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**HEDGEHOG SIGNALING IN RHABDOMYOSARCOMA:
ROLE OF GLI FACTORS AND SPLICE VARIANTS IN
SIGNAL TRANSDUCTION**

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To my beloved family

ABSTRACT

Rhabdomyosarcoma (RMS) is the most common soft-tissue childhood cancer. Deregulation of the Hedgehog (HH) signaling pathway, which is essential for proper embryonic development, has been implicated as a driving force for this cancer. In paper I, we have shown that sporadic human RMS have an overactive HH signaling pathway, and exhibit loss of heterozygosity of the two tumor suppressor genes, *PTCH1* and/or *SUFU*, indicating a role for the promotion of rhabdomyoblastic tumor development. Moreover, we also identified a novel *PTCH1* germ-line mutation in a patient suffering from the Nevoid basal cell carcinoma syndrome and also demonstrated that fetal rhabdomyoma (RM), a benign rhabdomyoblastic tumor, is a true component of this disorder. We analysed 12 RM/RMS tumors and 5 E-RMS cell lines for the presence of mutations in *PTCH1*, but none were detected. To evaluate the functional importance of the deregulated HH pathway in specifically in embryonal RMS (E-RMS), we analysed the E-RMS cell lines for their dependence on HH activity (Paper II). All cell lines expressed HH signaling components and displayed upregulated HH target gene expression. Inhibition of HH signaling activity by the use of two small molecule antagonists, cyclopamine and GANT61, led to reduced proliferation of the cell lines. The effect of GANT61 was specific as HH target gene expression was reduced, whereas cyclopamine gave off-target effects. GANT61 induced apoptosis, and significantly reduced tumor growth in an *in vivo* model. Knockdown of the GLI transcription factors, the ultimate effectors of HH signaling, revealed that GLI1 and GLI3 were important for cell proliferation, whereas GLI2 was dispensable. As GANT61 inhibits GLI1/GLI2 transcriptional activity, the inhibition of E-RMS growth is likely to be mediated through GLI1.

The HH pathway is a very complex and highly regulated pathway, and the complexity is further increased by the presence of several isoforms of HH pathway components. In paper III, we identified and analysed a novel GLI1 splice variant, which is generated by skipping exons 2 and 3 and encodes an N-terminal truncated GLI1 protein (GLI1 Δ N). The expression of this variant is downregulated in tumor tissues compared to normal samples. GLI1 Δ N was upregulated by HH signaling to the same extent as full-length GLI1, but generally had a weaker capacity to activate transcription.

Another negative regulator of HH signaling, SUFU, has also several isoforms. In paper IV, we have analysed a C-terminal truncated variant, SUFU- Δ C, for its impact on HH signal transduction. SUFU- Δ C mRNA was expressed at similar levels as SUFU-FL, but on the protein level only very low amounts of SUFU- Δ C could be detected in E-RMS cell lines. Although SUFU- Δ C was shown to be less stable than SUFU-FL, it possesses an equal ability to repress GLI2 and GLI1 Δ N, but not GLI1FL transcriptional activity. Co-transfection of SUFU- Δ C and SUFU-FL resulted in increased protein expression levels relative to individual transfections, implying a protein stabilizing capacity of the SUFU variants.

In conclusion, we have shown a major role for the HH signaling pathway in the establishment and maintenance of RMS tumors. The analyses of the GLI1 and SUFU splice variants reveal increased complexity, and suggest novel regulatory mechanisms in the HH signaling pathway, in RMS but also in other HH pathway-related tumors.

LIST OF PUBLICATIONS

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

- I. **Tostar U**, Malm CJ, Meis-Kindblom JM, Kindblom LG, Toftgård R and Undén AB. Deregulation of the hedgehog signaling pathway: a possible role for the *PTCH* and *SUFU* genes in human rhabdomyoma and rhabdomyosarcoma development. *J Pathol.* 2006, 208; 17-25
- II. **Tostar U**, Toftgård R, Zaphiropoulos PG and Shimokawa T. Reduction of human embryonal rhabdomyosarcoma tumor growth by inhibition of the hedgehog signaling pathway. *Genes & Cancer*, 2010, in press.
- III. Shimokawa T, **Tostar U***, Lauth M*, Palaniswamy R*, Kasper M, Toftgård R and Zaphiropoulos PG. Novel human glioma-associated oncogene1 (GLI1) splice variants reveal distinct mechanisms in the terminal transduction of the Hedgehog signal. *J. Biol. Chem.* 2008, 283, 14345-54
- IV. **Tostar U**, Finta C, Zaphiropoulos PG, Shimokawa T. A suppressor of fused carboxy terminal variant and its impact on hedgehog signal transduction. (2010) Manuscript.

