From The Department of Biosciences and Nutrition
Center for Biosciences
Karolinska Institutet, Stockholm, Sweden

THE ROLE OF SUPPRESSOR OF FUSED IN DEVELOPMENT AND TUMORIGENESIS IN THE MOUSE

Karin Heby-Henricson

Karolinska Institutet

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Cover illustrations:

Upper left: whole mount *in situ* hybridization with *Shh* riboprobe on a *Sufu*$^{-/-}$ E9.5 embryo.
Upper right: hematoxylin & eosin staining of a *Sufu*$^{+/-}$*;Trp53*$^{+/-}$ skin lesion in association with a hair follicle showing aberrant morphology.
Lower left: alkaline phosphatase staining of *Sufu*$^{-/-}$ embryonic stem cell clones growing on a feeder layer of mouse embryonic fibroblasts.
Lower right: hematoxylin & eosin staining of a teratoma generated from *Sufu*$^{-/-}$ embryonic stem cells, showing a predominance of neuroectodermal tissue and two endodermal cysts outlined by Goblet and ciliated cells.

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Till mor och far,
mina barn
& Jacob

POPULÄRVETENSKAPLIG SAMMANFATTNING

Livet börjar när en spermie möter och smälter samman med en äggcell. Det befruktade ägget delar sig och de nya cellerna som uppstår kommunicerar med varandra med hjälp utav olika signalproteiner. Det finns ett antal olika signalkaskader som är väldigt viktiga under embryonalutvecklingen; en av dem är hedgehog-signalvägen. Signalproteinet hedgehog bildas och utsöndras av vissa celler, för att sedan kännas igen av receptorer på närliggande cellers ytor. Den huvudsakliga receptorn för hedgehog heter patched (PTCH). I frånvaro av hedgehog verkar PTCH genom att inhibera signalvägen. Men när hedgehog binder till PTCH, upphör inhiberingen och signalen skickas vidare. Detta leder till uppreglering av flera olika målgener som styr celldelning och differentiering, d.v.s. utvecklingen av icke specialiserade celler till mer specialiserade celler, t.ex. nerv-, lever- eller broskceller. Det är av största vikt att de olika signalkaskaderna regleras korrekt. Minsta fel kan leda till allvarliga missbildningar eller cancer senare i livet.

I min avhandling har jag studerat Suppressor of fused (Sufu) i mus. Sufu är ett viktigt protein i hedgehog-signalvägen där det, liksom PTCH, fungerar genom att hålla signalvägen avstängd i frånvaro av hedgehog. I det första delarbetet visade vi att om man slår ut båda kopiorna (allelerna) av genen som kodar för Sufu så dör musfostren redan 9.5 dagar efter befruktningen. De uppvisar grava missbildningar i huvudregionen och i neuralröret, som senare ska utvecklas till ryggrad och nervsystem. Vi visade också att möss som bara saknar den ena allelen av Sufu-genen (Sufu-heterozygota möss), med tiden utvecklar en huddefekt som liknar den vanligaste formen av cancer i västvärlden, nämligen basalcellscarcinom (BCC). I människa är det känt att mutationer som leder till onormal uppreglering av hedgehog-signalvägen i första hand förorsakar BCC, men även andra former av cancer. Vanligast är mutationer i genen för PTCH, men mutationer i andra komponenter av hedgehog-signalvägen kan också förekomma. Människor som från födseln bär på en muterad PTCH-allel utvecklar Gorlins syndrom, vilket karaktäriseras av ett flertal missbildningar såsom käkcystor och polydaktyli (se figur 7). Dessa patienter visar även en kraftig predisponering för cancer, i synnerhet medulloblastom i hjärnan och multipla BCC. Vi kunde i vår studie påvisa förekomst av käkcystor i de Sufu-heterozygota mössen, och nyligen identifierades en familj med Gorlins syndrom som orsakats av en nedärvd mutation i SUFU-genen. Dessa upptäckter gör vår musmodell väldigt intressant att använda för studier av detta syndrom.

I den andra studien som ingår i min avhandling korsade vi de Sufu-heterozygota mössen med möss som saknar genen för p53. Genen för p53 är väldigt ofta muterad i cancer och därför var det angeläget att undersöka om de BCC-liknande hudförändringana i våra möss skulle utvecklas till fullskaliga BCC när p53 också var borta. Det visade sig att 57 % av dessa möss i stället utvecklade medulloblastom, och de övriga fick maligna lymfom till följd av avsaknaden av p53. Men vi kunde inte se några förändringar i hudlesionerna under den tid mössen levde. Slutsatsen är att olika vävnader är olika känsliga för kombinerad frånvaro av Sufu och p53, vilket skulle kunna vara kopplat till utvecklingsfas och celldelningsförmåga i dessa olika vävnader.

I den tredje studien använde vi oss av embryonala stamceller (ESC) som jag preparerat från tidiga musembryon, innan de implanterats i livmodern. ESC har potential att utvecklas till vilka andra celler som helst, och genom att injicera dem

under huden på nakna möss, börjar de spontant att utveckla en typ av godartade tumörer kallade teratom. Dessa teratom består av celltyper som representerar de tre groddlagren: ektoderm (bildar hud och nerver), endoderm (bildar inre organ såsom lever och njurar) och mesoderm (bildar muskler, brosk och skelett). Vi kunde visa att teratom som härstammar från celler som saknar Sufu mestadels består av outvecklade nervceller. Endodermala strukturer förekom, men mesoderm i form av brosk och ben saknades helt.

För att kunna studera hur fullständig avsaknad av Sufu påverkar olika utvecklingsskeden och cancer i olika vävnader har jag även arbetat med att ta fram en s.k. konditionell musmodell. Med hjälp av denna kan jag bestämma när och var Sufu ska slås ut. Vi har initierat studier där vi utnyttjar de konditionella Sufu-mössen för att få fram möss som saknar Sufu specifikt i huden.

Sammantaget understryker upptäckterna som presenteras i min avhandling den vitala funktion Sufu har i hedgehog-signalvägen när nervsystemet, brosk- och benvävnad utvecklas, men också dess betydelse för uppkomsten av hjärntumörer och hudcancer.

ABSTRACT

Embryonic development is a process that involves a number of evolutionarily well-conserved signaling cascades, including the hedgehog pathway. Mutations in components of this pathway have been identified in certain developmental disorders, and in many different kinds of cancers. In fact, the most common cancer in the Western World, basal cell carcinoma (BCC) of the skin, is due to mutations that cause aberrantly activated hedgehog signaling. This thesis focuses on a protein known as Suppressor of fused (Sufu), which is an essential tumor suppressor within the hedgehog pathway. In **PAPER I**, we made the surprising observation that Sufu actually plays a fundamental role in the mammalian hedgehog signaling pathway, in contrast to its role in fruit flies and even zebrafish. In these organisms, Sufu plays an insignificant part in normal hedgehog signaling, since its absence only results in minor phenotypic changes. However, in the mouse, we showed that loss of Sufu leads to embryonic death in mid-gestation with the embryos exhibiting severe cephalic defects and an open neural tube. We also demonstrated that the *Sufu* loss-of-function phenotype was due to ligand-independent activation of the hedgehog signaling pathway.

In humans, Gorlin syndrome is a rare developmental disorder that in the majority of cases is due to inactivating mutations in the gene that encodes the hedgehog receptor, PTCH1. This leads to overactivated hedgehog signaling, since PTCH1 is no longer able to inhibit the signal transducer, Smoothened (SMO). Gorlin syndrome involves an array of different developmental defects, but it also leads to increased tumor susceptibility, especially in the form of multiple BCCs. In PAPER I we discovered that mice, heterozygous for the *Sufu* gene, develop features of Gorlin syndrome, including a skin phenotype with BCC-like attributes, in addition to developmental aberrations in the form of jaw keratocysts. In addition, we showed that the extent of epidermal skin changes correlated with increased hedgehog pathway activation.

The BCC-like lesions in $Sufu^{+/-}$ mice are reminiscent of basaloid follicular hamartomas (BFH), which are more benign lesions than BCCs. In **PAPER II**, the aim was to investigate whether the $Sufu^{+/-}$ skin lesions would develop into full-blown BCCs if *Trp53* was knocked out simultaneously. *Trp53* is a well-known tumor suppressor gene that can enhance hedgehog-driven tumors, and is often mutated in sporadic BCCs, sometimes in combination with *PTCH1* mutations. We showed that $Sufu^{+/-}$ mice on a *Trp53* null background develop medulloblastomas and rhabdomyosarcomas, which is consistent with previous reports. Surprisingly, however, the $Sufu^{+/-}$ skin phenotype was not altered in the absence of *Trp53*, and showed no changes in latency, multiplicity, cellular phenotype or proliferative capacity during the lifespan of the mice. This finding suggests a differential, tissue-specific sensitivity to *Sufu* and *Trp53* gene loss, possibly linked to developmental phase and proliferative potential in specific tissues.

In **PAPER III**, we studied developmental and differentiation processes in the absence of *Sufu*, using embryonic stem cells (ESCs) derived from $Sufu^{-/-}$ pre-implantation embryos. $Sufu^{-/-}$ ESCs were found to express typical pluripotency markers, but the activity of the hedgehog pathway was increased only modestly compared to wild-type ESCs, as indicated by *Gli1* target gene expression. The $Sufu^{-/-}$ ESCs formed embryoid bodies *in vitro*, which, in later stages, were smaller than their wild-type counterparts, suggesting a deficiency in proliferation. To test the differentiation capacity of the $Sufu^{-/-}$ ESCs *in vivo*, the cells were injected subcutaneously into nude mice to form teratomas. Teratomas from $Sufu^{-/-}$ ESCs developed at efficiencies and latencies equivalent to teratomas from wild-type ESCs, yet in stark contrast to wild-type, $Sufu^{-/-}$ teratomas were dominated by neuroectodermal

tissues and were deficient in the mesodermal derivatives, cartilage and bone. These findings call attention to the central role played by *Sufu* in the hedgehog signaling pathway, and propose a function for *Sufu* in ectodermal-mesodermal cell fate decision.

In a **PRELIMINARY STUDY**, we have generated conditional *Sufu* mutant mice with the aim of deleting *Sufu* in specific tissues at specific time-points. These studies are ongoing, and experiments to create mice with complete loss of *Sufu* in the K5 (basal cell) compartment of the skin have been initiated.

In summary, the studies in this thesis highlight an essential role for *Sufu* in the hedgehog signaling pathway during development and tumorigenesis in mammals.

LIST OF PUBLICATIONS

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

I. Svärd J*, **Heby-Henricson K***, Persson-Lek M, Rozell B, Lauth M, Bergström A, Ericson J, Toftgård R, Teglund S.
Genetic elimination of Suppressor of fused reveals an essential repressor function in the mammalian Hedgehog signaling pathway.
Developmental Cell (2006) 10:187-97.

II. **Heby-Henricson K**, Bergström Å, Rozell B, Toftgård R and Teglund S.
Loss of *Trp53* promotes medulloblastoma development but not skin tumorigenesis in *Sufu* heterozygous mutant mice.
Molecular Carcinogenesis (2011) DOI: 10.1002/mc.20852.

III. **Heby-Henricson K**, Hoelzl MA, Rozell B, Kasper M, Toftgård R, Teglund S.
Loss of *Suppressor of Fused* Restricts the Differentiation Potential of Murine Embryonic Stem Cells.
Manuscript

Related publications:

Shimokawa T, Svärd J*, **Heby-Henricson K***, Teglund S, Toftgård R, Zaphiropoulos PG.
Distinct roles of first exon variants of the tumor-suppressor Patched1 in Hedgehog signaling.
Oncogene (2007) 26:26:4889-96.

*These authors contributed equally to this work.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

BCC	Basal cell carcinoma
BOC	Brother of CDO
CDO	Cell adhesion molecule-related/down-regulated by oncogenes
ci	Cubitus interruptus
CK1	Casein Kinase 1
cos2	Costal 2
Dhh	Desert hedgehog
Disp	Dispatched
Drosophila	*Drosophila melanogaster*
E	Embryonic day
ESC	Embryonic stem cell
fu	Fused
Gli	Glioma-associated protein
GS	Gorlin syndrome
GSK3β	Glycogen synthase kinase 3β
HE	Hematoxylin and eosin staining
Hh	Hedgehog
HHIP	Hedgehog interacting protein
IFE	Interfollicular epidermis
Ihh	Indian hedgehog
ICM	Inner cell mass
LOH	Loss of heterozygosity
MB	Medulloblastoma
MEF	Mouse embryonic fibroblast
NBCCS	Nevoid basal cell carcinoma syndrome
PKA	Protein kinase A
ptc	Patched (Drosophila)
Ptch1	Patched 1 (mammals)
Ptch2	Patched 2 (mammals)
RMS	Rhabdomyosarcoma
Shh	Sonic hedgehog
Smo	Smoothened
Sufu	Suppressor of fused

Human proteins are capitalized (e.g. SUFU), mouse proteins have an initial capital letter (e.g. Sufu) and *Drosophila* proteins are written in lower case (e.g. sufu). Genes are indicated with italics (e.g. *Sufu*).

1 INTRODUCTION

This thesis focuses on the role of a protein known as Suppressor of fused (Sufu), which is an essential component of the mammalian hedgehog signaling pathway. This pathway is already important in the earliest stages of development when the nervous system evolves, and it continues to be involved in multiple developmental processes. However, in adult individuals, the pathway plays a limited role and can cause cancer when activated aberrantly.

1.1 DEVELOPMENTAL BIOLOGY

Two of the studies included in this thesis (PAPERS I and III) concern the essential role of Sufu in embryonic development and tissue differentiation. In order to provide the requisite background for my research I have summarized only the most relevant aspects of developmental biology, as it is an extensive topic.

1.1.1 PRE-IMPLANTATION BIOLOGY

Life begins with a single diploid cell or zygote, which is produced during fertilization - when a haploid sperm cell fuses with a haploid egg cell (Gilbert, 2006). The zygote goes through a number of cleavages, or mitotic divisions, generating a sphere of numerous smaller cells (blastomeres). After the third division, the sphere consists of eight loosely arranged blastomeres and the process of compaction is initiated. Compaction occurs when the blastomeres increase their surface contact, become polarized and develop into two groups of cells; the inner cell mass (ICM) and the trophectoderm. The cells of the ICM are those that will eventually become the embryo proper. The cells in the outer layer develop into the trophectoderm, which is the structure that gives rise to extraembryonic tissues such as the embryonic membranes and the placenta. At the ICM/trophectoderm stage, the embryo is called a blastocyst. At this point the trophectoderm expands to form a vesicle-like structure with a fluid-filled cavity (blastocoel) and the ICM is a compact structure at one end of the blastocyst. Thereafter, the embryo hatches, meaning that it releases itself from the zona pellucida (a protective glycoprotein membrane surrounding the egg and the pre-implantation embryo) and implants into the uterine wall. Implantation is followed by gastrulation, an extraordinary cell-rearrangement process, during which the cells of the embryo are rearranged to establish the multilayered body plan. At this point, future tissues are specified by the three germ layers: 1) the ectoderm, which gives rise to the nervous system and skin - these cells are spread over the outside surface of the embryo; 2) the endoderm, which develops into internal organs such as the gut, liver and lungs - these cells are brought inside the embryo and 3) the mesoderm, which becomes bone, cartilage and muscle - these cells form the intermediate layer.

The actual anatomical position of a cell within the embryo is very important during development since it gives the cell a particular identity. There are several directional terms to describe cellular positions, and in vertebrates the following directional terms of the body plan are used: anterior-posterior (head to tail), dorso-ventral (back to stomach), and left-right.

Differentiation is the process during which less specialized cells become more specialized. The process is stepwise, often with several intermediate stages/stem cells (SCs), and frequently involves physical changes in cell size and morphology, as well as metabolic changes. In addition, differential gene expression patterns may cause

altered responsiveness to various signals; hence, the process is dependent on tight gene control.

All these complex events have to be well orchestrated to function properly, and, in summary, this involves four fundamental processes: cell proliferation, specialization, movement and cell-cell communication. The central signaling pathways that control these processes are: TGFβ (transforming growth factor β), JAK/STAT (Janus kinase/signal transducer and activator of transcription), RTK (receptor tyrosine kinases), NOTCH (notch wing phenotype in *Drosophila*), nuclear hormone, WNT (Wingless and Integration 1 hybrid in *Drosophila*), and HH (hedgehog) (Pires-daSilva and Sommer, 2003). Through a branching chain of interactions with downstream molecules and, in some cases, with each other, target gene transcription is regulated and development and differentiation can proceed.

1.1.2 EMBRYONIC STEM CELLS

A totipotent cell is a one that can become any other type of cell (Evans, 2011). Only the zygote and the blastomeres have this capacity since they can become both trophectoderm and ICM cells. The ICM contains embryonic stem cells (ESCs), which exhibit the capacity to self-renew, and are pluripotent, meaning that they can become any type of cell within the organism, apart from trophectoderm cells. These characteristics have transformed ESCs into a useful research tool for use in the study of early development and inherited diseases, as well as in regenerative medicine. ESCs can be derived *in vitro* from the ICM and are capable of differentiating into a wide range of fetal tissues in culture (Rossant and McKerlie, 2001) or *in vivo*, in chimeric embryos (Nagy et al., 2003). Depending on the influence of different signaling molecules that can be added to the culture medium *in vitro*, or are excreted by neighboring cells *in vivo*, the ESCs are induced to differentiate towards a particular cell lineage.

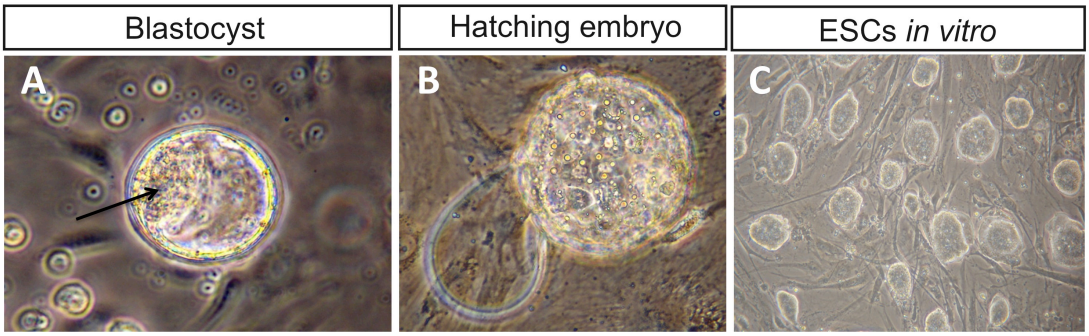

Figure 1. (A) A mouse blastocyst at E3.5 with the ICM visible at one end (indicated by arrow). (B) Hatching of a mouse embryo. The zona pelucida is visible at the lower left. (C) Mouse ESC clones growing on a feeder layer of MEFs *in vitro*. © Karin Heby-Henricson

1.1.3 NEUROGENESIS

Neurulation takes place shortly after gastrulation, and involves the formation of the neural tube, which will develop into the brain and most of the spinal cord. Neurulation is initiated when the mesoderm signals to a group of ectodermal cells on the dorsal surface of the embryo, instructing them to become neuroectodermal cells and form the neural plate. The neural plate undergoes anterior-posterior lengthening, then folds and finally fuses into a hollow cylinder, known as the neural tube. As a result of molecular

gradients created by secreted signaling molecules, the neural tube becomes specified into distinct domains, which contain the precursors for the different areas of the central nervous system (CNS) (Colas and Schoenwolf, 2001; Smith and Schoenwolf, 1997). During this process, the original ectoderm is divided into three cellular areas: 1) the neural tube, 2) externally positioned cells that will form the epidermis of the skin and 3) neural crest cells that will migrate to form peripheral neurons, glia and the pigmented melanocytes of the skin.

