ptch1* is expressed in the stroma of many tissues and these mice can therefore be used to investigate the function of *Ptch1* in the stromal compartment. In addition, loss of *Ptch1* can be induced at different time points during embryogenesis to investigate at what steps *Ptch1* is essential. Finally, these mouse models could also be excellent tools to test the effects of drugs that target components of the Hh signalling pathway.

SAMMANFATTNING PÅ SVENSKA

Utvecklingen från embryo till en vuxen individ är strikt reglerad. För att celler ska kunna veta vilken typ av vävnad de ska bilda måste de kommunicera med varandra. Denna kommunikation kan ske genom att en cell producerar signalproteiner som utsöndras från cellen så att det kan binda till proteiner på en annan cells yta och därmed orsaka förändringar i denna cell. Hedgehog (Hh) är ett sådant signalprotein som är viktig under embryoutvecklingen i organismer från bananflugan *Drosophila* till människor. Eftersom Hh talar om för celler om de ska dela sig eller inte så kan skador (mutationer) i gener relaterade till Hh orsaka cancer. Mutationer i Hh-relaterade gener har hittats i en rad olika cancerformer. Några exempel är hudcancerformen basalcellscancer och hjärntumören medulloblastom. Jag har försökt förstå molekylärbiologin bakom utvecklingen av dessa tumörformer. Förhoppningen är att genom att identifiera avgörande steg i tumörprocessen lära oss hur vi ska förebygga och behandla cancer.

Denna avhandling handlar om de två Hh-relaterade generna *Patched1* och *Suppressor of Fused (Sufu)* vars funktion är att hindra celldelning i frånvaro av Hh. Om det uppstår mutationer i någon av dessa gener så kan det bidra till att cellerna kan dela sig okontrollerat och ge upphov till cancer.

I arbete I har vi visat att möss utan *Sufu* dör under embryoutvecklingen med en överaktiv Hh signalering. Vuxna möss med bara en genkopia för *Sufu* utvecklar lesioner i huden som liknar basalcellscancer. *Sufu* behövs således under embryoutvecklingen i möss och fungerar som tumörhämmare för basalcellscancer.

I arbete II har vi studerat om frekvensen eller spektrat av tumörer som utvecklas av möss som saknar en genkopia vardera av *Sufu* och *Patched1* skiljer sig från möss som saknar en genkopia av *Patched1* eller en genkopia av *Sufu*. Vi fann att frekvensen av basalcellscancerliknande lesioner är högre i möss som saknar en genkopia vardera av *Sufu* och *Patched1* jämfört med möss som saknar en genkopia av *Patched1* eller en genkopia av *Sufu*. Frekvensen av andra tumörtyper, bla medulloblastom, skiljer dock inte mellan möss som saknar en genkopia vardera av *Sufu* och *Patched1* och möss

som saknar en genkopia av Patched1. Utvecklingen av medulloblastom i dessa möss är främst beroende av förlust av Patched1.

I arbete III har vi studerat olika varianter av Patched1. Vi fann att dessa har olika förmåga att nedreglera Hh signalering. Deras stabilitet skiljer sig avsevärt mellan varandra och vissa varianter är mer instabila i närvaro av Hh protein. De olika varianterna har olika uttrycksmönster i musembryon. Förekomsten av dessa olika varianter av Patched1 ger en cell möjlighet att finreglera aktiviteten av Hh signalvägen genom att välja vilken variant av Patched1 som uttrycks.

I arbete IV har vi studerat möss som saknar Patched1 i prostata och i huden. Vi fann att avsaknad av Patched1 i prostata inte har någon effekt i mössen. Avsaknad av Patched1 i huden leder däremot till uppkomst av basalcellscancerliknande lesioner som har flera likheter med hårfolliklar. Detta stöder teorin att basalcellscancer är en form av felaktig hårfollikelutveckling.

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