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LIST OF ABBREVIATIONS

A-RMS	Alveolar rhabdomyosarcoma
BCC	Basal cell carcinoma
BOC	Brother of CDO
CDO	Cell adhesion molecule-related/down-regulated by oncogenes
DHH	Desert hedgehog
E-RMS	Embryonal rhabdomyosarcoma
FRM	Fetal rhabdomyoma
GANT	GLI-antagonist
GAS1	Growth arrest-specific 1
GLI	Glioma-associated oncogene
HH	Hedgehog
HHIP	Hedgehog interacting protein
IHH	Indian hedgehog
LOH	Loss of heterozygosity
NBCCS	Nevoid basal cell carcinoma syndrome
PTCH	Patched
RM	Rhabdomyoma
RMS	Rhabdomyosarcoma
SHH	Sonic hedgehog
SMO	Smoothened
SUFU	Suppressor of fused
TGF- β	Transforming growth factor beta

INTRODUCTION

CANCER

Cancer is a collective term for a large group of diseases in which abnormal cells divide without control and are able to invade other tissues. Cancer is a leading cause of death worldwide, it accounted for 13% of all deaths in 2004 (WHO, 2009). There are more than 100 different types of cancer, and they can affect any part of the body. Most cancers are named from the organ or type of cell in which they originate, for example a cancer that originates from melanocytes in the skin is called melanoma.

The body is made up of many types of cells, and cellular growth is a highly regulated process. The genetic material (DNA) of a cell can get damaged or changed, producing mutations that affect normal cell growth and division. These abnormal cells have lost their responsiveness to normal growth controls and continue to divide and may form a mass of tissue called a tumor. However, only one mutation is not enough to create a tumor, cancer is a multistep process involving numerous alterations in cells and their physiological control mechanisms. The complexity of this process is reflected in the long time periods required for most human cancers to develop. Genetic abnormalities found in cancer typically affect two general classes of genes; the activation of oncogenes and/or the inactivation of tumor suppressor genes. Genetic alterations can occur at many levels. Entire chromosomes can be gained or lost or a single DNA nucleotide can be mutated. Epigenetic events can regulate gene silencing at the chromatin level. Carcinogens, such as tobacco smoke, radiation, chemicals or infectious agents, can cause genetic changes, which may also be inherited, or acquired through errors in DNA replication.

In the year 2000, Hanahan and Weinberg suggested that all tumors harbour six essential alterations in cell physiology -“The hallmarks of cancer”- that dictate malignant growth. These capabilities include: self-sufficiency in growth signals; insensitivity to antigrowth signals; evasion of apoptosis; possession of limitless replicative potential; sustainment of angiogenesis; facilitation of tissue invasion and metastasis (Hanahan and Weinberg, 2000). Now, there is emerging evidence that two additional hallmarks should be added, at least for some tumors; deregulation of cellular energetics and avoidance of immune destruction (Zitvogel et al., 2006; Acebo et al., 2009). Tumors depend on their surrounding stroma cells to provide blood supply and connective tissue support, and the microenvironment is therefore crucial for the growth of the tumor. Thus, cancer is now viewed as a heterogenous complex tissue with many interactions between malignant cells and their dynamic microenvironment (Kenny et al., 2007).

The majority of tumors arising in humans are benign; they grow locally and do not invade adjacent tissues, and are usually harmless. Malignant tumors on the other hand are cancerous. They invade nearby tissues and give rise to metastases that spread to other sites in the body through the blood and lymph system, and are responsible for almost all deaths from cancer.

Oncogenes

An oncogene is a gene whose protein products can help to transform a normal cell into a tumor cell. A proto-oncogene is a gene whose normal activity promotes cell proliferation. A proto-oncogene can function as an oncogene if aberrantly activated through mutations, amplifications, chromosomal rearrangements or overexpression. The oncogenic mutations are dominant, “gain-of-function”, which means that only one allele needs to be altered for oncogenic effects. Proto-oncogenes are classified as: growth factors, growth factor receptors, signal transducers, transcription factors and regulators of cell death. Examples include *RAS*, *MYC*, *MDM2* and *GLI* (Kinzler et al., 1987; Croce, 2008).