1.1.4 SKIN AND HAIR FOLLICLE DEVELOPMENT

The outermost cells of the embryo will form the skin, which begins as a one-layered structure then develops into two layers. The inner, basal layer forms the true epidermis while the outer layer, or periderm, is a temporary structure that is shed once the epidermis is formed. Several layers of cells (keratinocytes) constitute the stratified epidermis, which is often referred to as the interfollicular epidermis (IFE) (Koster and Roop, 2007). The basal cells proliferate to form spinous cells, which subsequently undergo further maturation into granular cells. Granular cells do not divide; instead, they differentiate and migrate outward to form the cornified layer (stratum corneum). In this layer, the cells have a high keratin content and are flattened, with the nucleus pushed to the edge of the cell. Dead cells in this layer start to shed soon after birth, but the proliferating basal cells will produce new keratinocytes continuously throughout life.

Keratins are the major structural proteins of the epidermis. They assemble as obligate heterodimers, and changes in their expression patterns characterize the stratified epidermis (Koster and Roop, 2004). The keratins, K8 and K18, are expressed by uncommitted surface ectoderm (Moll et al., 1982), then the expression of K5/K14 heterodimers is induced as these uncommitted cells commit to an epidermal fate (Byrne et al., 1994). Finally, the initiation of terminal differentiation results in K1/K10 expression in the suprabasal cells (Bickenbach et al., 1995; Fuchs and Green, 1980). Filaggrin and loricrin are epidermal filament-associated proteins, and are markers for the granular layer, in which they are expressed (Steven et al., 1990).

Hair follicle formation in the embryonic epidermis is initiated by the underlying dermis at embryonic day 12.5 (E12.5) in the mouse (Alonso and Fuchs, 2006). It can be seen as a local thickening of the epithelium, called a placode, at E14.5, and it is followed by the condensation of fibroblasts within the dermis, which form a structure known as the dermal condensate. In the dermis, the proliferating epidermal cells of the hair follicle, envelope the dermal condensate, generating the dermal papilla enclosed within the hair bulb. Molecular communication between the placode and the dermal papilla results in proliferation of the basal epithelium that becomes elongated and invaginates into the underlying dermis. The hair bulb structure continues to travel downwards together with the epithelial invagination, and the inner cells of the long, rod shaped invagination, begin to differentiate to form the actual hair (hair shaft) and the inner root sheet (IRS), which surrounds the hair shaft. The outer layer differentiates into the outer root sheet (ORS), which is continuous with the epidermis and is surrounded externally by the basement membrane. When the bulb reaches the bottom of the dermis, the hair follicle is fully mature, but the proliferative cells at the base of the follicle continue to divide to produce the hair shaft. To produce new hair, the hair follicles undergo cycles of growth (anagen) when the follicle generates an entirely new hair shaft, regression (catagen) and rest (telogen), during which the follicle resets and prepares for a new growth phase.

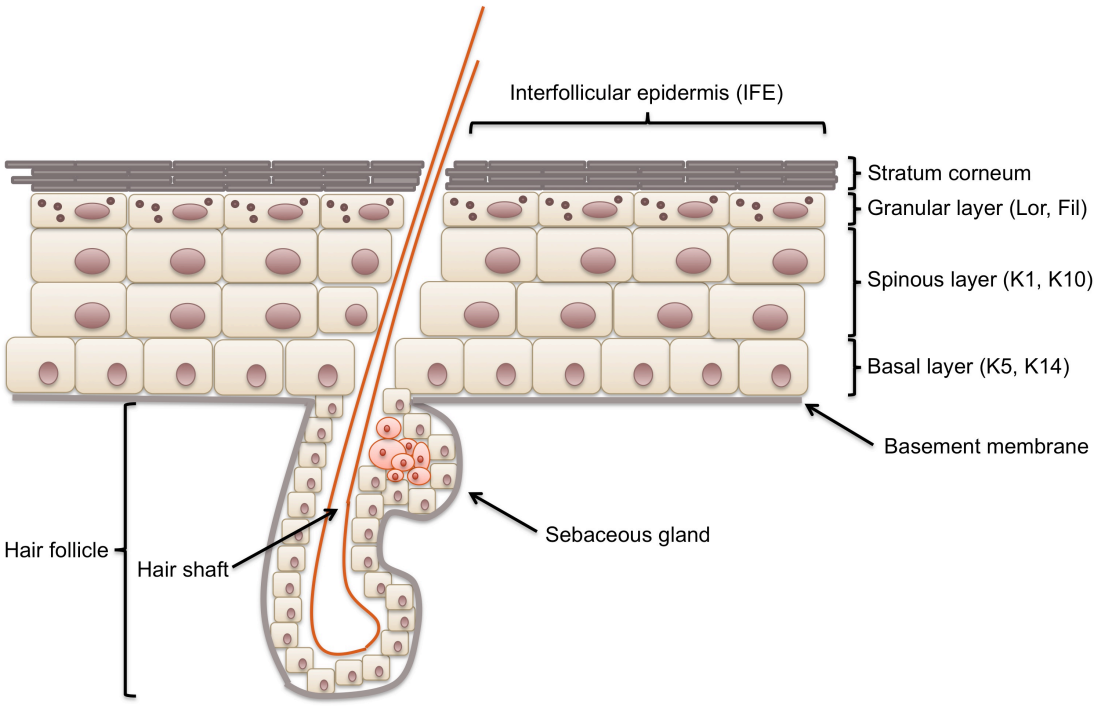

Figure 2. Gross morphology of the skin. A hair follicle with sebaceous gland is surrounded by stratified IFE. The different keratinocyte compartments are indicated together with their characteristic protein markers. Lor, loricrin; Fil, filagrin. © Karin Heby-Henricson

1.1.4.1 SKIN AND HAIR FOLLICLE STEM CELLS

SCs that reside within different tissues of the body are undifferentiated, generally slow cycling cells. SCs in the skin have the ability to divide asymmetrically, thereby generating one daughter SC with the ability to self-regenerate and one transit-amplifying cell, which contributes to the rapidly dividing progenitor population (Blanpain and Fuchs, 2009). Since the IFE is continuously losing cells as a result of shedding, there is a constant need for new cells, which come primarily from the actively proliferating basal layer. In contrast to the IFE, hair follicles undergo cycles of growth and rest, and rely on hair follicle SCs to replenish them with new cells in every cycle (Jaks et al., 2010). The resting hair follicle in mouse consists, almost entirely, of different keratinocyte SC populations, which overlap to some extent. Each of these SC populations appear to have its own distinct gene-expression profile, and its own defined tasks.

Hair follicles are present, more or less, all over the skin, and they play an important role in physiological tissue renewal and wound healing. In a wound situation, epidermal and dermal cells fill in and replace the injured skin tissue; however, cutaneous wounds also stimulate the proliferation and migration of keratinocytes in adjacent hair follicles, and these keratinocytes move along the hair follicle to the site of the lesion.

Signaling pathways of importance in regulating the different SC populations in the skin and hair follicles are the Wnt and Sonic hedgehog (Shh) pathways (Haegebarth and Clevers, 2009; Jiang and Hui, 2008). Shh is one of the earliest genes to be expressed in the hair placode (Bitgood and McMahon, 1995), while the aberrant activation of Shh in the IFE results in tumors expressing follicular markers (Youssef et al., 2010).

1.2 CANCER

The term 'cancer' represents a range of different diseases, all of which exhibit uncontrolled cell division, tissue invasion and spreading as common characteristics. By deregulating the control of growth-promoting signals, normal cells turn into tumor cells whose ability to sustain chronic proliferation may have several different causes. Some tumor cells may produce growth-stimulatory factors themselves, others signal to the tumor-associated stroma, which responds by supplying the tumor cells with growth promoting factors. Other tumors that develop are benign, meaning that they do not metastasize. Metastasis is a characteristic of malignant neoplasms and true cancers and occurs when cells from the primary tumor spread through the blood and lymphatic systems and implant into a new tissue environment, thus generating new tumor-foci distal to the primary tumor (Liotta and Kohn, 2003). However, tumorigenesis is a multistep process that relies on genome instability and inflammation, and multiple genetic alterations are needed to drive the progression of normal cells into highly malignant cancer cells. Douglas Hanahan and Robert A. Weinberg summarized the capabilities acquired during tumorigenesis in the "hallmarks of cancer" (Table 1) (Hanahan and Weinberg, 2011).

Table 1. The hallmarks of cancer

1.	Sustaining proliferative signaling	
2.	Evading growth suppression	
3.	Enabling replicative immortality	The six original hallmarks of cancer
4.	Activating invasion and metastasis	(Hanahan and Weinberg, 2000)
5.	Inducing angiogenesis	
6.	Resisting cell death	
7.	Deregulating cellular energetics	Emerging hallmarks
8.	Avoiding immune destruction	
9.	Genome instability and mutation	Enabling characteristics
10.	Tumor-promoting inflammation	

(Hanahan and Weinberg, 2011)

As shown in Table 1, genome instability and mutations are involved in tumorigenesis. Genetic alterations occur in many different ways: from the gain or loss of a single nucleotide (point mutation), to genomic translocations, or the gain or loss of entire chromosomes (aneuploidy). Another possibility is epigenetic changes, such as aberrant DNA methylation of promoters, which may result in transcriptional silencing of the affected gene, and lead to cancer. These changes in the genome can be induced by various factors including UV-irradiation and viral infections, or carcinogens such as the chemicals found in tobacco smoke and many other common products. Genetic alterations can also be inherited and are then present in all cells of the organism. There are two classes of genes that very often are altered in cancer cells – oncogenes and tumor suppressor genes, and these are discussed below.

1.2.1 ONCOGENES AND TUMOR SUPPRESSOR GENES

Proto-oncogenes represent genes that, under normal circumstances, are very often involved in cell cycle regulation and growth control (Pelengaris, 2006). This category of genes includes growth factors and their corresponding receptors, signal transducers and transcription factors, but also cell death regulators. When proto-oncogenes are activated aberrantly due to mutations, or due to an abnormal increase in expression, they turn into cancer promoting agents and are termed oncogenes (Bishop, 1996). The

transformation can be caused by a relatively small change in the original function of the proto-oncogene, but always results in enhanced function of the gene product, the oncoprotein. Activating, or gain-of-function mutations in proto-oncogenes are typically dominant, meaning that the tumor-inducing mutation only affects one of the two alleles of the gene. Examples of proto-oncogenes are *RAS* and *MYC*, but also the hedgehog signaling pathway components *SMO*, *GLI1* and *GLI2*.

In contrast to oncogenes, tumor suppressor genes are inactivated in cancer cells (Pelengaris, 2006). This results in the loss of the original function of the inactivated gene. The classical description of a tumor suppressor gene is that it must be fully inactivated in order to participate in tumor initiation and progression. Therefore, mutations in these genes are expected to act in a recessive manner, meaning that both alleles of the tumor suppressor gene have to be inactivated. This phenomenon was first described by Alfred Knudson in his 'two-hit hypothesis' (Knudson, 1971). Knudson hypothesized that an inherited germline mutation in a tumor suppressor gene (the first 'hit') would only cause cancer if the function of the other allele was subsequently compromised, either by a second mutation or by loss of heterozygosity (LOH) of the allelic region (the second 'hit'). Unfortunately, cancer genetics are more complex; in many cases heterozygotes display intermediate phenotypes to that of the wild-type and homozygote mutants (Berger and Pandolfi, 2011). In such cases, the remaining wild-type allele is not capable of producing the right amount of gene product for normal cellular function to be maintained, thus one copy of the gene is insufficient for proper function, a situation known as 'haploinsufficiency'.

Tumor suppressor genes can be divided into three general classes: gatekeepers, caretakers and landscapers (Kinzler and Vogelstein, 1997, 1998; Macleod, 2000). Gatekeeper tumor suppressor genes, such as the *RB* gene, function by restraining proliferation directly as cell cycle inhibitors, or by negatively regulating pro-proliferative pathways, such as the *PTEN* and *APC* genes. Additional gatekeeper tumor suppressor genes with haploinsufficiency characteristics include *PTCH1* and *SUFU*, which are both involved in the hedgehog signaling pathway, the latter being the subject of this thesis. The caretaker tumor suppressor genes prevent or repair DNA damage, thereby averting new mutations and cancer progression. The tumor suppressor gene *TP53* appears to act both as a gatekeeper and a caretaker, and also has typical features of haploinsufficiency. Landscaper tumor suppressor genes act as modulators of the microenvironment in which tumor cells grow, and loss-of-function mutations in these genes promote neoplastic conversion of adjacent epithelia and tumor growth.

1.2.1.1 TRANSFORMATION RELATED PROTEIN 53, Trp53

Transformation related protein 53, referred to as TP53 in humans (*TP53* gene) and p53 in mice (*Trp53* gene), is known to play key roles in situations of cellular stress. TP53 is activated in response to DNA damage caused by UV irradiation, for example, or by various chemical compounds, and the protein has several mechanisms of action: it can activate the DNA repair machinery, induce growth arrest by restraining the cell cycle G1 to S transition, and initiate apoptosis in situations when the DNA damage is irreparable. The *TP53* gene is mutated in at least 50% of human cancers (Hollstein et al., 1991; Soussi and Beroud, 2001), and LOH occurs in a fraction of tumors that harbor *TP53* mutations (Levine et al., 1991). However, the inherited loss of one copy of the *TP53* gene results in Li-Fraumeni syndrome (LFS), which is characterized by an increased susceptibility to a wide variety of cancers (Varley et al., 1997). Since approximately 60% of the tumors in LFS patients have LOH in the *TP53* locus, many of the remaining tumors are believed to arise due to haploinsufficiency of the *TP53* gene. The most common tumors in LFS patients are bone- and soft tissue sarcomas, acute leukemia, early-onset breast cancer, brain tumors and adrenocortical tumors. The

corresponding *Trp53*$^{+/-}$ mouse model also develops tumors that in many cases do not have LOH (Donehower et al., 1992; Venkatachalam et al., 1998). The *Trp53*$^{+/-}$ mice have a phenotype intermediate between wild-type and *Trp53*$^{-/-}$ mice, the latter developing multiple tumors and having more rapid tumor progression. Mutations in *TP53* often coexist with mutations in other genes, and have also been shown to enhance tumor progression in mouse models. In PAPER II of this thesis, the *Trp53* mutant mouse model was used to study co-operativity with hedgehog signaling in skin tumorigenesis.

1.3 THE HEDGEHOG SIGNALING PATHWAY

The hedgehog gene (*hh*) was initially discovered by Christiane Nüsslein-Volhard and Eric Wieschaus in a large screen for embryonic patterning defects in the larval body of the fruit fly, *Drosophila melanogaster* (Nüsslein-Volhard and Wieschaus, 1980). Vertebrate homologs of hh and most of the components in the hh signaling pathway have since been identified (Ingham et al., 2011). The Hh pathway in vertebrates plays an essential role in the development of the central and peripheral nervous systems, the skeleton, limbs, skin, hair, lungs, gut, germ cells and many other tissues, and relatively minor changes in the concentration of the Hh ligand have dramatic effects on the cellular response. The high sensitivity is needed to fine tune normal development and homeostatic processes in adult organisms. Briefly, Hh ligands are translated and modified in the signaling cell and are then released into the extracellular space. Receptor molecules present on surrounding cells interact with the ligands and the signal is transferred through a complex chain of events involving inhibitor and activator proteins, kinases and phosphatases, and eventually transcriptional repressors and activators. Altered Hh signaling, due to mutations in some of the components of the pathway, is associated with birth defects as well as cancers (Teglund and Toftgard, 2010). Several human diseases and various mouse models have contributed to our understanding of the roles played by this pathway, although it is still best understood in *Drosophila*.

1.3.1 PRODUCTION AND SECRETION OF HEDGEHOGS

The full-length Hh proteins are synthesized as 45 kDa two-domain proteins, each composed of a 19 kDa amino-terminal domain (Hh-N) containing a signal peptide sequence, and a 25 kDa carboxy-terminal domain (Hh-C), which has the promotion of autocatalytic cleavage between the two domains as its only known function (Burglin, 2008; Lee et al., 1994). The autocatalytic cleavage process, which takes place in the endoplasmic reticulum of the signaling cell, involves self-splicing through a nucleophilic reaction, that depends on cholesterol as the electron donor (Beachy et al., 1997). The cleavage event results in the covalent attachment of the cholesterol moiety to the C-terminal part of Hh-N. Thereafter, the protein undergoes palmitoylation at the N-terminus of Hh-N, a reaction promoted by the acyl transferase, HHAT (Chen et al., 2004). This unique processing of the Hh protein is important for its proper release, extracellular movement and, hence, its range of action.

Dispatched (Disp) is a large 12-pass transmembrane protein that is required exclusively in hh producing cells to facilitate the correct release of lipidated Hh-N (Amanai and Jiang, 2001; Burke et al., 1999). In *Drosophila*, it has been shown that, once the lipidated hh-N molecule has been transported by Disp to the outside of the cell, it stays connected to the plasma membrane bilayer through interactions with the glypican proteins, dally and dally-like, which are physically anchored to the membrane and are necessary for hh release (Callejo et al., 2011; Panakova et al., 2005). Dally and

dally-like are believed to recruit lipophorins to the lipidated hh-N. This recruitment facilitates the assembly of larger, multivalent hh-N-lipoprotein particles, which are then released upon cleavage of the dally and dally-like anchoring motifs, a process possibly mediated by the hydrolase, notum (Ayers et al., 2010). In mammals, GPC3, a corresponding member of the glycosylphosphatidylinositol (GPI)-linked glypican subfamily of heparan sulfate proteoglycans, appears to be involved in Hh ligand distribution. GPC3 also seems to compete with the receptor, Ptch, for Hh binding, thereby negatively regulating the pathway (Capurro et al., 2008; Hacker et al., 2005).

There are three hh homologues in mammals: Sonic hedgehog (Shh), Desert hedgehog (Dhh) and Indian hedgehog (Ihh), which are processed, modified and released in a similar way as *Drosophila* hh. Despite their high sequence similarity to other vertebrate and invertebrate Hh proteins, functional differences exist, for example, not all interactions with their cell-surface receptors are conserved, as discussed in the following chapters.