Tumor suppressor genes

Tumor suppressor genes are genes whose normal function is to inhibit or control cell division, and thereby prevents tumor formation. Tumors often have inactivating mutations in tumor suppressor genes. In general, both alleles of a tumor suppressor gene must be inactivated in order to observe “loss-of-function” of the tumor suppressor protein product and to change the behaviour of the cell. This is called the Knudson’s two-hit hypothesis (Knudson, 1971), in which an inherited germ-line mutation in a tumor suppressor gene will only cause cancer if the other allele is also inactivated. Such an event may occur by deletion or chromosomal rearrangements later in life and is referred to as loss of heterozygosity (LOH). However, there are some exceptions to the “two-hit” rule for tumor suppressors. It has been shown that only one mutated allele can contribute to cancer development, the phenomenon of haploinsufficiency, which is the case for the *p27* gene (Fero et al., 1998). Dominant negative mutations, in which the mutated protein product can prevent the function of the normal protein from the un-mutated allele, have also been found in tumor suppressor genes, such as *p53* (Baker et al., 1990). Other typical examples of tumor suppressor genes are the *adenomatous polyposis coli* (*APC*), the retinoblastoma (*RB*) and *PTCH1* genes (Vogelstein and Kinzler, 2004).

Treatment

The standard therapeutic treatments for cancer include surgery, radiotherapy or chemotherapy. They are usually used in combinations depending on the type and site of the tumor. As the basic understanding of the cause of different cancers expands, new possibilities for drug targets arise. In the last decade many targeted therapies have been developed. The targets are manifold and include inhibiting angiogenesis, promoting apoptosis, stimulating immune defence mechanisms and so on. However, clinical trials have generally shown modest responses to targeted therapies when used alone. As a result targeted therapies are now used in combination with radiation and/or chemotherapy. Examples of successful targeted therapies used clinically include the tyrosine kinase inhibitor Gleevec and the estrogen receptor antagonist tamoxifen (Le Tourneau et al., 2008).

Many cancer patients are cured from their tumors. However, a majority of tumors (depending on cancer type) continue to develop, due to drug resistance, toxic side-effects and/or infections, which are limiting factors for continuous treatment, as well as

acquisition of new mutations leading to further growth advantages. This leads to metastasis formation and eventually kills the patient.

CANCER AND DEVELOPMENT

During the development from an embryo to an adult organism, cells need to proliferate and differentiate in a highly regulated process. It is the communication between cells that co-ordinates these processes. Surprisingly, it is only a few types of cellular signals that are used and these include Wnt, Notch, transforming growth factor- β (TGF- β), platelet derived growth factor (PDGF), fibroblast growth factor (FGF) and hedgehog (HH) (Dreesen and Brivanlou, 2007; Barakat et al., 2010). These secreted proteins signals direct cell proliferation, cell fate determination, epithelial-mesenchymal transitions and the rearrangement of cells by motility and adhesion changes. These signaling pathways are often dysregulated in cancer; by activating a single signal transduction pathway, tumors can grow, recruit a blood supply and invade adjacent tissues. Tumors associated with misregulated signaling typically arise from tissues where the signaling molecule was normally expressed during development.

HEDGEHOG SIGNALING IN DEVELOPMENT AND DISEASE

The hedgehog (HH) gene was first identified in a genetic screen for mutations disrupting the patterning of the *Drosophila* larvae (Nusslein-Volhard and Wieschaus, 1980). The HH gene was named so because of the larvae “spiky” appearance. The HH signaling pathway is fundamental in embryonic development, and regulates both cell fate and proliferation. It is involved in patterning of a diverse range of vertebrate structures, including the brain, neural tube, lungs, skin, axial skeleton, teeth, hair, mammary gland, skeletal muscle and limbs (McMahon et al., 2003). Additionally, HH signaling also functions as a regulator of cell proliferation, cell differentiation, and tissue regeneration and repair in adult tissue.

Dysregulation of the HH pathway during embryonic development leads to severe birth defects. Holoprosencephaly, polydactyly, craniofacial defects and skeletal malformations, are all linked to mutations in HH pathway components. Abnormal re-activation of HH signaling in the adult can lead to tumor formation, which will be discussed more thoroughly in a later section.

SIGNAL TRANSDUCTION OF THE HEDGEHOG PATHWAY

The HH pathway is evolutionary conserved from flies to human, and although the core components are retained, gene duplications and additional signaling events have added complexity to the pathway in mammals. This thesis focuses on vertebrate HH signaling.

HH signaling involves multiple inhibitory interactions. Binding of the HH ligand to its receptor, patched (PTCH), removes the inhibitory action of PTCH on the

transmembrane protein smoothened (SMO). The activated SMO initiates an intracellular signal transduction cascade that leads to the inhibition of formation of GLI transcriptional repressors and stimulation of the GLI transcriptional activators. Of course, the intracellular signaling mechanism is much more complex, and new components of the pathway continue to be identified. In particular, in vertebrates a specialized organelle, the primary cilium, is essential for HH signaling. Primary cilia are solitary microtubule-based extensions that protrude from the plasma membrane of most cells, considered to be non-motile. They function as “cellular antennae” sensing external factors and regulating different signal transduction pathways (Eggenchwiler and Anderson, 2007). Assembly and continued function of cilia is controlled by a group of proteins that are involved in “intraflagellar transport” (IFT) (Pedersen and Rosenbaum, 2008), and when these are mutated the observed phenotype is similar to that of HH pathway-mutants (Huangfu et al., 2003). An overview of vertebrate HH signaling is given in Figure 1.

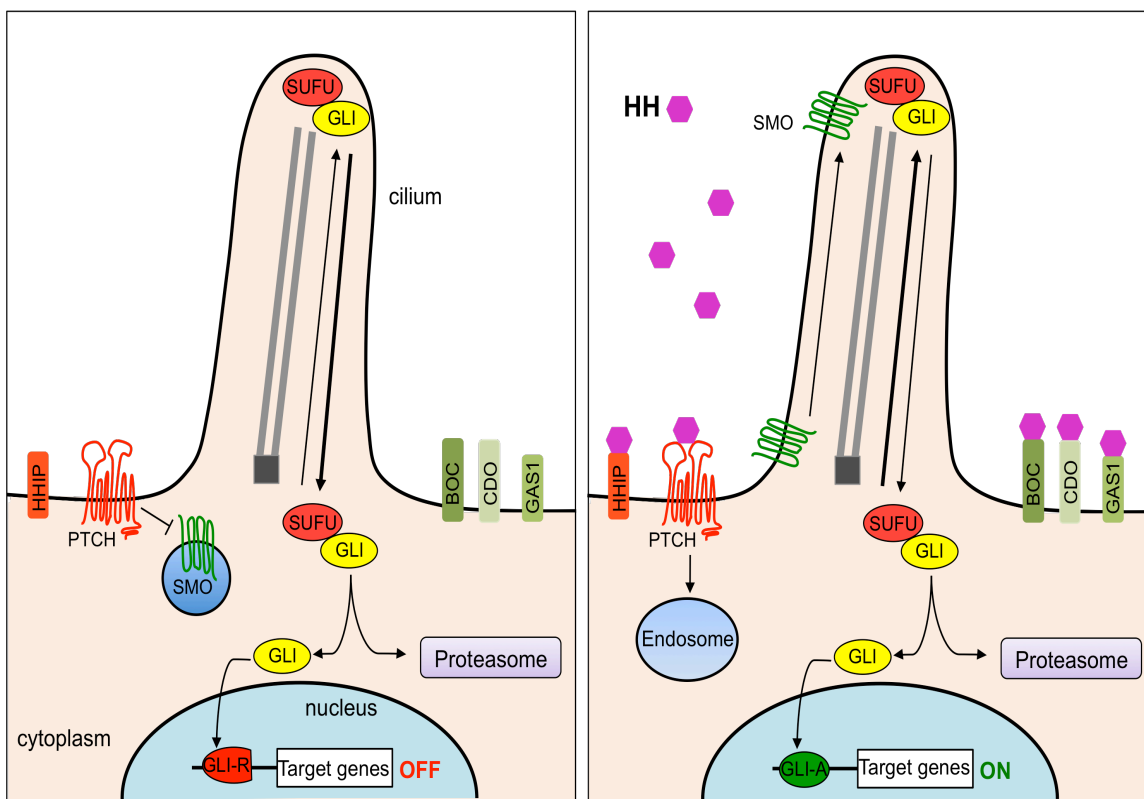


Figure 1. A schematic model of the HH signaling pathway. In the absence of HH ligands (left panel), PTCH is located at the base of the cilium and suppresses the function of SMO by preventing its entry into the cilium. Full-length GLI proteins (yellow), mainly GLI3, are converted to a C-terminally truncated repressor form (GLI-R, red), and mediate transcriptional repression of HH target genes. In the presence of HH ligands (right panel), binding inhibits PTCH’s function, which results in the movement of SMO into the primary cilium. The HH/PTCH complex internalizes and subsequently gets degraded. SMO becomes activated and promotes the activation of full-length GLI proteins (GLI-A, green), mainly GLI2, which enters the nucleus and promotes transcription of target genes. The cell-surface protein HHIP competes with PTCH for binding to HH ligands and limits their range of action, while the cell surface proteins GAS1, CDO and BOC positively affect the HH signaling output.