1.3.2 HEDGEHOG RECEPTION

In mammals, the cell surface receptor for Hh signaling, Patched, has two homologues Ptch1 and Ptch2. These homologues show differential expression during development, suggesting different functionality (Carpenter et al., 1998; Frohlich et al., 2002; Motoyama et al., 1998a; Motoyama et al., 1998c). *Ptch1* is a tumor suppressor gene that maps to chromosome nine in humans and 13 in mice. It encodes the major receptor for Hh; a 12-pass membrane spanning protein with a sterol-sensing domain and homology to proton-driven bacterial transporters (Fuse et al., 1999). In the absence of an Hh-N signal, Ptch1 inhibits another seven-pass membrane protein, with homology to G-protein coupled receptors, known as Smoothened (Smo) (Taipale et al., 2002). Since Smo is indispensable for Hh signaling, the activation of Hh target genes ultimately relies on the proper inhibition of Ptch1 (Zhang et al., 2001). The interaction of Hh-N with the two large extracellular domains of Ptch1 releases the inhibition of Smo (Beachy et al., 2010). This interaction is promoted by three transmembrane proteins: CAM-related/downregulated by oncogenes (Cdo), brother of Cdo (Boc) and growth arrest-specific 1 (Gas1) (Allen et al., 2007; Martinelli and Fan, 2007a; Tenzen et al., 2006)and recent data have shown that, in mammals, Cdo, Boc and Gas1 function in several tissues as essential mediators of multiple cellular reactions in response to Hh (Allen et al., 2011; Izzi et al., 2011).

Vertebrate Hh-N proteins appear to interact directly with Ptch1 (Fuse et al., 1999; Marigo et al., 1996; Stone et al., 1996), but no such direct interaction has been found between the corresponding *Drosophila* hh-N and ptc proteins (Zheng et al., 2010). In *Drosophila*, the co-receptor homologues of Cdo and Boc (ihog and boi [brother of ihog] respectively) interact directly with ptc and appear to be essential for *Drosophila* hh-N binding. It has been suggested that, together, these proteins constitute the *Drosophila* hh-N receptor.

Hedgehog interacting protein (Hhip) is a cell surface protein that negatively affects Hh signaling in vertebrates by binding to Hh-N, thereby competing with Ptch1 for the Hh-N ligand while simultaneously modulating Hh-N ligand distribution. Transcription of both *Ptch1* and *Hhip* is activated upon Hh signaling, but so far, Hhip does not seem to have a role in signal reception or transduction.

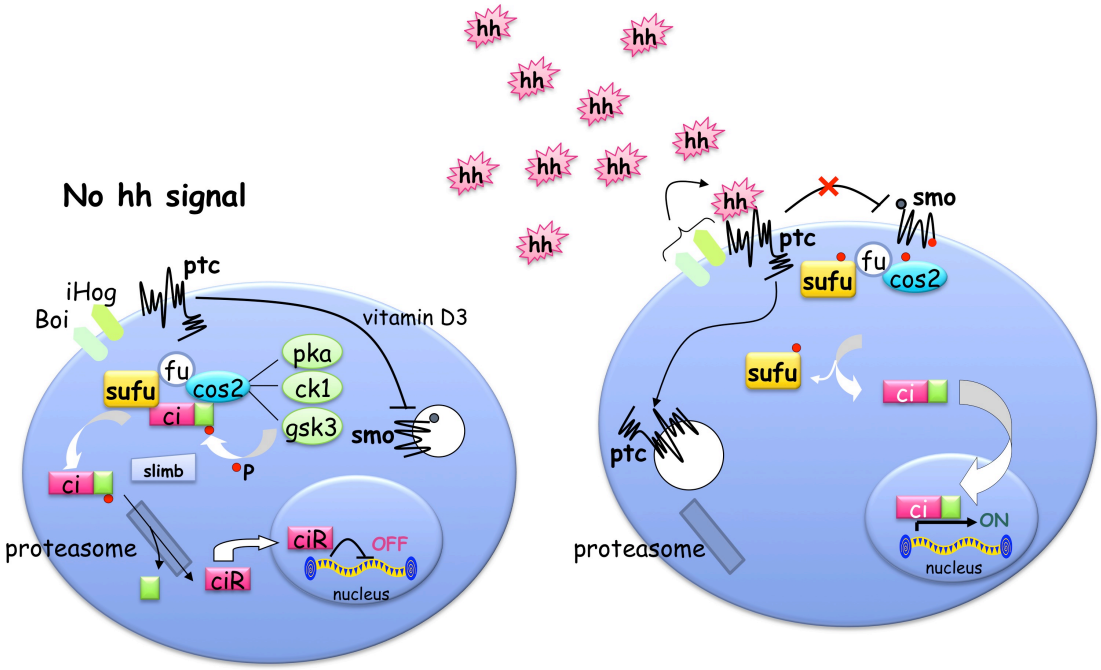

Figure 3. The hh signaling pathway in *Drosophila*. In the absence of the hh ligand, ptc inhibits smo. The HSC (cos2, fu, sufu, ci, pka, ck1 and gsk3) promotes the partial degradation of ci to a transcriptional repressor. In the presence of the hh ligand, the inhibition of smo is relieved resulting in disassembly of the HSC and inhibited ci degradation. Full-length ci becomes a transcriptional activator that initiates target gene transcription. © Karin Heby-Henricson

In the absence of an Hh ligand, Ptch1 inhibits Smo sub-stoichiometrically, and is thus capable of inhibiting a large excess of Smo (Denef et al., 2000; Ingham et al., 2000; Taipale et al., 2002). This indicates that the interaction between Ptch1 and Smo is indirect and may occur via some kind of mediator. Since the Ptch1 protein shares structural similarities with proton-driven bacterial transporters it has been suggested that it operates as a transporter of small molecule inhibitors or activators. The finding that several synthetic molecules can modulate Smo activity by binding to, and either activating or inhibiting Smo, supports this view. Examples of an Hh agonist and an Hh antagonist that function in this context are purmorphamine (Sinha and Chen, 2006) and cyclopamine (Chen et al., 2002), respectively.

Other small molecules such as oxysterols, derived from cholesterol in the sterol biosynthesis pathway, have been shown to exert a positive effect on Hh signaling upstream of Smo (Dwyer et al., 2007). In addition, it has been demonstrated that Ptch1 induces the secretion of vitamin D3, which inhibits Smo directly (Bijlsma et al., 2006). Rather confusingly, oxysterols are produced downstream of vitamin D3, and although this discrepancy does not yet have an explanation, it has been suggested that, in the absence of an Hh ligand, vitamin D3 levels are high and oxysterol levels are low, leading to operative Smo inhibition. In the presence of an Hh signal, increased synthesis and transport of oxysterols take place, resulting in Smo activation (Wang et al., 2007).

Recent data from *Drosophila* have implicated another molecule, phospholipid phosphatidylinositol-4-phosphate (PI4P), in the regulatory mechanism of ptc and smo (Yavari et al., 2010). PI4P activates Smo in mammalian fibroblasts. The contention is that Ptch1 inhibits PI4P levels by controlling the kinase activity that promotes PI4P synthesis. Increased PI4P levels are then thought to mediate intracellular trafficking of a speculative lipid modulator of Smo. Despite these findings, the Ptch1/Smo inhibitory mechanism is still considered as largely unresolved.

1.3.3 SIGNALING FROM SMO TO GLI

In the absence of Hh, Smo is localized to the membranes of intracellular, endocytic vesicles. In response to Hh signaling in vertebrates, Smo shuttles from these vesicles to the membrane of the primary cilium. This event represents one of the important pathway divergences between invertebrates and vertebrates. While most invertebrate cells lack a primary cilium, this organelle is ubiquitous in most vertebrate cells (except bone marrow cells and the intercalated cells of the kidney collecting duct) and is a prerequisite for Hh signaling (Praetorius and Spring, 2005).

Several important molecules are involved in transferring the signal between Smo and the Gli transcription factors via the cilia. One of these molecules, suppressor of fused (Sufu), is the focus of this thesis. In *Drosophila*, sufu has an insignificant role in the hh pathway (Ohlmeyer and Kalderon, 1998; Preat, 1992), and it produces only a very weak phenotype in zebrafish upon genetic ablation or morpholino knock-down (Koudijs et al., 2005; Tay et al., 2005; Wolff et al., 2003). In contrast, we, and others, have shown that Sufu is indispensable to the Hh signaling pathway in mammals, where it has acquired a new essential repressor function (**PAPER I**) (Cooper et al., 2005; Svard et al., 2006). In order to describe Sufu properly, I will begin by presenting the Gli transcription factors.

1.3.3.1 THE GLI TRANSCRIPTION FACTORS

Gli1, Gli2 and Gli3, the three Gli protein homologues in mammals, are zinc finger-containing transcription factors in the Hh pathway. In *Drosophila*, only one transcription factor, Cubitus interruptus (ci) mediates the hh signal, and its activity is basically regulated in two ways: firstly, in the absence of an hh signal, ci is partially degraded into a transcriptional repressor (Aza-Blanc et al., 1997); secondly, upon hh signaling, the partial processing of ci is inhibited, and full-length, active ci is produced. As an inhibitory mechanism to limit the hh response, the full-length ci activators are completely degraded after transcriptional activation of their target genes (Kent et al., 2006; Zhang et al., 2006). The processing, or degradation, of ci is carried out by the proteasome, which usually degrades substrates completely. During the unusual, partial, degradation of ci, the proteasome degrades the C-terminal, transactivation domain of ci, and leaves the DNA-binding N-terminal domain of the protein intact. The cleavage occurs in the absence of an hh/smo signal and is promoted by phosphorylation of specific motifs within the C-terminal domain of ci (Price and Kalderon, 2002). Protein kinase A (pka) primes the phosphorylation sites for further phosphorylation by two serine/threonine kinases; glycogen synthase kinase 3β (gsk3β) and casein kinase 1 (ck1). The F-box protein, slimb (βTrCP in mammals), recognizes the phosphorylated motifs and catalyses the sequential ubiquitination of the C-terminal domain that is subsequently degraded (Jia et al., 2005). In addition, a 'simple sequence' of a few amino acids that affects proteasome processing, has been identified adjacent to the zinc fingers in ci. It has been suggested that 'simple sequences' weaken the binding of the proteasome to the protein that is being processed, thereby allowing for partial escape from degradation.

Gli3 is the mammalian homologue that most closely resembles ci (Tempe et al., 2006; Wang et al., 2000a). In the absence of an Hh signal, Gli3 is processed into a truncated repressor that counteracts the transcriptional activity mediated by the full-length Gli activator proteins. Gli2 is similar to Gli3, but the processing of Gli2 into its repressor form is less efficient (Pan et al., 2009). Gli1 is not directly activated by Hh signaling, but is a transcriptional target gene of Gli2 and Gli3, and it is not processed into a transcriptional repressor, but is fully degraded in the absence of Hh (Dai et al., 1999; Kaesler et al., 2000).

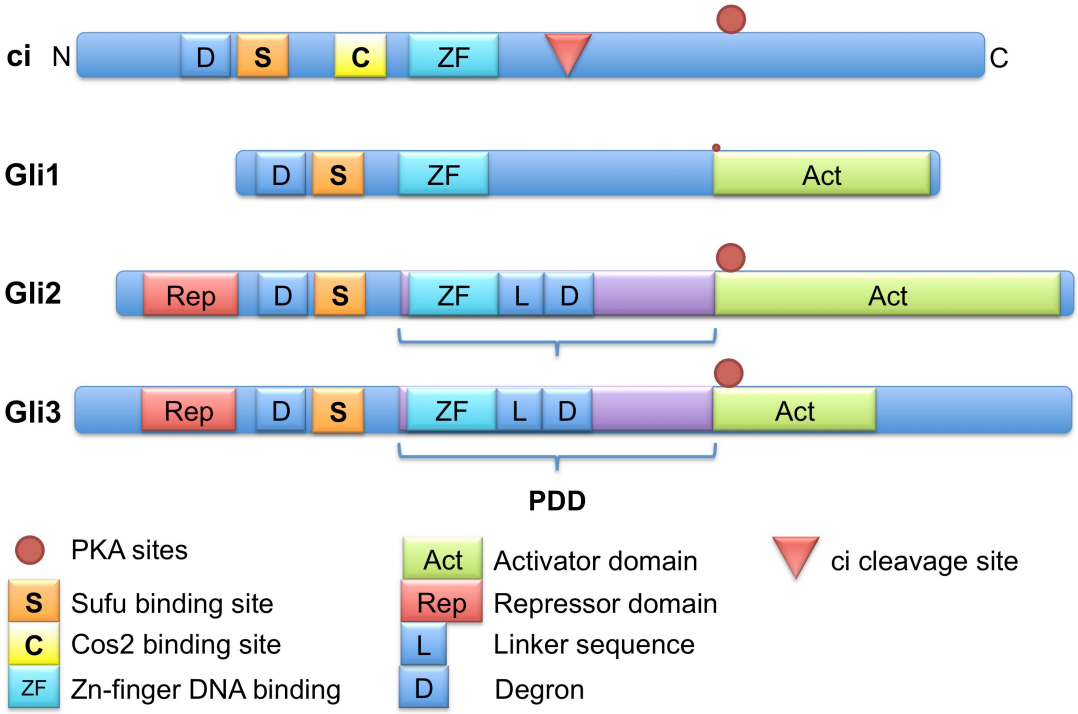

Figure 4. Schematic drawing of ci and the three Gli proteins. Gli2 functions as the primary activator of Hh signaling in mammals, Gli3 mainly works as a repressor and is the Gli protein most similar to ci. The processing determinant domain (PDD) includes the Zn-finger DNA binding domain, a linker sequence (L) and a degron (D), all of which are essential for proper degradation of Gli2 and Gli3 to transcriptional repressors. The PDD is lost in Gli1, which only works as a secondary activator of transcription. Gli1 also lacks some of the PKA binding sites present in Gli2 and Gli3, indicated by a smaller red dot in the figure. © Karin Heby-Henricson

Although the Gli1 and Gli2 oncogenes have very similar DNA binding specificities and overlapping regulator properties, some of their activities are clearly distinct (Eichberger and Frischauf, 2006). Gli2 and Gli3 are essential genes in mammals, while Gli1 represents a secondary mediator of Hh signaling, and is dispensable to embryonic development. Recently, a region within Gli2 and Gli3, often referred to as the processing determinant domain (PDD) (Pan and Wang, 2007), was found to consist of three components responsible for processing: the zinc finger domain, an adjacent linker sequence and a degron sequence (Figure 4) (Schrader et al., 2011). The processing of Gli3 was completely abolished if any one of the three components was disrupted. In addition, Gli1 seemed to have lost the linker sequence and the degron. Moreover, the Gli1 region corresponding to the degron region in Gli3, lacked several of the Pka and ubiquitination sites. In addition to Pka, Gsk3β and Ck1, the protein kinases, Cdc2l1, Dyrk2 and Map3K10, have also been identified as regulators of Gli activity (Evangelista et al., 2008; Varjosalo et al., 2008).

The transfer of the Hh signal from Smo to the Gli transcription factors is best understood in *Drosophila*. In the absence of an hh/smo signal, the hedgehog signaling complex (HSC) assembles, promoting the partial degradation of ci to its ci repressor form (Robbins et al., 1997; Sisson et al., 1997). The HSC consists of ci, fu, sufu, pks, gsk3, ck1 and cos2. Costal-2 (cos2) is an orthologue of a member of the kinesin family of motor proteins, which recruits the other components of the HSC (Farzan et al., 2008). Fused (fu) is a serine/threonine kinase, and a positive regulator of the hh pathway (Liu et al., 2007; Ruel et al., 2007). Upon hh signaling, smo is phosphorylated by pka and ck1 (Apionishev et al., 2005; Jia et al., 2004; Zhang et al., 2004), and transduces the signal by interacting with fu and cos2 (Ascano and Robbins,

2004; Jia et al., 2003; Liu et al., 2007; Lum et al., 2003; Ogden et al., 2003; Ruel et al., 2003). New data indicate that smo recruits the HSC to the plasma membrane by interacting with cos2 (Zhang et al., 2011), which induces the dimerization of fu, and its subsequent auto-phosphorylation and promotes further phosphorylation of cos2 and sufu. Consequently, the HSC becomes partially dissociated.

Drosophila sufu, is a negative regulator of hh signaling that binds to and stabilizes ci even upon HSC disassembly. Since this occurs in the cytoplasm, sufu can restrain ci nuclear import (Methot and Basler, 2000; Wang et al., 2000b). However, once sufu is phosphorylated by fu, the sufu/ci interaction is abrogated, leading to the nuclear import of ci and ci target gene activation. More recent data show that fu can also stabilize full-length ci via the phosphorylation of cos2, and can promote the activation of ci independently of sufu (Zhou and Kalderon, 2011). In *Drosophila*, the absence of fu results in segment polarity defects, but loss of sufu alone has no phenotype (Preat, 1992). However, if both sufu and fu are deleted, the fu phenotype is restored, hence, sufu got its name from its function as a suppressor of *fu* mutations in flies.

1.3.3.2 SUPPRESSOR OF FUSED

We, and others, have shown that in mammals, in contrast to flies, Sufu is a major negative regulator of the Hh signaling pathway (Cooper et al., 2005; Svard et al., 2006), and Sufu loss-of-function mutations in mouse cause expansive ligand-independent activation of Hh target genes. Sufu has long been considered as a puzzling protein due to the lack of similarities with other known proteins, making it difficult to predict its precise function. Interestingly, however, Sufu is the most conserved member of the Hh pathway in mammals and it interacts with all three Gli proteins, playing a role in controlling their processing/degradation, and thereby in cell fate decision (Ding et al., 1999; Stone et al., 1999).