HH ligand production and secretion

The HH proteins are signaling molecules that are secreted from the producing cell and function both on nearby and distant cells in developing tissues. In the producing cell the full-length HH precursor proteins undergo autocatalytic cleavage to release an N-terminal fragment with a cholesterol moiety covalently linked to its C-terminus (Porter et al., 1996), and a palmitate moiety at the N-terminus (Pepinsky et al., 1998). The lipid modifications are essential for the proper movement and reception of the ligands. The release of the lipidated HH from the secreting cell requires Dispatched (Disp), a transmembrane protein that is structurally related to patched (Caspar et al., 2002; Ma et al., 2002).

In mammals there are three different HH proteins, Sonic (SHH), Indian (IHH) and Desert (DHH) HH. SHH is broadly expressed and is involved in the development of many organs (Ingham and McMahon, 2001). IHH has been implicated in regulation of cartilage, bone and gut (Vortkamp et al., 1996; St-Jacques et al., 1999; van den Brink, 2007), whilst DHH mainly acts in the developing germ-line (Bitgood et al., 1996).

Reception of the HH signal

The HH signal is received and transduced at the cell membrane by the receptor patched (PTCH). PTCH is predicted to have 12-transmembrane domains, two extracellular loops where HH binds, one intracellular domain (Marigo et al., 1996), and a sterol-sensing domain (Loftus et al., 1997), which is likely to play a role in regulating transduction of the HH signal. There are two patched homologs in humans, PTCH1 and PTCH2, of which PTCH1 is ubiquitously expressed (Hahn et al., 1996a), whereas PTCH2 is expressed preferentially in skin and testis (Carpenter et al., 1998). *PTCH1* is located on chromosome 9q22, and contains 23 exons (Hahn et al., 1996b; Johnson et al., 1996). Furthermore, PTCH shares structural motifs with members of the resistance-nodulation-division (RND) family of bacterial transmembrane pumps (Taipale et al., 2002). Based on the structure, PTCH has been proposed to function as a pump transporting small molecules across the membrane that regulates the activity of SMO (Taipale et al., 2002).

Several other molecules are involved in regulating the HH reception. Hedgehog interacting protein (HHIP) acts as a negative regulator as it competes with PTCH for HH ligand binding (Chuang and McMahon, 1999; Chuang et al., 2003). There is also positive regulation; CDO, BOC and GAS1 are also able to bind HH and act as co-receptors and enhance the binding of HH to PTCH (Tenzen et al., 2006; Allen et al., 2007).

Signaling from SMO to GLI

Smoothed (SMO), a seven-transmembrane protein, acts as a G-protein-coupled receptor (GPCR) that transmits the HH signal upon stimulation (Stone et al., 1996). When HH binds PTCH, the PTCH-HH complex becomes internalized to endosomes, and this allows SMO to move into the cilium. The precise mechanism whereby PTCH signals to SMO is still unclear. However, transport of small molecules has been suggested. Several possible candidates are reported, including pro-vitamin D3, which

has a negative effect on HH signaling by directly inhibiting SMO (Bijlsma et al., 2006) and oxysterols, derivatives of cholesterol, which exert positive stimulation of HH signaling and induce localization of SMO to the cilium (Corcoran and Scott, 2006; Rohatgi et al., 2007). However, ciliary localization is not enough to activate SMO fully, and a second separate event is required for its complete activation (Rohatgi et al., 2009; Wang et al., 2009). The GPCR kinase GRK2 phosphorylates the C-terminus of SMO, which positively affects HH signaling, and possibly mediates a conformational switch of SMO (Chen et al., 2004; Meloni et al., 2006). Ultimately, SMO promotes downstream pathway activation by shifting the processing of the GLI transcription factors in favour of activator forms.