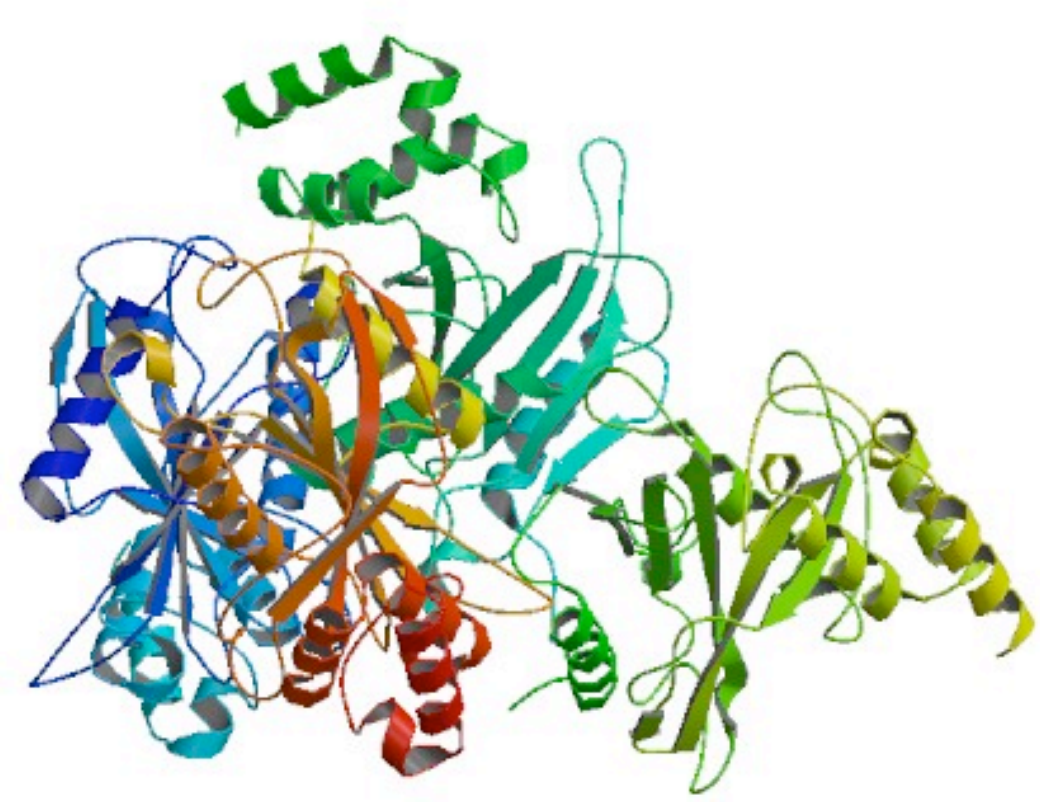

Figure 5. N-terminal 3D-structure of the human SUFU protein, published in the web-based protein data bank. http://www.pdb.org/pdb/explore/explore.do?structureId=1M1L (Merchant et al, 2004)

The *Drosophila* sufu gene encodes a protein consisting of 468 amino acids with little homology to any other protein. The only notable exception is a PEST sequence (a sequence rich in proline [P], glutamic acid [E], serine [S] and threonine [T]) in the carboxy-terminal portion of the protein, which is associated with rapid protein degradation (Pham et al., 1995). The human *SUFU* gene encodes a protein of 484 amino acids that is 37% identical to the *Drosophila* sufu protein, and 97% identical to mouse Sufu, which also has 484 amino acids (Kogerman et al., 1999; Stone et al., 1999). Human *SUFU* is located on chromosome 10 and contains 12 exons (Grimm et al., 2001), while the mouse *Sufu* gene also contains 12 exons, and maps to chromosome 19. As with the *Drosophila* protein, human and mouse Sufu share little homology with other known proteins, except for the PEST sequence and four consensus target sites for Pka, whose role is still unclear. Although both human and mouse Sufu proteins contain a PEST sequence, the actual sequence and its location in the Sufu protein differ between the two species (Stone et al., 1999). Stability measurements of Sufu in mouse embryonic fibroblasts (MEFs) have shown that it has a half-life of 24 hours in low Hh conditions, but upon activation of Shh signaling the Sufu half-life is decreased to four hours tentatively due to degradation in the proteasome (Yue et al., 2009). Crystallization of the full-length SUFU protein has been difficult, but a 3D-structure of an N-terminal fragment (amino acids 27 to 268) of SUFU has been solved (Figure 5) (Merchant et al., 2004). It consists of six amphipathic α-helices, seven antiparallel β-barrels in a core bundle, and has a concave surface with an acidic patch that overlaps with its GLI1 binding region.

It is believed that SUFU binds to all GLI transcription factors in a head-to-tail manner in a 1:1 ratio. The Sufu-binding site on Gli1 is located N-terminal to the Zn finger (DNA-binding) domain. This region is conserved in all three Gli proteins, is universal among vertebrates, and is also found in the ci protein of *Drosophila*. The binding site contains an SYGH motif of four amino acids that is recognized by the C-terminal domain of Sufu. In addition, several alternative splice variants of human SUFU have been identified, but two of these have lost their ability to interact with Gli1 (Dunaeva et al., 2003; Grimm et al., 2001). A third splice variant, also found in mice, has 485 amino acids and is expressed relatively abundantly.

Mammalian Sufu is constitutively expressed in most adult tissues, at various developmental stages, and is found both in the cytoplasm and in the nucleus (Barnfield et al., 2005; Kogerman et al., 1999). Several nuclear export signals have been identified in the C-terminal region of Sufu, leading to the suggestion that Sufu shuttles between the cytoplasm and the nucleus, facilitating the nuclear export and cytoplasmic tethering of Gli, and hampering Gli transcriptional activity. However, following our discovery that in Sufu null MEFs, Gli1 remained localized in the cytoplasm, this scenario became less likely (Svard et al., 2006). Now it is believed that Sufu's role in the cytoplasm is to promote the production of Gli repressors (Humke et al., 2010), and that its role in the nucleus is to suppress the transcriptional activity of Gli through the recruitment of co-repressors.

SAP18 is a nuclear protein and a binding partner of the mammalian homolog of the yeast Sin3A protein (mSin3A), which together with histone deacetylase (HDAC) forms a corepressor of transcription (Cheng and Bishop, 2002; Zhang et al., 1997). SAP18 was shown to interact specifically with Sufu, and a complex composed of Sufu, SAP18, mSin3A and Gli1 was found to bind to the Gli-binding element (5′-GACCACCCA-3′) in DNA ((Kinzler and Vogelstein, 1990). It was also shown that Sufu represses Gli-mediated transcription by recruiting the mSin3-HDAC co-repressor complex to promoters containing the Gli-binding element. These studies clearly suggest a nuclear role for Sufu in repressing the transcriptional activity of Gli.

It has been shown in *Drosophila* that loss of sufu causes destabilization of ci, and that overexpression of sufu stabilizes ci (Ohlmeyer and Kalderon, 1998; Zhang et al., 2006). The conclusion from these discoveries was that sufu regulates the function of both full-length ci and ci repressors. In vertebrates, the absence of Hh leads to stabilization of the full-length Gli2 and Gli3 proteins followed by proteolytic processing of the majority of Gli3, which becomes a transcriptional repressor (Wang et al., 2010; Wen et al., 2010). Only a small fraction of Gli2 is processed into a transcriptional repressor. Hh signaling inhibits the processing of the full-length Gli proteins and converts Gli2 and Gli3 into transcriptional activators. Gli2 is the primary transcriptional activator of Hh signaling, and its activation results in the transcription of *Gli1*, which functions as a secondary activator, further boosting transcriptional activity.

1.3.3.3 THE ROLE OF PRIMARY CILIA

Primary cilia are organelles that protrude from the surface of nearly all vertebrate cells. These solitary, non-motile antennas have multiple functions, sensing both mechanical and chemical changes in the environment (Hoey et al., 2011). Primary cilia have the same basic structure as motile cilia with a core bundle of nine microtubule pairs that protrude from the basal body up to the ciliary tip. The primary cilium is dependent on the intraflagellar transport (IFT) machinery for the transport of particles along the microtubules (Goetz and Anderson, 2010), and the IFT facilitates the correct construction, maintenance and functioning of the organelle. Anterograde (from base to tip) transport depends on the motor protein, kinesin-2, while retrograde (from tip to base) transport depends on the cytoplasmic motor, dynein-2. Disruption of either of these motors results in perturbed ciliary function and assembly. Interestingly, it has been demonstrated that Hh signaling in mammals is dependent on proper ciliary function (Huangfu et al., 2003), and in mouse, all major Hh pathway components are associated with the primary cilium; however, in *Drosophila* this organelle is absent.

In the absence of Hh, Ptch1 is located at the base of the cilium (Corbit et al., 2005; Rohatgi et al., 2007). Smo is located on intracellular endocytic vesicles and is not associated with the primary cilium at this stage, possibly due to an indirect blocking signal from Ptch1. Kif7, the vertebrate homolog of cos2, is also localized at the cilium base together with Gli, promoting the formation of Gli repressor forms (Endoh-Yamagami et al., 2009; Liem et al., 2009). However, Gli is also located in the cilium tip, together with Sufu, where Sufu is believed to promote the formation of Gli repressors and antagonize the activator forms of Gli, partly through the recruitment of Gsk3β (Haycraft et al., 2005; Kise et al., 2009). Upon Hh signaling, Ptch1 is inhibited, and, together with the ligand, leaves the ciliary space and becomes internalized into endosomal vesicles (Corbit et al., 2005; Rohatgi et al., 2007). Simultaneously, Smo is allowed to localize to the cilium, resulting in the accumulation and dissociation of the Sufu-Gli complex in the cilium tip (Tukachinsky et al., 2010). This abrogates the cleavage of Gli proteins and promotes the accumulation of their full-length forms, which are transported in a retrograde fashion through the cilium. Subsequently, full-length Gli is activated by an as yet unknown mechanism, and is transported into the nucleus where it initiates target gene transcription.

Although Sufu co-localizes with Gli and Smo to the cilium and the formation of Gli repressors is compromised in cells lacking a primary cilium (Haycraft et al., 2005; Huangfu and Anderson, 2005; Liu et al., 2005), some data suggest that the inhibitory effects of Sufu on Gli activity are independent of the cilium (Chen et al., 2009; Jia et al., 2009). This cilium-independent function may involve the speckle-type POZ protein (Spop), whose *Drosophila* homolog, hib, forms a complex with ci and cullin3, which is associated with E3 ubiquitin ligase (Zhang et al., 2006), and consequently, ci becomes ubiquitinated and degraded. In *Drosophila*, sufu interferes

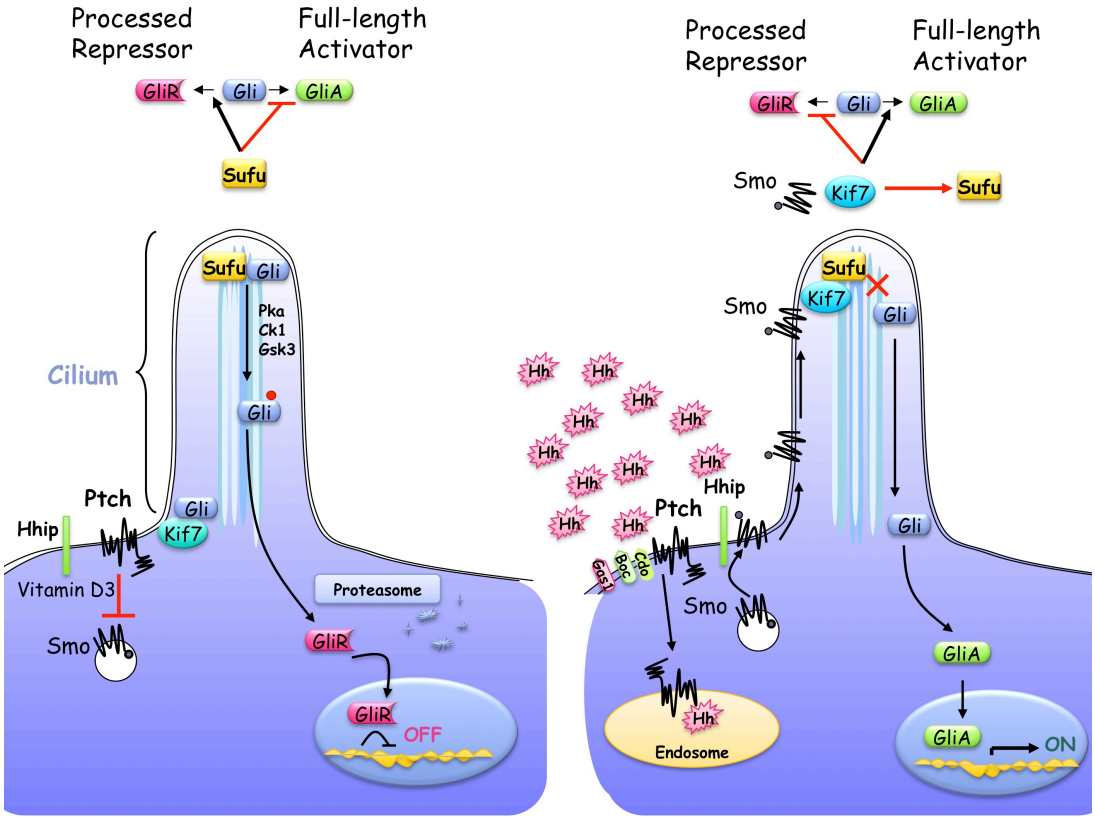

Figure 6. Schematic drawing of Hh signaling in mammals. Left-hand illustration: in the absence of the Hh ligand, Ptch is situated at the base of the primary cilium, inhibiting Smo via small molecule inhibitors such as vitamin D3. Kif7 and Sufu bind to and restrict the ciliary and nuclear localization, respectively, of full-length Gli. Sufu also promotes the partial degradation of full-length Gli to a transcriptional repressor. Right-hand illustration: Hh ligand binding to Ptch and additional co-receptors (Cdo, Boc and Gas1) results in Ptch-Hh internalization into endosomes, thus eliminating the inhibition of Smo, which can then translocate to the cilium. In the cilium, Smo and Kif7 inhibit Sufu, which can no longer keep the full-length form of Gli in the ciliary tip or promote the degradation of Gli to its repressor form. Instead, full-length Gli becomes activated and translocates into the nucleus where it initiates target gene transcription. © Karin Heby-Henricson

with this process and rescues ci from degradation by competing with hib for ci binding. Wang et al. showed that mouse Sufu promotes stabilization of the full-length forms of Gli2 and Gli3, whereas Spop promotes their degradation and the processing of Gli3 into a transcriptional repressor (Wang et al., 2010). These authors also found that Spop and Sufu oppose each other by competitive binding to the same regions on Gli2 and Gli3, and that the Gli3 repressor can function independently of Sufu.

Unlike the situation in *Drosophila*, there does not seem to be direct contact between Smo and Sufu, or between Smo and Kif7 in mammals, since Smo lacks the major binding site for cos2/Kif7 (Varjosalo et al., 2006). In addition, the functional loss of Fu in mouse has no influence over Hh signaling, but instead is important for motile cilia formation (Chen et al., 2005; Merchant et al., 2005; Wilson et al., 2009). Kif7 was also believed to be dispensable for Hh signal transduction, although recent studies have proved otherwise. In fact, Kif7 is essential for mouse Hh signaling and is associated with the primary cilium (Cheung et al., 2009; Endoh-Yamagami et al., 2009; Liem et al., 2009).

In *Drosophila*, cos2 is the link between the HSC and the microtubules in an Hh-dependent manner, but it lacks the kinesin motor function since it has lost its ability to bind ATP (Farzan et al., 2008). It has also been suggested that Kif7 links the Hh pathway and the microtubules of primary cilia, but unlike cos2, Kif7 retains all the

kinesin motor motifs (Endoh-Yamagami et al., 2009; Liem et al., 2009). In the absence of an Hh signal, Kif7 is located at the base of the cilium, which is enriched in proteasomes and PKA, allowing control over the processing of Gli, while in the presence of an Hh ligand, Kif7 moves to the ciliary tip. It is possible that Kif7 also participates in the directional anterograde transport of Hh pathway components, such as the Gli proteins; however, it does not seem to be required for Gli2 and Gli3 accumulation in the cilium tip upon Hh signaling, since Gli2 and Gli3 appear to localize at the tip even in the absence of Kif7 (Hsu et al., 2011). In *Drosophila*, cos2 plays dual functions in the sense that it promotes the degradation of ci to become a repressor of hh signaling in the absence of a ligand, but also permits high levels of hh pathway activation by antagonizing sufu. Kif7 seems to play a similar role; when the pathway is off Kif7 prevents the accumulation of Gli2 (and Gli3) in the cilium tip, thereby preventing the formation of their activator forms, and when the pathway is on, Kif7 positively regulates Gli-mediated transcription by downregulating Sufu protein levels, but it also seems to act negatively by inhibiting Gli-mediated transcription through a Sufu-independent mechanism (Hsu et al., 2011). Conclusively, primary cilia mediate a dual role by enabling both activation and repression of the Hh signaling pathway.

1.3.4 HEDGEHOG TARGET GENES

The Gli proteins bind to and regulate Hh target gene transcription. Both the Gli activator and repressor forms bind to the same promoter regions, which all have a common consensus binding sequence: 5´-GACCACCCA-3´ (Kinzler and Vogelstein, 1990). However, there is also a widespread presence of evolutionarily conserved nonconsensus Gli binding sites in the enhancer sequences of the Hh target genes (Parker et al., 2011; Winklmayr et al., 2010). These sequences seem to be important for target gene regulation in intermediate levels of Hh signaling. The Hh gradient produces opposing gradients of Gli activator and repressor forms which compete for the same genomic binding sites. Their high or low Gli binding affinity in turn characterizes these binding sites. Genes that respond broadly across the Hh gradient should have high-affinity Gli binding sites and genes activated only by strong Hh signaling should have low-affinity sites. Some of the components within the Hh pathway itself are among the most well documented direct targets for Hh signaling and include *Gli1* (Dai et al., 1999; Ikram et al., 2004; Lee et al., 1997), *Ptch1*, *Ptch2* and *Hhip* (Chuang and McMahon, 1999; Rahnama et al., 2004; Yoon et al., 2002), which act either as pathway activators (Gli1) or inhibitors (Ptch1, Ptch2 and Hhip). Thus, the outcome of Hh signaling reflects the levels of Hh ligand, depends on the affinity for Gli binding in the enhancer regions of the target genes, and involves both negative and positive feedback loops that either inhibit or enhance the signaling output.

Recalling the broad spectrum of Hh involvement in various biological events, it is likely that Hh regulates a large number of genes in addition to those already mentioned. To give some examples, HH target genes have been implicated in processes such as cell cycle control [*CyclinD* (Duman-Scheel et al., 2002; Yoon et al., 2002)], proliferation [*N-Myc* (Kenney et al., 2003; Oliver et al., 2003)], cell fate decision [*Nkx2.2* (Lei et al., 2006) and *FoxA2* (Sasaki et al., 1997)], cell survival [*Bcl2* (Bigelow et al., 2004; Regl et al., 2004b)] and cell cycle progression [*E2F1* (Regl et al., 2004a)]. Additional candidate target genes are constantly being identified through systemic screenings (Eichberger et al., 2006; Hallikas et al., 2006; Vokes et al., 2007; Vokes et al., 2008; Xu et al., 2006). In addition, the Hh pathway is also involved in crosstalk with several other important signaling pathways such as Wnt/β-catenin, TGF-

β/Bmp, Notch and FGF. Together, they build up a complex network of direct and indirect communication in order to coordinate essential processes during development.

1.3.5 HEDGEHOG IN DEVELOPMENT

Differential concentrations of hedgehog regulate the relative amounts of the repressor and activator forms of Gli, resulting in specific patterns of target gene expression and thereby affecting cell fates (Ogden et al., 2004). A good example is the patterning of the developing limb-bud where Shh is needed to polarize the anterior-posterior axis (Benazet and Zeller, 2009). Another similar example is the developing neural tube in vertebrates (Dessaud et al., 2008), Shh is secreted from the notochord and floorplate of the neural tube, and a long-range gradient is established along the dorsal-ventral axis, with the highest concentration of Shh in the ventral region. This gradient results in a specific pattern of gene expression, which is determined by the negative or positive transcriptional regulation of each gene by a precise concentration of Shh. This targets the cells to differentiate into a specific neuronal subtype. The pathway is also important during cartilage and bone development through the action of Ihh, which coordinates various aspects of chondrocyte and osteoblast development through interaction with parathyroid hormone-related peptide (PTHrP) (St-Jacques et al., 1999; Vortkamp et al., 1996). The third hedgehog homolog, Dhh, is involved in the development of testes and external genitalia (Bitgood et al., 1996).

1.3.5.1 HEDGEHOG AND DEVELOPMENTAL DISORDERS

Since the HH signaling pathway is involved in so many different aspects of development, it is not hard to imagine that there will be serious consequences if the pathway is not functioning properly. For example, holoprosencephaly is a condition characterized by CNS and facial anomalies with a range of severity from cyclopia to cebocephaly, microcephaly and hypotelorism with cleft palate and/or lip (Cohen, 2010). Holoprosencephaly may be the result of HH pathway inhibition due to inactivating mutations in *SHH* or *GLI2*, or to inhibition of SMO by cyclopamine. Impaired cholesterol biosynthesis can also cause human holoprosencephaly (Haas and Muenke, 2010).

1.3.5.1.1 GORLIN SYNDROME (GS)

Gorlin syndrome (GS), also called nevoid basal cell carcinoma syndrome (NBCCS) or basal cell nevus syndrome (BCNS), is an autosomal dominant disorder. GS is characterized by multiple basal cell carcinomas (BCCs) in the skin of young people, and also epidermal cysts and palmar and plantar pits (Gorlin, 2004). Although BCC tumors are rarely malignant and are among the least fatal, the multiplicity of the tumors in some GS patients can cause severe tissue destruction by local invasion. GS patients also show a high incidence of other tumors including medulloblastoma (MB), meningioma, ovarian and heart fibroma, fetal rhabdomyoma and, rarely, rhabdomyosarcoma (RMS). In addition, GS patients can also suffer from several developmental abnormalities including calcification of falx cerebri, skeletal malformations such as bifid ribs, craniofacial features with macrocephaly, hypertelorism, cleft lip/palate, and odontogenic keratocysts.

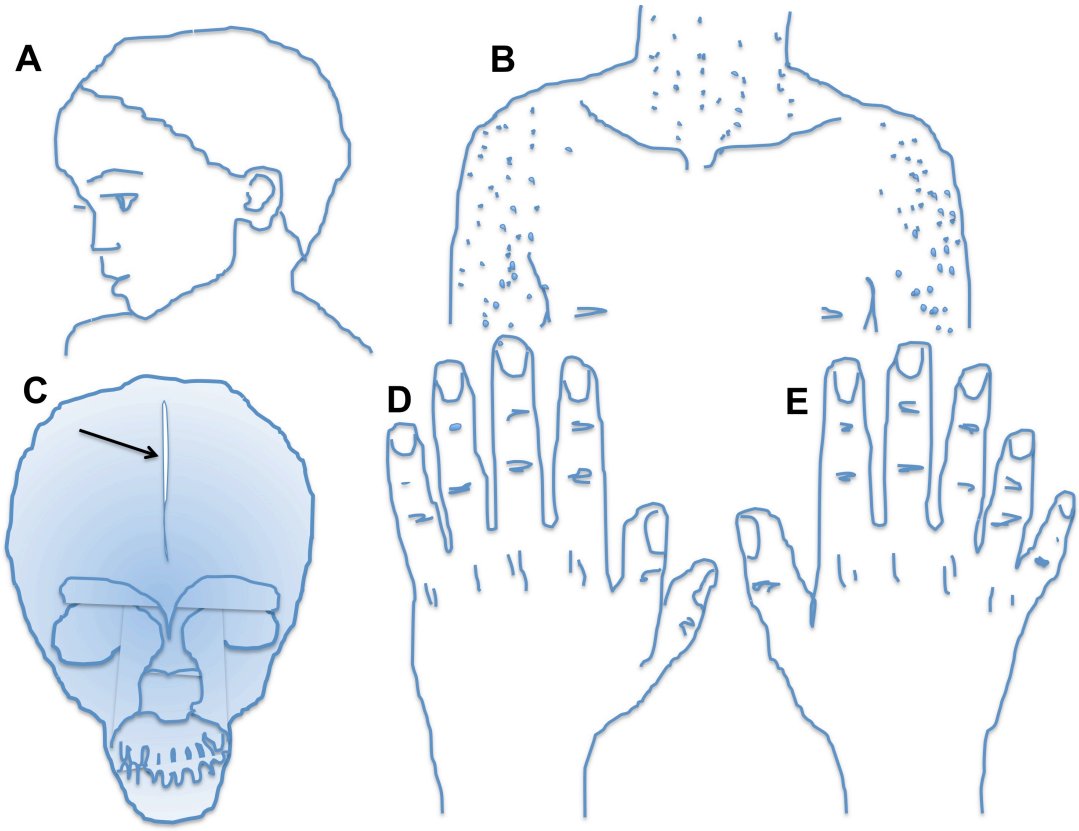

Figure 7. Gorlin syndrome involves many different developmental defects as well as different kinds of tumors. These are representative drawings of some of these features. (A) Frontal bossing and macrocephaly, (B) multiple BCCs predominantly on sun-exposed areas of the skin, (C) calcification of falx cerebri depicted by arrow, (D) preaxial and (E) postaxial polydactyly. © Karin Heby-Henricson

The syndrome is predominantly caused by heterozygous, inactivating mutations of *PTCH1*, which result in constitutive HH pathway activation (Gailani et al., 1992; Hahn et al., 1996; Johnson et al., 1996). Somatic inactivation of the remaining *PTCH1* allele has been shown in BCCs from GS patients, indicating that *PTCH1* acts as a classical tumor suppressor gene. However, it is likely that most of the developmental defects are due to haploinsufficiency of *PTCH1*, indicating the importance of finely-tuned gene-dosage in the pathway.

The first reports of genetic heterogeneity in GS have recently been published. One GS family were reported to carry a mutation in *PTCH2* (Fan et al., 2008), and another family had a germline truncating mutation in *SUFU* (Pastorino et al., 2009). It is worth noting that the family with the *SUFU* mutation did not develop BCCs, instead the affected individuals suffered from MB or calcification of the falx cerebri, and palmar and plantar pitting, and no *PTCH1* mutations were detected. In another study, two families were identified with germline frameshift mutations in *SUFU*. Of the 25 individuals identified as carriers of the mutation, seven developed MB, but none had typical signs of GS (Yoshida et al., 2003).

1.3.5.1.2 OTHER HEDGEHOG-RELATED SYNDROMES

There are several other syndromes related to malfunctions in HH signaling; Greig cephalopolysyndactyly syndrome is an autosomal dominant disorder, like GS, characterized by limb anomalies such as polydactyly, ocular hypertelorism, a broad, prominent forehead and mental retardation in more severely affected patients (Balk and

Biesecker, 2008). The syndrome is caused by large deletions, translocations, frameshifts or other mutations in the first third of the GLI3 gene resulting in functional haploinsufficiency. If the GLI3 gene is mutated in its second-third, it results in a truncated GLI3 repressor protein and another syndrome known as Pallister-Hall syndrome (Biesecker, 2006), which is characterized by hypothalamic hamartoma, bifid epiglottis and insertional polydactyly, in addition to the features of Greig cephalopolysyndactyly syndrome.

Another example of an HH-related developmental disorder is the autosomal recessive Carpenter syndrome, caused by mutations in RAB23 (Jenkins et al., 2007). RAB23 is a negative regulator of HH signaling, acting downstream of SMO, but upstream of GLI2 and GLI3 to inhibit formation of the GLI2 activator. Carpenter syndrome is characterized by craniosynostosis, short fingers, polydactyly, obesity and congenital heart defects.

In addition to the above mentioned syndromes, defects associated with perturbation of ciliary proteins, with the common name ciliopathies, can give rise to a broad range of phenotypes (Baker and Beales, 2009). This includes features associated with malfunctioning HH signaling e.g. polydactyly.

1.3.6 HEDGEHOG IN CANCER

HH is a very important player during development; however, in adult individuals the role of HH signaling is limited. Re-activation of the pathway has been implicated in a variety of sporadic tumors including BCC (Gailani et al., 1996; Lacour, 2002), medulloblastoma (Pomeroy et al., 2002), digestive tract tumors (Berman et al., 2003), pancreatic cancer (Thayer et al., 2003), small-cell lung cancer (Watkins et al., 2003) and prostate cancer (Karhadkar et al., 2004; Sheng et al., 2004), some of which will be discussed in this chapter.

1.3.6.1 SKIN CANCER

Skin cancer includes several different cancers that may develop from the various cell types present within the skin. In addition to the IFE and its keratinocytes, melanocytes, hair follicles and sebaceous glands are also part of the skin and different kinds of tumors may develop in each of these compartments. The following sections focus on skin tumors that are related to aberrant HH signaling.

1.3.6.1.1 SQUAMOUS CELL CARCINOMA AND MALIGNANT MELANOMA

Squamous cell carcinoma (SCC) of the skin, arises from the squamous epithelium, and most often localizes to the head and neck region (Li et al., 2011). A link between HH and SCC is unlikely (Eklund et al., 1998), although there are examples of studies that have shown overexpression of HH pathway components in SCC skin samples (Schneider et al., 2011). Also, nuclear *GLI1* expression was shown as an independent predictor of early relapse and poor overall survival in esophageal SCC after chemoradiotherapy (Zhu et al., 2010).

So far, no genetic alterations in components of the HH pathway have been found in malignant melanoma, but both GLI1 and GLI2 seems to play important roles in this disease, since both correlate with melanoma progression, invasiveness and metastasis (Alexaki et al., 2010; Das et al., 2009).

1.3.6.1.2 BASAL CELL CARCINOMA

Sporadic BCC of the skin is the most commonly occurring tumor in Caucasians, with approximately 1 million new cases each year in the US, and 40,000 new cases in Sweden. Fortunately, BCC rarely metastasizes and is therefore one of the least fatal tumors, but it is still capable of causing morbidity and the risk of developing further BCCs is high. In rare cases (<0.1%), the clinical course of the disease may be aggressive and regional metastases in other tissues, such as the lymph nodes, can occur (Malone et al., 2000). However, distant metastases can also develop, for example in the lungs, or more rarely in bone and other internal organs (Jarus-Dziedzic et al., 2000; Lo et al., 1991; Tavin et al., 1995). The majority of BCCs arise on sun-exposed areas of the skin, but they can also be caused by ionizing radiation, chemical carcinogens and infection by human papilloma viruses (Bastiaens et al., 1998). BCC is characterized by a broad phenotypic variability and is classified into the following histological subtypes; nodular, infiltrative, superficial, and mixed (Rippey, 1998).

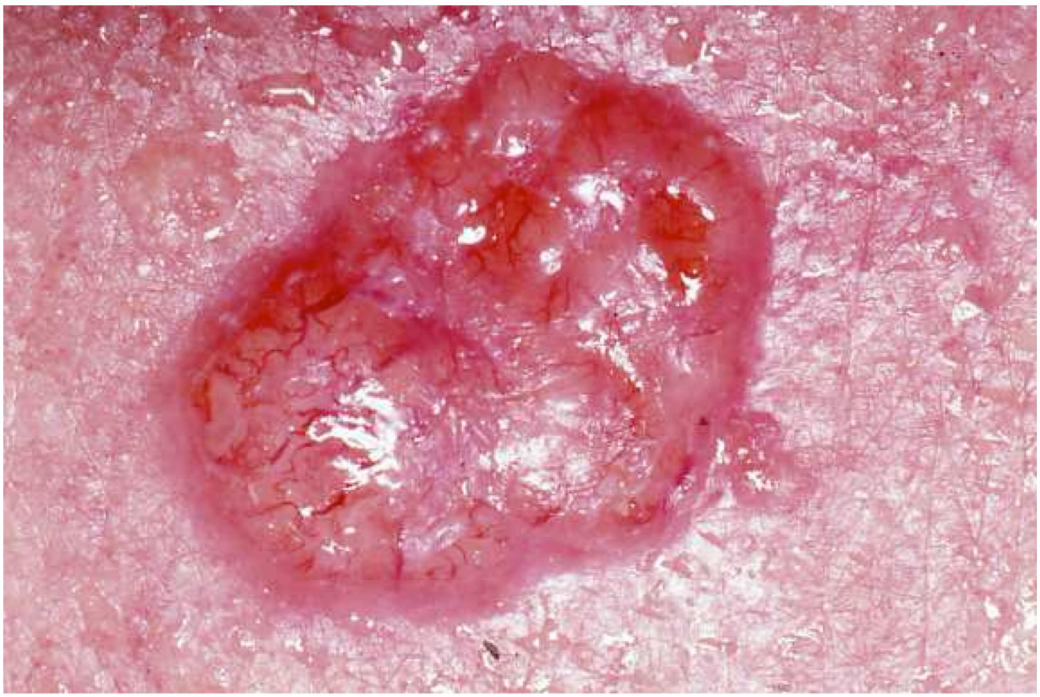

Figure 8. A picture of a BCC on a human back (John Hendrix, MD).

Almost all BCCs show evidence of HH pathway activation, with approximately 90% of the tumors associated with inactivating mutations of the *PTCH1* gene, often in combination with loss of heterozygosity (Epstein, 2008; Gailani et al., 1996; Kim et al., 2002). However, mutations in other components of the HH pathway have also been detected, for example, gain-of-function mutations in *SMO* that may account for the remaining 10% of sporadic BCCs, and inactivating mutations of *SUFU*, although these are quite rare (Epstein, 2008; Ng and Curran, 2011; Reifenberger et al., 2005). All of these mutations result in overactive HH signaling, and thus it is believed that overactive HH signaling is a prerequisite for BCC development. In terms of molecular events, it is known that the tumor suppressor gene, *TP53*, is also mutated in skin cancers. *TP53* mutations are present in >50% of human BCCs and 70% of these are likely to be UV-induced and often coincide with *PTCH1* mutations.

The cells that constitute a BCC histologically resemble the basal cells of hair follicles, sebaceous glands and the IFE. BCCs also express cytokeratins similar to

those normally expressed in these regions (Donovan, 2009). In normal adult skin, HH signaling is limited to the follicles (Dahmane et al., 1997; Oro et al., 1997), which is why it has been suggested that BCCs may originate from multipotent stem/progenitor cells derived from the outer root sheet, or bulge, of the hair follicle. Several studies support this hypothesis; although recently, two separate investigations, using transgenic mice, have come up with contradictory results. In one study, SMO was constitutively activated in different compartments of the skin, including the hair follicle bulge stem cells (Youssef et al., 2010). The authors found that BCCs in this mouse model did not develop from the hair follicle stem cells but from long-term resident progenitors of the IFE and upper infundibulum. In the second study, BCCs were induced by X-ray irradiation of *Ptch*$^{+/-}$ mice that also had conditional loss of *Trp53* (Wang et al., 2011). This treatment augmented the level of BCC carcinogenesis, particularly from the hair follicle bulge, but also from the IFE, which was explained by enhanced SMO expression in the IFE. One possible reason for the discrepancy between these two studies is that Ptch1 and SMO may have functions that are separate from the canonical HH pathway, and these functions may influence tumorigenesis. Ptch1, for example, has been shown to inhibit cell cycle progression by sequestering cyclin B1 in the cytoplasm (Barnes et al., 2001). Consistent with this theory, strong nuclear localization of cyclin B1 was observed in BCCs from *Ptch1*$^{+/-}$ mice, but not in mice expressing activated SMO (Wang et al., 2011). These data indicate that activation of the HH pathway through loss of Ptch1, or overactivation of Smo, is mechanistically distinct. Recent data from our lab confirmed that BCCs could originate from both the hair follicle bulge and the IFE in mice (Kasper et al., 2011).

1.3.6.1.3 BASALOID FOLLICULAR HAMARTOMA

Basaloid follicular hamartoma (BFH) is a rare cutaneous neoplasm, associated with hair follicles, that closely resembles BCC (Saxena et al., 2007). However, while BCC is a low-grade malignancy that can ulcerate and destroy local tissue, BFH does not and is considered benign. Unfortunately, it is difficult to differentiate between the two types of tumors, both clinically and histologically. BFH and BCC are generally presented histologically as epithelial proliferations of basal cells, although BFH is only seen where normal hair follicles are present, and does not involve the IFE or deeper reticular dermis (Requena et al., 1999). Immunohistochemical staining for proliferation markers, such as PCNA (co-factor for DNA polymerase δ) and Ki67 (associated with mitosis), may be helpful in differentiating between the lesions since they tend to stain less in BFH than in BCC, reflecting the lack of progressive growth in BFH (Jih et al., 2003; Naeyaert et al., 2001).

HH target genes are only modestly upregulated in mouse and human BFHs, which is in stark contrast to the high levels detected in BCCs (Grachtchouk et al., 2003). In BCCs, high levels of the G1 cyclins D1 and D2 are also detected. Possibly, the level of HH pathway activation and G1 cyclins are key determinants of skin tumor phenotype.

BFH is considered benign, but its premalignant potential has not yet been determined (Saxena et al., 2007; Yoshida et al., 2003). BCCs have been reported to arise within a BFH, and patients with BFH have an increased risk of developing BCC. Therefore, it has been suggested that BFH and BCC are the same entity, but this is still under debate. Clearly, it is important to be able to identify and distinguish between malignant and benign lesions in order to treat patients appropriately and to spare them unnecessary surgery or costly and stressful therapies.

1.3.6.2 BRAIN TUMORS

In 1987, a new gene was found to be amplified more than 50-fold in brain tumors arising from glial cells, known as malignant gliomas (Kinzler et al., 1987). The gene was named *GLI* after the tumor, and was thought to be an oncogene. Later studies revealed that gliomas or glioblastomas very seldom harbor *GLI* amplifications (Bigner et al., 1988; Mao and Hamoudi, 2000), but the HH pathway may still be important in the maintenance of these tumors since the tumor cells are responsive to HH ligands secreted by the tumor stroma (Becher et al., 2008; Clement et al., 2007; Ehtesham et al., 2007).

1.3.6.2.1 MEDULLOBLASTOMA

The first suggestion that *SUFU* might be a tumor suppressor gene occurred when *SUFU* mutations were found in a group of children with medulloblastoma (MB) (Taylor et al., 2002). The patients harbored germline truncating or missense mutations in *SUFU* in combination with LOH. In one case an interstitial deletion that included the *SUFU* locus was found on chromosome 10q24. The child suffered from abnormal facial features and cognitive delay, in addition to MB. This indicated that *SUFU*, like *PTCH1*, also acts as a classical tumor suppressor gene.

MB is the most common malignant brain tumor in children affecting approximately 1 in 200,000, and it accounts for 15 to 20% of all pediatric brain tumors (Ellison, 2002; Gajjar et al., 2004; Gilbertson and Ellison, 2008). Despite improved survival rates in recent years, this disease is still incurable in one-third of patients, while current treatments cause considerable damage to survivors. About 30% of MBs exhibit HH pathway activation, and 50% of these are associated with *PTCH1* or *SUFU* loss-of-function mutations, or with *SMO* gain-of-function mutations (Kool et al., 2008; Northcott et al., 2009; Thompson et al., 2006). However, interestingly, the remaining 50% exhibit HH activation without evidence of mutations in these genes. The incidence of MB in GS is approximately 5%, with onset within the first two years of life (Cowan et al., 1997) compared to seven to eight years of age in the general population. Other genes are also involved in MB, including *TP53* (Cogen and McDonald, 1996), *APC* (Huang et al., 2000), *PTEN* (Rasheed et al., 1997), *CTNNB1* (Eberhart et al., 2000; Zurawel et al., 1998) and *MYC* (Brown et al., 2000).

MB has long been thought of as a neuroepithelial tumor that develops in the posterior fossa of the cerebellum, although MB includes a range of distinct brain tumor subtypes. The 2007 World Health Organization (WHO) classification of CNS tumors is based on the histological appearance of MB tumors, and lists the tumors in four subtypes (Gilbertson and Ellison, 2008): 1) the classic MB subtype is composed of small, uniform cells with a high nuclear-to-cytoplasmic ratio; 2) the nodular/desmoplastic subtype consists of nodules with differentiated, slow growing neurocytic cells surrounded by desmoplastic, more proliferative cells; 3) pleomorphic cells with a polyhedral shape and high growth fraction characterize the third anaplastic MB subtype; 4) the large-cell MB, which consists of large, uniform cells with vesicular nuclei.

In addition to the histopathologic features of MB, there are also cytogenic and molecular factors to consider when optimizing treatment and prognosis (Cho et al., 2011; Monje et al., 2011; Northcott et al., 2011). There are at least four different molecular subtypes, and the subtype with the most favorable prognosis is associated with *CTNNB1* mutations and activated WNT signaling. In general, these tumors have a classic histology and can occur in all age groups. The HH pathway active subgroup has some heterogeneity in age, occurring primarily in young infants and adults, and these tumors have a characteristic nodular/desmoplastic histology, and a good prognosis. A

poor prognosis is associated with *c-MYC*-amplifications in the third molecular subtype, and these tumors often have a large-cell/anaplastic histology, primarily affecting children aged three to ten years. The remaining subtype seems to occur in all age groups, has a fair prognosis and is distinguished from the others by not expressing *MYC*. Elevated *MYC* expression is seen in the WNT and HH subgroups (Northcott et al., 2011), probably due to the fact that *MYC* is a target gene of both WNT and HH signaling; however, the level of *MYC* expression in these subtypes and its effect on tumor biology is still unclear.

The cerebellum starts to develop around embryonic day 10 in the mouse (Gilbertson and Ellison, 2008), when it originates from two germinal zones within the rhombic lip. Granule neuron precursor cells (GNPCs) come from the second germinal zone, and migrate rostrally to form the external germinal layer (EGL). Under normal conditions the EGL persists until post-natal day 15 in mice, and the second post-natal year in humans. It is a structure that harbors actively proliferating progenitor cells, which, under tight regulation, become postmitotic, differentiate and migrate inward to form the mature granule neurons of the internal granule layer (IGL). When the GNPCs move inward, leaving the EGL to form the IGL, they migrate past Shh-secreting Purkinje cells. During this migration, the Ptch1-expressing GNPCs proliferate in response to Shh (Dahmane and Ruiz i Altaba, 1999; Lewis et al., 2004), and mouse models have shown that the HH-related MB subtype arises from GNPCs (Schuller et al., 2008; Yang et al., 2008). Although activation of the HH pathway in neural stem cells also results in MB formation, the stem cells must first make the transition to committed granule neuron progenitors. Recently, it was shown that the WNT MB subtype exhibits a distinct pattern of gene expression and actually arises in the brain stem where the HH pathway is inactive (Gibson et al., 2010). Interestingly, some MBs harbor both HH and WNT pathway mutations simultaneously, which may indicate some overlap among the cells of origin (Parsons et al., 2011).

1.3.6.3 OTHER HEDGEHOG-RELATED TUMORS

The HH pathway is also involved in the development of muscle tissues (Brand-Saberi, 2005; Bryson-Richardson and Currie, 2008). Although HH is normally turned off in adult musculature, SHH is reactivated during muscle tissue regeneration, for example, after injury (Straface et al., 2009). Under such circumstances, SHH exerts a regulatory function in committed adult muscle progenitor cells by inducing proliferation and preventing differentiation (Collins et al., 2005; Koleva et al., 2005). The mesenchymal tumor, rhabdomyosarcoma (RMS), is believed to develop as a consequence of mutations in muscle progenitor cells, and is characterized by immature skeletal muscle differentiation. There are two variants of RMS: an embryonal and an adult type, both of which have been more or less associated with aberrant HH signaling. Of the reported embryonal RMS tumors, 33% have LOH in *PTCH1* (Bridge et al., 2000), 18% have LOH in *SUFU* (Bridge et al., 2002) and 49% have GLI1 gain-of-function mutations, although in most cases, these aberrations are also associated with either p53 or retinoblastoma (RB) pathway signatures (Rubin et al., 2011). There are reports of RMS in GS patients (Gorlin, 2004; Tostar et al., 2006), and the *Ptch1*$^{+/-}$ mouse model for GS showed an increased incidence of RMS (Hahn et al., 2004), though this was strongly influenced by the genetic background of the mouse strain.

1.3.6.4 ROUTES OF HEDGEHOG SIGNALING IN CANCER

In principle, in HH-related cancers, there are three modes of HH signaling or pathway activation, which exploit distinct oncogenic functions of the HH pathway (Rubin and de Sauvage, 2006; Scales and de Sauvage, 2009).

1) Ligand-independent, cell-intrinsic signaling. Tumors with this kind of pathway activation have genetic alterations downstream of the HH ligand. This includes all *PTCH1* mutations, and thus involves GS and the majority of sporadic BCCs and MBs. Other mutations in this category include *SMO* activating mutations and loss-of-function mutations in *SUFU*.
2) Ligand-dependent, autocrine or juxtacrine signaling, meaning that tumor cells both produce and respond to the same HH ligands. Tumors with this type of pathway activation have no identified somatic mutations in HH pathway components; rather, they demonstrate elevated HH ligand expression and/or ectopic PTCH and GLI expression in the tumor compartment.
3) Ligand-dependent, paracrine signaling. This mode of (long-range) action, when epithelial cells secrete HH ligands that interact with adjacent mesenchymal/stromal cells, is common during normal development and is important for proper patterning (Hooper and Scott, 2005; Ingham and McMahon, 2001; Ingham and Placzek, 2006). In a tumor situation, HH pathway activation is found in the surrounding stroma and not in the tumor itself (Fan et al., 2004; Tian et al., 2009; Yauch et al., 2008). The HH ligands produced by the tumor cells are received by the stroma, which signals back to the tumor, creating a favorable microenvironment for the tumor by supplying soluble growth and survival factors (Theunissen and de Sauvage, 2009).

Recently, a reversed variant of the third type of HH signaling was recognized (Theunissen and de Sauvage, 2009). In this reverse paracrine signaling mode, HH is secreted from the stroma and is received by the tumor cells, providing the appropriate microenvironment for tumor growth. So far, this type of signaling has only been observed in hematological malignancies, where stroma-secreted HH seems to be essential for cancerous B-cell survival via the upregulation of *BCL2* (Dierks et al., 2008; Hegde et al., 2008; Scales and de Sauvage, 2009).

1.3.6.5 TREATMENT OF HEDGEHOG-RELATED CANCER

An estimated 25% of all human tumors may depend on growth activation by the HH pathway (Lum and Beachy, 2004). As a consequence, disruption of the pathway may be of great therapeutic importance (Ng and Curran, 2011), and several early stage clinical trials of inhibitors that block both intrinsic signaling in the tumor, and extrinsic signaling to stromal cells, have shown reduced tumor growth. Since all canonical HH signaling requires SMO, small molecule inhibitors of SMO, such as cyclopamine, completely block HH pathway signaling, regardless of the ligand, and provide valuable tools both for HH signaling research and for the development of targeted cancer therapies. In fact, all HH pathway therapeutics in current clinical trials function by inhibiting SMO. Unfortunately, several naturally occurring SMO inhibitors, such as cyclopamine, have poor solubility, low potency, rapid clearance, are unstable and display nonspecific toxicity (Lipinski et al., 2008). However, a range of novel compounds that inhibit SMO and block its signal have been identified during the last decade (Mas and Ruiz i Altaba, 2010), and many of these compounds have structures distinct from that of cyclopamine. Promising preclinical data has come from studies using the benzimidazole, HhAntag, as a SMO inhibitor to treat MB in *Ptch1*$^{+/-}$*;Trp53*$^{-/-}$ mice (Romer et al., 2004). Oral delivery of the drug eliminated a large, spontaneous MB in this mouse model. However, HhAntag was shown to cause growth defects in bone when given to mice during the first 10 days of postnatal development (Kimura et al., 2008).

The most widely clinically tested SMO inhibitor is Vismodegib (GDC-0449), which was developed by Curis and Genentech (Graham et al., 2011; LoRusso et al., 2011; Robarge et al., 2009). Recently, positive results from a phase II trial with Vismodegib in patients with advanced BCCs were reported from Genentech. The first

phase I trial of this inhibitor revealed that 19 out of 33 patients with BCC showed a good response, and 50% of the patients with metastatic BCC also responded well (LoRusso et al., 2011; Von Hoff et al., 2009). In addition, one adult patient with metastatic MB in the trial showed a dramatic, although transient, response to Vismodegib (Rudin et al., 2009). Unfortunately, the patient relapsed due to spontaneous mutations in the drug-binding site of SMO. This relapse reflects a problem with previous chemotherapy and/or radiation therapy of patients with advanced disease, as these treatments are highly mutagenic, and increase the likelihood of the patient developing resistance to subsequent targeted therapies.

A general drawback with SMO inhibitors is that presumably they are ineffective against ligand-independent tumors with mutations downstream of SMO. Specific targeting of GLI would have wider applications, and several groups, including our own (Lauth et al., 2007), are attempting to develop such drugs. Thus, depending on the route of HH signaling that is involved in a particular tumor situation, different kinds of treatment have to be considered. For type two signaling, tumor growth may be suppressed either by SMO antagonists or pathway inhibitors such as HH neutralizing antibodies (Gupta et al., 2010). In case of ligand-dependent, paracrine activation of tumor stroma, xenograft mouse models have shown that treatment with SMO inhibitors can slow tumor growth (Yauch et al., 2008). In another mouse model of pancreatic cancer, inhibition of Smo enhanced the response to chemotherapy by depleting tumor-associated stromal tissue (Olive et al., 2009).

1.4 THE MOUSE AS A MODEL FOR HUMAN DISEASE

Much of what we know about the HH signaling pathway today comes from studies in model organisms, such as the fruit fly and mouse. Mouse studies have helped us to understand the biology of different human conditions, ranging from developmental disorders to cancer. Various mouse models carrying loss-of-function mutations, or transgene overactivation modifications in components of the Hh pathway are listed in Table 2.

The *Ptch1* heterozygous mouse model produces a phenotype equivalent to human GS, and shows many of the characteristic features of the syndrome, including MB, RMS and developmental defects (Goodrich et al., 1997; Hahn et al., 1998). Interestingly, $Ptch1^{+/-}$ mice also develop skin proliferations, but these are more similar to BFHs than BCCs (Aszterbaum et al., 1999). These mice only develop full-blown BCC lesions resembling those in humans following ionizing or UV radiation.

Lesions strikingly similar to human BFHs also develop in mice expressing mutated human SMO (SMO-M2), which is largely resistant to inhibition by PTCH1, in the K5- (basal cell) compartment of the epidermis (*K5-SMO-M2*) (Grachtchouk et al., 2003; Xie et al., 1998). Full-blown BCCs do not develop in this mouse model either, and investigation of target gene transcription levels indicated that the Hh pathway is only modestly upregulated in BFHs. Interestingly, overexpression of human *GLI1* or mouse *Gli2* under control of the K5 promoter in mouse results in rapidly growing BCC-like tumors. This indicates that a threshold level of Hh pathway activation is required for the development of BCCs. Heterozygous loss of *Sufu* also results in BFH formation, although this is five times more frequent than in $Ptch1^{+/-}$ mice (Svard et al., 2006; Svard et al., 2009). Compound $Sufu^{+/-};Ptch1^{+/-}$ mice exhibit a further increase in BFHs, but still do not develop BCCs. Thus, the level of Hh pathway activation seems to be an important determinant for tumor development, and may represent the criterion for differentiating between BFH and BCC.

As mentioned previously, mutations in *TP53* are quite common in human BCCs and often coexist with mutations in *PTCH1* (Ling et al., 2001; Reifenberger et

al., 2005). It has been shown that the tumor suppressor, p53, can inhibit the activity, reduce the levels and prevent nuclear localization of GLI1, and in turn GLI1 represses p53 (Stecca and Ruiz i Altaba, 2009). In this way a GLI1-p53 inhibitory loop is established, which may be important in tumorigenesis when p53 is lost, leading to enhanced GLI1 activity. It is possible that the loss of p53 may be an important determinant for BFH and BCC development, and indeed, loss of *Trp53* enhances tumor development in mice with heterozygous loss of either *Ptch1* or *Sufu* (Heby-Henricson et al., 2011; Lee et al., 2007; Wetmore et al., 2001). These mice develop MB and RMS, but surprisingly, show no sign of aggravation of the BFH skin phenotype, most likely due to the fact that these mice have to be sacrificed because of other more severe tumors, before any change in the skin can be noticed.

Table 2. A selection of mouse models for major Hh pathway components.

Gene	Mouse model	Defect	Reference
Shh	*Shh*$^{-/-}$	Lethal	(Chiang et al., 1996)
	K14-Shh	BCC	(Oro et al., 1997)
Ihh	*Ihh*$^{-/-}$	Lethal	(St-Jacques et al., 1999)
Dhh	*Dhh*$^{-/-}$	Male infertility	(Bitgood et al., 1996)
Hhip	*Hhip*$^{-/-}$	Lethal	(Chuang and McMahon, 1999)
	Hhip$^{+/-}$	RMS	(Gerber et al., 2007)
Disp	*Disp*$^{-/-}$	Lethal	(Kawakami et al., 2002)
Ptch1	*Ptch1*$^{-/-}$	Lethal	(Goodrich et al., 1997; Hahn et al., 1998)
	Ptch$^{+/-}$	BFH, MB, RMS	(Goodrich et al., 1997; Hahn et al., 1998)
	Ptch$^{+/-}$*;Trp53*$^{-/-}$	MB, RMS	(Lee et al., 2007; Wetmore et al., 2001)
	Ptch1$^{fl/fl}$*;K14-Cre*	BCC	(Siggins et al., 2009)
	Ptch1$^{+/-}$*;Ptch2*$^{-/-}$	MB	(Lee et al., 2006)
Ptch2	*Ptch2*$^{-/-}$	Viable	(Lee et al., 2006; Nieuwenhuis et al., 2006)
Smo	*Smo*$^{-/-}$	Lethal	(Zhang et al., 2001)
	K5-SMO-M2	BFH	(Grachtchouk et al., 2003; Xie et al., 1998)
Kif7	*Kif7*$^{-/-}$	Lethal	(Cheung et al., 2009; Endoh-Yamagami et al., 2009)
Fu	*Fu*$^{-/-}$	Lethal	(Chen et al., 2005; Merchant et al., 2005)
Sufu	*Sufu*$^{-/-}$	Lethal	(Cooper et al., 2005; Svard et al., 2006) **PAPER I**
	Sufu$^{+/-}$	BFH	(Svard et al., 2006) **PAPER I**
	Sufu$^{+/-}$*;Trp53*$^{-/-}$	BFH, MB, RMS	(Heby-Henricson et al., 2011; Lee et al., 2007) **PAPER II**
	Sufu$^{+/-}$*;Trp53*$^{+/-}$	BFH	(Heby-Henricson et al., 2011) **PAPER II**
	Sufu$^{+/-}$*;Ptch1*$^{+/-}$	BFH, MB, RMS	(Svard et al., 2009)
Gli1	*Gli1*$^{-/-}$	Viable	(Park et al., 2000)
	K5-GLI1	BCC	(Nilsson et al., 2000)
Gli2	*Gli2*$^{-/-}$	Lethal	(Motoyama et al., 1998b)
	K5-Gli2	BCC	(Grachtchouk et al., 2000)
Gli3	*Gli3*$^{-/-}$	Lethal	(Motoyama et al., 1998b)

Note: BCC, basal cell carcinoma; BFH, basaloid follicular hamartoma;
MB, medulloblastoma; RMS, rhabdomyosarcoma.

In summay, the Hh pathway has numerous implications in development and tumorigenesis. In this thesis I have used the mouse as a model to study the function of Sufu within the Hh pathway, during embryonic development and tissue differentiation, as well as in skin tumorigenesis.

2 MATERIALS AND METHODOLOGY

The materials and methods employed in the various studies undertaken are described in each of the individual papers. The aim of this section is to explain specific methodological issues and to give a more detailed description of some central methods and techniques.

2.1 WHOLE MOUNT *IN SITU* HYBRIDIZATION

In **PAPER I**, whole mount *in situ* hybridization was used to investigate the expression of certain Hh pathway components. Embryos were dissected at E8.5 and E9.5 in cold phosphate buffered saline (PBS), and the yolk sac was removed and used for PCR genotyping. Embryos were fixed in freshly prepared 4% paraformaldehyde (PFA) (Sigma) in PBS on ice for one to two hours. Thereafter the embryos were dehydrated by passing through a graded methanol series (25%, 50%, 75%) and stored at -20°C in 100% methanol.

Non-radioactive riboprobes (antisense and sense) were made from 1 µg linearized and purified cDNA plasmid templates together with DIG RNA labeling mix (Roche), SUPERaseIN RNase inhibitor (Ambion) and RNA polymerase T3, T7 or SP6 (all from Roche).

Embryos of the same age and genotype were pooled and rehydrated in the reverse graded methanol series mentioned above. Bleaching of embryos was performed in 6% hydrogen peroxide, followed by proteinase K (10 µg/ml, Roche) treatment. Embryos were re-fixed in 0.2% glutaraldehyde/4% PFA (both from Sigma) and afterwards incubated in hybridization solution [50% formamide (Invitrogen), 1.3X SSC, pH 5.0, 5 mM EDTA, 50 µg/ml yeast RNA type III (Sigma), 0.2% Tween-20 (Sigma), 0.5% CHAPS (Sigma), 100 µg/ml heparin (Sigma) in DEPC-H_2O] at 70°C for one to three hours with gentle rocking in a hybridization oven. Hybridization with 1 µg/ml DIG-labelled riboprobe (DIG RNA labeling mix, Roche) was performed overnight at 70°C. Subsequent washing was performed in pre-warmed hybridization solution, whereafter the embryos were transferred to RNase buffer (0.5 M NaCl, 10 mM Tris-HCl, pH 7.5, 0.1% Tween-20 in DEPC-H_2O) containing 100 µg/ml RNase A (Qiagen) for 30 minutes at 37°C. After RNase A treatment, embryos were washed in washing solution (50% formamide, 2X SSC, 1% SDS in DEPC-H_2O) at 65°C, and rinsed in MAB-T solution (150 mM NaCl, 100 mM maleic acid, 0.5% Tween-20 in DEPC-H_2O). Pre-blocking was performed for one to two hours in blocking solution [150 mM NaCl, 100 mM maleic acid, 10% sheep serum, 0.1 g/ml Roche blocking reagent, 2 mM levamisole (Sigma)], which was subsequently replaced by blocking solution containing anti-DIG AP-conjugated antibody (diluted 1:2000), and incubated overnight at 4°C. Thorough washing of embryos was performed in MAB-T solution overnight at 4°C. The MAB-T solution was removed and NTMT (100 mM NaCl, 100 mM Tris-HCl, pH 9.5, 50 mM $MgCl_2$, 0.1% Tween-20 in DEPC-H_2O) containing 2 mM levamisole was added. This solution was replaced by NBT/BCIP reaction mix [4.5 µl NBT (75 mg/ml in 70% dimethylformamide) and 3.5 µl BCIP (50 mg/ml in 100% dimethylformamide) both from Roche, per 1 ml NTMT containing levamisole]. When the reaction was complete, embryos were re-fixed in 0.1% glutaraldehyde/4% PFA. To enhance visualization of the staining, embryos were 'cleared' in 80% glycerol/PBS.

2.2 DERIVATION OF MOUSE EMBRYONIC STEM CELLS

For the derivation of mouse embryonic stem cells (ESCs) used in the studies in **PAPER III**, we combined and modified two previously published protocols (Bryja et al., 2006; Meissner et al., 2009). Time-mated *Sufu*$^{+/-}$ females were sacrificed at E3.5 by cervical dislocation. Uteri were dissected and blastocysts were flushed from the uterine horns in Dulbecco's Modified Eagle's Medium (DMEM) containing 10 mM HEPES buffer solution (both from GIBCO, Invitrogen AB) under a dissection microscope. Blastocysts were collected and transferred separately to wells containing a mitotically inactivated feeder layer of mouse embryonic fibroblasts (MEFs) in KSRES consisting of KnockOut-DMEM (K-DMEM), 20% KnockOut-Serum Replacement (K-SR), 2 mM L-glutamine, 0.1 mM nonessential amino acids, 0.1 mM 2-Mercaptoethanol, 10 μg/ml gentamicin (all from GIBCO, Invitrogen AB), and 1,000 U/ml recombinant leukemia inhibitory factor [LIF (ESGRO), Millipore], supplemented with 50 μM of the MEK1 inhibitor PD98059 (GIBCO, Invitrogen AB) (Meissner et al., 2009) and incubated at 37°C in 6.8%, CO_2 and high humidity. Blastocysts were allowed to attach to the feeders and hatch spontaneously (Figure 1). After five to six days, the ICMs, containing the ESCs, were picked under a microscope and trypsinized in TrypLE Express (GIBCO, Invitrogen AB) for five minutes at 37°C. The ICMs were dissociated mechanically, with extreme care, into individual cells and small cell clumps under a microscope. The cell suspensions were transferred to wells containing MEFs and KSRES+PD98059, and after several days typical ESC colonies (Figure 1) could be detected in 40% of the wells. ESCs were maintained on a single layer of mitotically inactivated MEFs in KSRES medium. During ESC derivation, and up to the second passage, KSRES medium was supplemented with PD98059. After the second split, growth medium was changed to KSRES without the inhibitor. ESCs were passaged as required, approximately after two to three days in culture. KSRES was changed daily. MEFs were isolated as previously described (Todaro and Green, 1963) and mitotically inactivated either by 30 Gy γ-irradiation before seeding, or by treatment with 10 μg/ml mitomycin-C (Sigma) for two to six hours after seeding.

2.3 GENE TARGETING

In **PAPER I** and in the **PRELIMINARY STUDY**, *Sufu* knock-out mice were generated by gene targeting *in vivo*. Gene targeting is defined as the introduction of site-specific modifications into the genome by homologous recombination, and is generally used for the production of mutant animals to study gene function *in vivo* (Torres, 1997). Replacement vectors are most frequently used as the substrate for homologous recombination, for gene targeting in ESCs. The replacement vector typically consists of two regions of DNA (a short and a long arm), 4 to 10 kb in total that are homologous to the genomic target locus. The two DNA regions are interrupted by a positive selection marker gene, for example, bacterial aminoglycoside phosphotransferase (neo), which is selected for by G418. As a negative selection marker, the thymidine kinase (tk) gene from the herpes simplex virus (HSV) is often used, which can be selected against using gancyclovir. This will select against ESC clones that have integrated the targeting vector at random.

Cre (causes recombination) recombinase originates from the P1 bacteriophage and recognizes and mediates site-specific recombination between loxP (locus of crossover (x) in P1) sites. The loxP sequence consists of two 13 bp inverted repeats interrupted by an 8 bp nonpalindromic sequence, which settles the orientation of the sequence. The Cre-mediated intramolecular recombination occurs when two loxP sites are placed in the same orientation on a linear DNA molecule, and the

sequence in-between the loxP sites (the floxed sequence) is excised. Cre/loxP-based gene targeting allows the introduction of subtle mutations into target loci and also the creation of large deletions resulting in conventional gene inactivation, leaving a single 34 bp loxP site in the target locus after Cre recombination.

Conditional gene targeting in mice can be defined as a gene modification that is restricted to certain cell types or developmental stages. A conditional mutant is generated by crossing a strain harboring a loxP-flanked segment of a target gene, with another strain expressing Cre recombinase under the control of a tissue-specific promoter. The modification is then restricted to the pattern of Cre expression in that particular Cre transgenic strain. Conditional gene inactivation is especially useful if lethality is anticipated in conventional mutants.

The Flp/frt system is based on the same principles as the Cre/loxP system. Just like Cre, the flipase (Flp) recombinase mediates a recombination event between two copies of the frt (Flp-recognition target) sequence. Any sequence between the frt sites will be deleted.

The targeting construct is transfected via electroporation into ESCs from a 129/SvJ mouse strain (agouti coat color). ESC clones are selected for neo resistance and screened for by DNA analysis using Southern blotting and PCR. Correctly targeted clones are injected into blastocysts from the C57Bl/6 mouse strain (black coat color). Then the blastocysts are transferred into pseudopregnant females that will give birth to chimeras composed of targeted cells and C57Bl/6 cells. If the sperm or eggs contain the targeted mutation, germline transmission will occur when the chimeras are backcrossed to C57Bl/6 mice. This results in offspring heterozygous for the targeted mutation. These mice should be backcrossed to C57Bl/6 for five to ten generations to get a congenic strain. Thereafter, they can be intercrossed to generate homozygous offspring. In the case of conditional gene targeting, at this stage, these mice can be crossed to Cre transgenic mice to produce the conditional knock-out mice.

In **PAPER I**, conventional *Sufu* knock-out mice were generated by replacing exon 1 in the *Sufu* gene with a neo cassette. In the **PRELIMINARY STUDY**, conditional *Sufu* knock-out mice were produced by flanking exons four to six with loxP sites.

3 AIMS OF THE THESIS

The hedgehog signaling pathway is associated with several human diseases when dysregulated; however, the individual components of the pathway, its normal functioning and its connection to these diseases, especially cancer, have to be investigated further. The general aim of this study was to investigate the role of *Sufu*, an important negative regulator of the Hh signaling pathway, in mice during embryonic development, tissue differentiation and tumorigenesis.

Specific aims:

PAPER I
To examine the role of *Sufu* in mammals by developing *Sufu* knock-out mice, and to characterize the *Sufu* loss-of-function phenotype.

PAPER II
To determine whether the $Sufu^{+/-}$ mouse skin phenotype could be aggravated upon the simultaneous loss of *Trp53*.

PAPER III
To characterize ESCs in *Sufu* loss-of-function conditions and to explore the differentiation capacity of $Sufu^{-/-}$ ESCs.

PRELIMINARY STUDY
This study was performed to enable time- and tissue-specific *Sufu* loss-of-function studies in the mouse, by developing conditional *Sufu* knock-out mice.

4 RESULTS AND DISCUSSION

4.1 PAPER I

Genetic elimination of Suppressor of fused reveals an essential repressor function in the mammalian Hedgehog signaling pathway

In order to study the role of *Sufu* in mammals we decided to create a *Sufu* knock-out mouse by gene targeting in embryonic stem cells. Despite the comparatively insignificant role played by *sufu* in *Drosophila* and zebrafish, studies in our lab had previously indicated that *Sufu* was a potent inhibitor of Hh signaling and Gli nuclear localization in mammalian cellular assays (Kogerman et al., 1999). In PAPER I we showed that loss of *Sufu* caused embryonic lethality, around day 9.5 post coitus, accompanied by severe forebrain defects, extensive apoptotic activity in the neuroepithelium and an open neural tube. In addition, the *Sufu*$^{-/-}$ embryos lacked forelimb buds and branchial arches, the latter possibly causing the hemorrhaging seen in the cephalic region (Figure 9), which may have been due to compromised circulation. The phenotype of the *Sufu*$^{-/-}$ embryos was morphologically very similar to the *Ptch1*$^{-/-}$ embryo phenotype (Goodrich et al., 1997), and both *Sufu*$^{-/-}$ and *Ptch1*$^{-/-}$ embryos were studied by whole mount *in situ* hybridization, which showed a significant increase in *Shh* and *Gli1* expression patterns, and in *Sufu*$^{-/-}$ embryos, *Ptch1* expression also showed a significant increase, compared to wild-type littermates. *Gli2* and *Gli3* were expressed at lower levels in *Sufu*$^{-/-}$ and *Ptch1*$^{-/-}$ embryos compared to wild-type, especially in the open cephalic region.

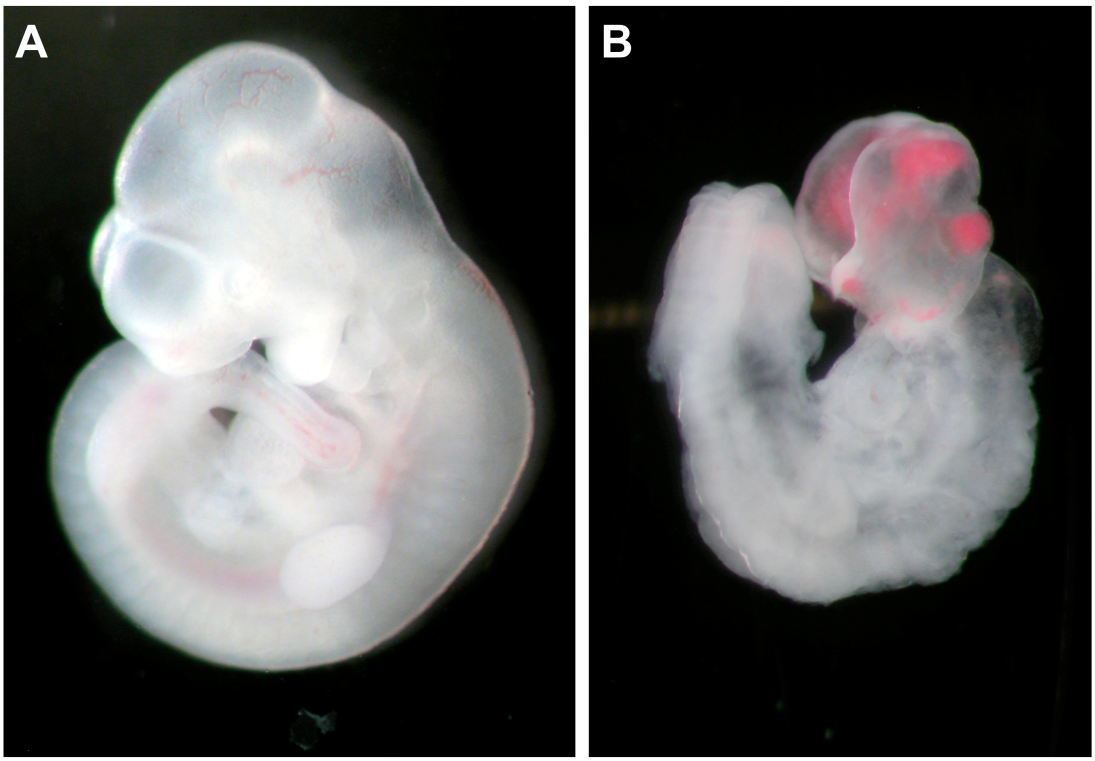

Figure 9. (A) Wild-type and (B) Sufu$^{-/-}$ embryo, both E9.75, showing the open neural tube and hemorrhaging in the cephalic region of the Sufu$^{-/-}$ embryo.

Shh is normally secreted from the notochord and floorplate of the developing neural tube, which creates a gradient that is important for dorso-ventral patterning. Transverse sections of the neural tube from *Sufu*$^{-/-}$ and *Ptch1*$^{-/-}$ embryos demonstrated that most neuronal cells had adopted a ventral fate. For instance, *FoxA2*, which is normally only expressed in the most ventral part of the neural tube, was expressed along the whole dorso-ventral axis. In contrast, the dorsal neuronal marker, *Pax6*, was barely detectable. All markers studied showed a similar pattern of expression in both *Sufu*$^{-/-}$ and *Ptch1*$^{-/-}$ embryos.

For this study, MEFs were also established from *Sufu*$^{-/-}$ embryos to investigate the Hh pathway *in vitro*. *Sufu*$^{-/-}$ MEFs showed increased expression of the target genes, *Ptch1* and *Gli1*, indicating constitutive activation of the pathway. These cells were transfected with a hedgehog responsive reporter construct (8xGliLuc), and relative to wild-type MEFs, *Sufu*$^{-/-}$ MEFs showed a 12- to 15-fold increase in reporter activity. We were able to show that the increase in reporter activity was dependent on functional Gli-binding sites, since a reporter construct with mutated Gli (8xGli^{mut}Luc) did not result in increased reporter activity. Furthermore, the increase in Gli activity could be inhibited by the transient expression of human SUFU, resulting in wild-type levels of reporter activity. This result indicated that the phenotype was Sufu-dependent, and also that human SUFU can substitute functionally for mouse Sufu. In addition, the *Sufu*$^{-/-}$ MEFs were treated with a Smo agonist (SAG) to increase the expression of Gli still further, in order to activate the Hh pathway fully; however, this did not change the signaling outcome. Neither did treatment of *Sufu*$^{-/-}$ MEFs with the Smo inhibitor, cyclopamine. Together, these data indicate that the pathway cannot be activated further or inhibited at the level of Smo when Sufu is absent.

Forskolin is a known activator of PKA, and PKA is an inhibitor of Hh signaling. When *Sufu*$^{-/-}$ and *Ptch1*$^{-/-}$ MEFs [the latter a kind gift from J. Taipale (Taipale et al., 2000)] were treated with forskolin a reduction in Gli reporter levels was detected. This reduction was stronger in *Ptch1*$^{-/-}$ MEFs compared to *Sufu*$^{-/-}$ MEFs, suggesting that the inhibitory effect exerted by PKA targets several different components in the pathway, that may have different effects on Hh signaling.

We also explored the subcellular localization of GLI1 in wild-type, *Sufu*$^{-/-}$ and *Ptch1*$^{-/-}$ MEFs via the transient transfection of an expression plasmid carrying an enhanced green fluorescent protein (EGFP)::GLI1 fusion protein. Since it has been suggested that Sufu plays a role in retaining Gli in the cytoplasm, we expected to see an increase in nuclear EGFP::GLI1 localization in the *Sufu*$^{-/-}$ MEFs. Surprisingly, the EGFP::GLI1 fusion protein was localized predominantly to the cytoplasm, even in *Sufu*$^{-/-}$ MEFs, and was only found in the nucleus upon treatment with the nuclear export inhibitor, Leptomycin B. This led us to suggest that the predominant role of Sufu is to inhibit Gli-dependent transcription in the nucleus as previously described (Cheng and Bishop, 2002; Zhang et al., 1997). More recent data have shown the importance of primary cilia in controlling the Hh signaling output, and that Sufu is located in the ciliary tip together with Gli, where Sufu is believed to promote the formation of Gli repressors and antagonize the activator forms of Gli (Haycraft et al., 2005; Kise et al., 2009). All signaling transmitted via Smo is dependent on primary cilia, but the inhibitory function of Sufu seems to be independent of this organelle (Jia et al., 2009).

In this study we also showed that *Sufu* heterozygous mice, which appeared normal at birth and were born at the expected Mendelian ratio, developed a skin phenotype with 100% penetrance. Macroscopically, the phenotype in older mice (one and a half to two years of age) appeared as ventral alopecia, increased pigmentation, and papules and nodules on the paws and tail. The earliest microscopic skin lesions were seen on the palmo-plantar aspect of the paws at around four to six months of age. These appeared as small basaloid evaginations arising from the basal epidermal cells,

which resembled dermal pits typically found in GS patients. The extent of the skin aberrations increased in older mice, and by two years of age they could be found in all skin areas. The *Sufu*$^{+/-}$ skin phenotype also included aberrant, and sometimes abortive, hair follicle morphology with branching, and hyperplastic sebaceous glands.

Proliferation of the cells within the skin of *Sufu*$^{+/-}$ mice was analyzed by Ki67 immunostaining, which indicated a relatively low number of positive cells. This result was consistent with the slow growth of the lesions, which resembled BFHs and early trichoblastomas. We also immunostained for keratin 5 (K5), which is a marker of epidermal basal cells, and found relatively uniform expression indicative of a basal cell origin for the lesions (Ramirez et al., 1994). Keratin 6 (K6) is a marker that is normally only present in the epidermis of the footpad and in the companion cell layer of hair follicles (Rothnagel et al., 1999). BCCs rarely express K6, although this marker has been associated with hyperproliferations within the IFE. The *Sufu*$^{+/-}$ lesions showed a heterogeneous K6 expression pattern, with weaker staining in the deeper, dermal portions, indicative of a mixed cell population within the lesions. Keratin 17 (K17) is a marker for the outer root sheet of hair follicles, but is occasionally expressed in the footpad epidermis (Panteleyev et al., 1997). K17 seems to be a direct target gene for Hh signaling, since there are Gli-binding sites in the K17 promoter region (Bianchi et al., 2005). This marker was strongly expressed in the *Sufu*$^{+/-}$ lesions, and we suggested that it was indicative of the expansion of primitive hair follicle-associated progenitor cells, which eventually resulted in the disturbed hair follicle architecture seen in these mice. RT-qPCR analysis showed that the skin lesions were associated with increased *Gli1* expression that became stronger in more severe lesions.

As well as the basaloid skin changes and pit-like lesions on the palmar aspects of the paws, the *Sufu*$^{+/-}$ mice also developed jaw keratocysts, all of which are features of GS. Thus, in addition to *Ptch1*$^{+/-}$ mice, the *Sufu*$^{+/-}$ mouse model represents a valuable complementary tool for GS studies.

In summary, in PAPER I, we showed that Sufu is an essential repressor of Hh signaling in mammals. Loss of *Sufu* resulted in ligand-independent activation of the Hh pathway leading to embryonic death in *Sufu*$^{-/-}$ fetuses and GS-like features in *Sufu*$^{+/-}$ mice that included basaloid proliferations of the skin, palmo-plantar pitting and jaw keratocysts. Taken together, our data showed that *Sufu* has gained a new, central, mammalian-specific role in the Hh signaling pathway during evolution. This was highlighted with a PaperPick in the 10-year anniversary issue of Developmental Cell: http://www.cell.com/developmental-cell/abstract/S1534-5807(11)00266-8

4.2 PAPER II

Loss of *Trp53* promotes medulloblastoma development but not skin tumorigenesis in *Sufu* heterozygous mutant mice

This study was performed to determine whether the skin phenotype in *Sufu*$^{+/-}$ mice, described in PAPER I, could be aggravated by the simultaneous loss of *Trp53*. Mutations in the human *TP53* gene are common in sporadic BCCs, where they often co-exist with activating mutations of the HH pathway. In addition, *Trp53* mutations have been shown to enhance HH driven tumorigenesis in *Ptch1*$^{+/-}$ mice (Wetmore et al., 2001).

We were able to show that *Sufu*$^{+/-}$ mice, which normally do not develop MB, developed 57% MB on a *Trp53*$^{-/-}$ background within six months of age, which confirmed previous studies (Lee et al., 2007). Malignant lymphomas associated with the *Trp53* null background, developed in 38% of the *Sufu*$^{+/-}$;*Trp53*$^{-/-}$ mice, and one of these mice (5%) suffered from RMS. In *Ptch1*$^{+/-}$ mice, the MB incidence increases

from 14% over a period of 10 months to >95% prior to 12 weeks of age on a *Trp53* null background (Wetmore et al., 2001). Since MBs are associated with LOH in the *Ptch* or *Sufu* loci, the difference in incidence between the two models may be explained by a more unstable *Ptch1* locus allowing for loss of the second allele. It may also be explained by different functional consequences of *Ptch1* and *Sufu* loss.

Surprisingly, however, the $Sufu^{+/-}$ skin phenotype was not altered in the absence of *Trp53* as long as the mice could be observed. The earliest skin proliferations became visible in histological sections of paw skin taken from both $Sufu^{+/-}$ and $Sufu^{+/-};Trp53^{-/-}$ mice at around two months of age. Statistical analyses showed that there was no difference in the latency or multiplicity of the lesions, and this did not change over time. We also studied the expression of epidermal markers within the paw skin lesions using immunohistochemistry in order to identify potential cellular differences between the $Sufu^{+/-}$, $Sufu^{+/-};Trp53^{+/-}$ and $Sufu^{+/-};Trp53^{-/-}$ skin lesions. Keratin 5 (K5) is a marker for the basal layer of the epidermis, and was found to be evenly expressed in all lesions, independent of genotype, indicating a basal cell origin of the lesions. In wild-type mice, keratin 6 (K6) is only expressed in the epidermis of the paw, and in the companion cell layer of hair follicles. Hyperproliferative cells within the IFE can also express K6, but this marker is rarely upregulated in BCCs. Paw skin lesions from $Sufu^{+/-}$, $Sufu^{+/-};Trp53^{+/-}$ and $Sufu^{+/-};Trp53^{-/-}$ mice had a very similar, heterogeneous K6 expression pattern, indicative of differences in cellular origin within the lesions and less chance that the lesions were BCCs. Keratin 10 (K10), a marker for the more differentiated cells of the suprabasal layer of the epidermis, predominantly stained the inner portions of the lesions, which indicated that differentiation had occurred within the lesions, and that they were similar to BFHs.

The transcription factor, p63, is expressed by transit amplifying cells in hair follicles, where it is important for stem cell maintenance. It is also a marker of the basal cell layer of the IFE, and we demonstrated that p63 was expressed in paw skin lesions from $Sufu^{+/-}$, $Sufu^{+/-};Trp53^{+/-}$ and $Sufu^{+/-};Trp53^{-/-}$ mice, again supporting a similar basal cell origin for the lesions. Finally, the proliferative activity of the lesions was examined using Ki67, a protein that is associated with rRNA transcription and is expressed in all active phases of the cell cycle. Lesions from all genotypes examined showed low Ki67 staining, which was in agreement with their slow growth and BFH-like characteristics.

These results were all interesting findings, since they suggested that the loss of *Trp53* on a $Sufu^{+/-}$ background has a different outcome in different tissues, in this case the cerebellum and the skin. The GNPCs in the EGL of the developing cerebellum are highly proliferative between embryonic day 10 and post natal day 15, whereafter they terminally differentiate and loose their proliferative capacity. GNPCs express Ptch1 and respond to Shh ligand binding, which is a tightly controlled process, and very small changes in the level of Shh signaling may have a drastic effect on the proliferative capacity of these cells. It is possible that the highest mutagenic risk for the cells in the brain occurs during this very narrow time-window, when the GNPCs are proliferating. The brain is naturally protected by its physical position and the blood-brain barrier, against environmental carcinogens such as UV irradiation and chemicals. Hence brain cells may not need the same molecular protection against DNA damage as, for example, cells within the skin. Not only are skin cells constantly exposed to environmental stress, they are also constantly proliferating. It is possible that the cells within the skin have several other mechanisms for protection, beyond the p53 pathway.

$Ptch1^{+/-}$ mice develop BCCs upon irradiation (Aszterbaum et al., 1999; Mancuso et al., 2004), and tumor progression can be enhanced by the simultaneous loss of *Trp53* (Wetmore et al., 2001). It is likely that irradiation would also enhance skin

tumorigenesis in *Sufu*$^{+/-}$ mice, but whether loss of *Trp53* could increase this further, remains to be determined. Conditional loss of *Trp53*, for example, under the control of the K5 or K14 promoter, would enable studies of the skin phenotype over a longer time period, beyond the four to six months the conventional *Sufu*$^{+/-}$*;Trp53*$^{-/-}$ mice are alive. A third alternative would be to transplant the skin from these mice to immunocompromised, nude mice for long-term studies.

In summary, our data demonstrated that the tumorigenic potential in *Sufu* heterozygous mice could be enhanced by the simultaneous loss of *Trp53*, but the cooperativity between the pathways seems to be linked to specific tissues or cell types, proliferative status and developmental stage.

4.3 PAPER III

Loss of *Suppressor of Fused* Restricts the Differentiation Potential of Murine Embryonic Stem Cells

Since *Sufu*$^{-/-}$ embryos have an embryonic lethal phenotype, it was decided to derive ESCs from *Sufu*$^{-/-}$ pre-implantation embryos to characterize further the effects of *Sufu* loss-of-function conditions during differentiation. Both human and mouse pluripotent ESCs express components of the Hh pathway, but the Hh signal activity is low (Maye et al., 2000; Wu et al., 2010; Wu et al., 2011). However, during differentiation into embryoid bodies (EBs) the pathway is upregulated and has a strong influence on neuroectodermal differentiation. The addition of Shh to undifferentiated ESCs in culture does not induce differentiation or affect pluripotency during EB formation. In this study, we showed that *Sufu*$^{-/-}$ ESCs exhibit normal ESC morphology, and can be kept in an undifferentiated state in culture, as confirmed by alkaline phosphatase staining and immunocytochemical analysis of the pluripotency markers SSEA-1, Oct3/4, Sox2 and Nanog. The expression levels of *Oct3/4*, *Sox2* and *Nanog* were also analyzed by RT-qPCR to determine whether loss of *Sufu* had any quantitative effects on these ESC markers. As expected, this analysis indicated very high expression of the markers in all ESC lines compared to expression in irradiated mouse embryonic fibroblasts (MEFs); however, loss of *Sufu* did not alter the expression levels of *Oct3/4*, *Sox2* or *Nanog* compared to wild-type ESCs.

RT-qPCR analysis was also performed to study the level of expression of some of the relevant Hh pathway components. The expression levels of the ligands *Shh*, *Ihh* and *Dhh* were very low, and were not altered significantly between wild-type and *Sufu*$^{-/-}$ ESCs. The expression of *Smo* and the transcription factors, *Gli2* and *Gli3*, was also unchanged. As reported in PAPER I, loss of *Sufu* causes high-level Hh pathway activation, therefore, the expression of *Ptch1* and *Gli1*, which are both target genes in the Hh pathway, and are widely used indicators of active Hh signaling, were also analyzed. Surprisingly, no obvious changes were detected in *Ptch1* expression, and only a moderate two-fold increase in *Gli1* expression was observed between wild-type and *Sufu*$^{-/-}$ ESCs. In contrast, the levels of *Ptch1* and *Gli1* in *Sufu*$^{-/-}$ MEFs, compared to wild-type MEFs, were 70- and 1800-fold higher, respectively. These results indicated that loss of *Sufu* was not sufficient to activate the Hh pathway fully in ESCs. Seemingly, mouse ESCs are not competent to mount a full Hh response, possibly due to the presence of ESC-specific factors that inhibit Hh signaling, and/or the absence of factors that are introduced when the ESCs start to differentiate.

The differentiation capacity of *Sufu*$^{-/-}$ ESCs was analyzed by EB formation *in vitro*. We found that *Sufu*$^{-/-}$ ESCs were able to form EBs, which were very similar to their wild-type counterparts in the early stages. However, after 18 days in suspension, the *Sufu*$^{-/-}$ EBs were clearly smaller than the wild-type EBs, indicating some kind of

growth inhibition. This finding led us to explore the growth and differentiation of the *Sufu*$^{-/-}$ ESCs further using an assay in which the ESCs were injected subcutaneously into immunocompromised nude mice to form teratomas *in vivo*. Tumors developed in 98% of the injected mice, and were mainly solid and well circumscribed.

Wild-type teratomas differentiated as expected, and contained cellular structures that represented the three germ layers. Ectoderm appeared as neuroepithelial rosettes, post mitotic cells and neuropil; mesoderm was represented by cartilage, bone and muscle tissues and endoderm was seen mainly as cystic structures delineated by ciliated cells, sometimes together with Goblet cells, and sometimes directly connected to a partly keratinized, stratified squamous epithelium.

Interestingly, we found that teratomas developing from *Sufu*$^{-/-}$ ESCs had a much more restricted differentiation pattern, and were dominated by neuroectodermal tissues that appeared mainly as rosette structures and neuropil. Endoderm was represented by similar cystic structures as seen in teratomas from wild-type ESCs, but mesoderm was rarely present, and then only as striated muscle fibers. Cartilage and bone tissues were not detected in any teratomas from *Sufu*$^{-/-}$ ESCs. The increased neuronal differentiation seen in teratomas from *Sufu*$^{-/-}$ ESCs relates to the discoveries described in PAPER I; in other words, that loss of *Sufu* enhances differentiation into ventral neurons due to increased Hh signaling. Others have also reported that overexpression of SHH in human ESCs resulted in augmented neuronal differentiation upon EB formation but did not effect endodermal or mesodermal differentiation (Wu et al., 2011). However, Ihh is known to be important for cartilage and bone development (St-Jacques et al., 1999). *Ihh* knockout mice display delayed, abnormal chondrocyte maturation and loss of mature osteoblasts. Recently, a chondrocyte-specific knockout of *Sufu* was shown to result in postnatal death, with pups displaying reduced body length and weight (Hsu et al., 2011). These data are in line with the present study. Since Sufu is a downstream factor for Ihh signal transmission, the absence of Sufu in our ESCs means that Ihh cannot coordinate cartilage and bone formation during teratoma development.

4.4 PRELIMINARY STUDY

Conditional *Sufu* loss-of-function studies

Since conventional *Sufu* knock-out mice are embryonic lethal, we wanted to create conditional *Sufu* knock-out mice in order to study the loss of *Sufu* in specific tissues and at specific time points. The targeting strategy was based on the Cre/loxP technique, and exons four, five and six of the *Sufu* gene were flanked by loxP sites (Figure 10). In addition, the neomycin selection cassette was flanked by frt sites to enable excision by Flp recombinase at a later stage. The construct was transfected into ESCs, and of approximately 900 ESC clones screened by Southern blotting, a single clone was positive for the modification. This clone was injected into blastocysts and transferred to pseudopregnant female mice to generate chimeras. After eight blastocyst injections three low chimeric mice were generated, one male and two females. Together these mice produced approximately 500 pups, but none was found to be heterozygous for the floxed *Sufu* allele.

Our next approach was to inject the positive ESC clone into eight-cell stage embryos, a recently developed method that allows for a higher degree of ESC contribution to the chimera (Poueymirou et al., 2007). In this case, germline transmission was achieved and heterozygously floxed (*Sufu*$^{+/fl}$) mice were crossed to 'Flp deleter' mice to excise the neomycin cassette.

Since our conventional *Sufu*$^{+/-}$ mice develop a skin phenotype with basal cell proliferations, our aim is to delete *Sufu* specifically in that tissue compartment. To this end, a basal cell layer-specific Cre mouse, K5-Cre, will be crossed with our conditional *Sufu* mouse. A breeding program to generate these mice is underway.

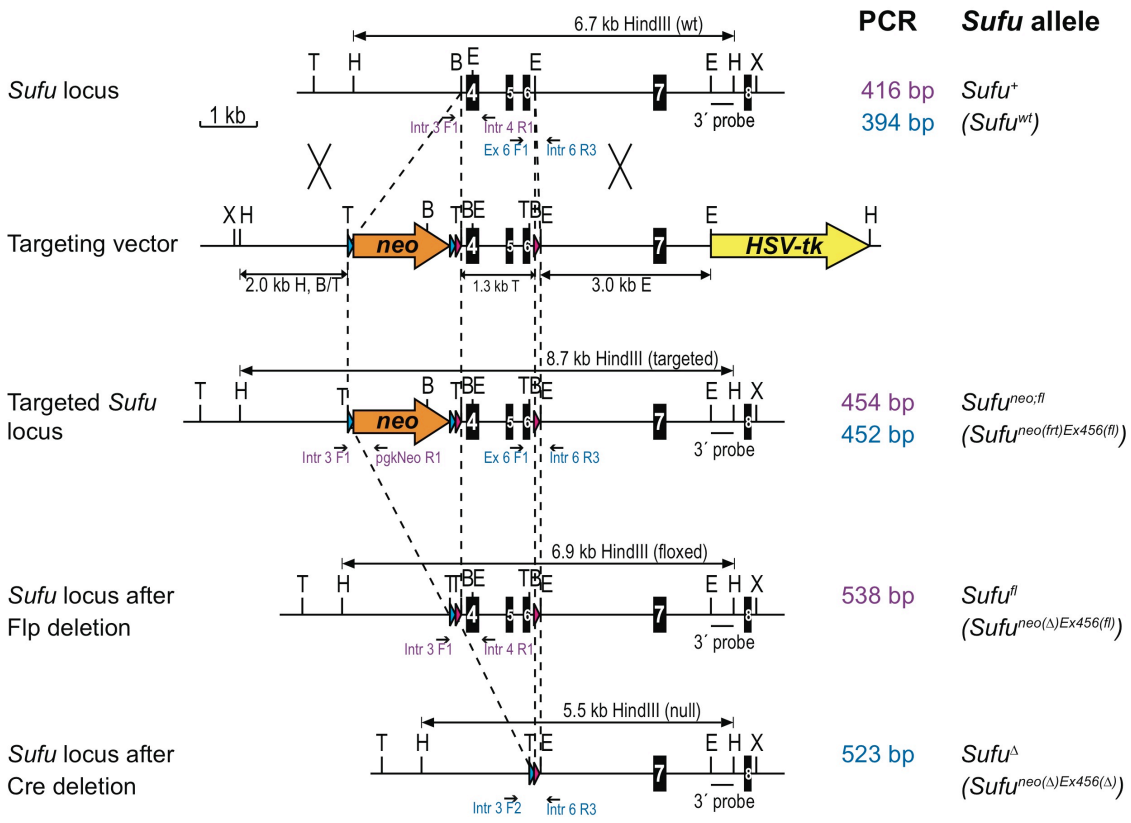

Figure 10. Gene targeting strategy for conditional *Sufu* loss-of-function studies. Exons 4, 5 and 6 are flanked by loxP sites (red arrow heads) to enable site-specific recombination, and excision of the intervening sequence, in the presence of Cre recombinase. A Neo cassette and an HSV-tk cassette were included for positive and negative selection, respectively. To be able to excise Neo *in vivo*, frt recombination sites (blue arrow heads) were introduced on either side of the Neo cassette. In the presence of Flp recombinase, the frt sites recombine, excising the intervening sequence.

5 CONCLUSIONS AND PERSPECTIVES

PAPER I describes a new and essential role for *Sufu* in mammals, which was discovered by knocking out the *Sufu* gene in mouse. This mouse model represents a valuable tool for further studies on the role of *Sufu* during development, tissue differentiation and tumorigenesis. **In PAPER II**, the *Sufu* heterozygous mouse model was used to investigate further the skin phenotype seen in these mice. Our aim was to determine whether the BFH and additional skin lesions could be aggravated by simultaneous loss of *Trp53*. Unfortunately, the $Sufu^{+/-};Trp53^{-/-}$ mice had to be sacrificed due to MB or malignant lymphoma, preventing detection of any possible progression of these lesions. A continuation of this project would be to cross the $Sufu^{+/-}$ mice to conditional $Trp53^{-/-}$ mice, and to knock out *Trp53* under the control of a skin-specific promoter. This would enable long-term studies on the cooperation between *Sufu* and *Trp53* in the skin. Another possibility would be to transplant skin from our $Sufu^{+/-};Trp53^{-/-}$ mice to immunocompromised nude mice for the same purpose. These studies could also be performed in combination with irradiation treatment to enhance tumor development.

In **PAPER III**, the aim was to investigate further how the loss of *Sufu* affects development and differentiation using $Sufu^{-/-}$ ESCs derived from $Sufu^{-/-}$ preimplantation embryos. This study is ongoing, but so far we can report that the loss of *Sufu* limits the capacity of ESCs to differentiate into cartilage and bone during teratoma formation *in vivo*. This novel finding will be investigated further and one future experiment will involve immunohistochemical analysis with tissue-specific antibodies to allow the identification of different cell types. It would also be interesting to visualize Hh pathway activity by analyzing expression of the Hh target genes, *Ptch1* and *Gli1*, within the teratomas. Since there is a limited access to reliable antibodies against these proteins, *in situ* hybridization of tissue sections could be used as an alternative. Since $Sufu^{-/-}$ EBs are clearly smaller than wild-type EBs after two to three weeks in suspension culture, we are also interested in additional investigations into this phenomenon. Is the smaller size due to inhibition of proliferation, increased apoptosis or other reasons? A quantitative analysis of the size difference has already been initiated.

Another ongoing project is the study of $Sufu^{-/-}$ ESCs during *in vitro* differentiation, meaning that ESCs are grown under certain conditions to promote differentiation towards a specific lineage. By studying the expression of tissue-specific markers we hope to investigate the role of *Sufu* and the effect of overactive Hh signaling with respect to differentiation of specific tissues. In addition, we are planning to create chimeric mice from the *Sufu* null ESCs. To distinguish the *Sufu* null cells from wild-type cells in these mice, the ESCs could be electroporated with a vector in which green fluorescent protein (GFP) is expressed under the control of the ß-actin promoter. This would provide insight into whether *Sufu* null cells can give rise to any adult tissues or organs, and whether these tissues/organs (if they arise) will develop tumors or other malformations. The study could also be performed on embryos at different time points during embryogenesis, which would be particularly useful if the chimeric mice were to die *in utero* or very soon after birth. Tissues and organs of interest include the skin, pancreas, prostate, lung and gut, which are all dependent on correct HH signaling during development, and have all been shown to have components of the HH signaling pathway mutated in human tumors.

Lastly, in a **PRELIMINARY STUDY** I have been working on a conditional *Sufu* knock-out mouse model that should allow the ablation of *Sufu* in particular tissues at specific time points, and which, if successful, would become the

model of preference over the chimeric mouse model mentioned above. Crossings between *Sufu* conditional mice and K5-Cre mice have been initiated, but it is still too early to analyze the results.

Finally, I hope that these studies will yield new insights into the effects of complete loss of *Sufu* in skin and other tissues. In addition, the mouse models may serve as useful tools in testing and evaluating new drugs that target the HH signaling pathway. Eventually, this may lead to better treatment and prognosis for patients that suffer from certain HH-related disorders and cancers.

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"Life is an emergent property of the millions of complex molecular interactions continually taking place in unfathomable coordination. In many cases the removal of one of these molecules can be the difference between a self sustaining-reproducible cell and a meaningless mess of chemicals" (Rinn, J., 2004). In a similar manner the many interactions, discussions, ideas, support and engagement from all of you have made this thesis possible.

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