The GLI transcription factors

The glioma-associated oncogene (GLI) gene family was identified by the amplification of human GLI1 in glioblastoma (Kinzler et al., 1987). They are zinc finger transcription factors (Ruppert et al., 1988) and mediate transcriptional responses to HH signaling. There are three GLI proteins in humans; GLI2 and GLI3 are direct effectors of the HH signaling pathway, whereas GLI1 is induced as a later event. GLI2 and GLI3 possess an amino-terminal repressor domain and a carboxy-terminal activator domain flanking the central DNA-binding zinc fingers (Sasaki et al., 1999). GLI1 however, lacks the amino-terminal repressor domain and functions only as a transcriptional activator. GLI2 similarly functions mainly as an activator of HH signaling, whereas GLI3 functions primarily as a repressor (Ruiz i Altaba, 1999). Ultimately it is the balance of the collective activator and repressor functions of these GLI transcription factors that determines the status of the HH transcriptional program.

Proteasome-mediated proteolysis regulates the abundance and transcriptional activity of the vertebrate GLI proteins. GLI2 and GLI3 are processed in three different ways. In the absence of the HH signal, they can be proteolytically cleaved to form transcriptional repressors, with GLI3 being the more significant repressor *in vivo* (Litingtung et al., 2002; Bai et al., 2004). This is achieved through sequential phosphorylations by protein kinase A (PKA), glycogen synthase kinase 3 β (GSK3 β) and casein kinase 1 (CK1), which promotes binding to β -TrCP and subsequent proteolytic processing in the proteasome (Tempe et al., 2006; Wang and Li, 2006). Another alternative in the absence of the HH signal, involves degradation. This process involves Spop, which promotes ubiquitination of GLI2 and GLI3 by the Culin3 E3 ubiquitin ligase and subsequent complete degradation through the 26S-proteasome (Chen et al., 2009). Upon activation of HH signaling both degradation of GLI2 and proteolytic processing of GLI3 into its repressive form are inhibited (Wang et al., 2000; Pan et al., 2006) thereby permitting GLI2 to function as a strong activator of the HH transcriptional program and allowing full-length GLI3 to serve as an activator in some circumstances (Bai et al., 2004; Wang et al., 2007)

All three GLI proteins, as well as SUFU, can localize to the cilium, suggesting that key GLI processing steps occur within the cilium itself (Haycraft et al., 2005).

Suppressor of fused

Suppressor of fused (SUFU) is a negative regulator of the pathway, and is named so because it was initially identified as a genetic suppressor of the fused kinase in drosophila wing development (Preat, 1992). SUFU deletion in mice causes increased HH signal activity and is embryonic lethal (Svärd et al., 2006). The human SUFU gene, which consists of 12 exons and is located in a region frequently deleted in tumors on chromosome 10q24, encodes a protein of 484 amino acids (Stone et al., 1999; Grimm et al., 2001).

SUFU binds all three GLI proteins (Stone et al., 1999; Dunaeva et al., 2003), and may control the processing and/or degradation of GLI and thus the GLI-A:GLI-R ratio (Dessaud et al., 2008). HH stimulation recruits SUFU-GLI complexes to cilia, and causes dissociation of the complex, allowing GLI to enter nucleus and activate transcription (Humke et al., 2010; Tukachinsky et al., 2010). SUFU may also act further downstream to control the cytoplasmic-nuclear shuttling of GLI and inhibit the entry into the nucleus. SUFU has a nuclear export signal (NES) and is thought to sequester GLI1 in the cytoplasm by restricting its nuclear localization (Kogerman et al., 1999; Merchant et al., 2004). Additionally, SUFU may act as a transcriptional co-repressor, since it can recruit and bind SAP18, a part of the mSin3-histone deacetylase (HDAC) transcriptional co-repressor complex, to SUFU-GLI complexes occupying GLI binding sites in the nucleus (Cheng and Bishop, 2002; Paces-Fessy et al., 2004).

HH target genes

GLI molecules can regulate target gene expression by direct binding to a specific consensus sequence located in the promoter region of the target genes (Kinzler and Vogelstein, 1990). The HH target genes include GLI1 (Lee et al., 1997; Dai et al., 1999; Ikram et al., 2004), which further amplifies the initial HH signal at the transcriptional level, and thus, *GLI1* mRNA levels are reliable indicators of pathway activity. Other ubiquitously expressed target genes include *PTCH1*, *PTCH2* and *HHIP* (Chuang and McMahon, 1999; Yoon et al., 2002; Rahnama et al., 2004), thereby creating negative feedback regulation of HH pathway activity via sequestration of HH.

The outcome of HH signaling varies depending on cell type, but it can include expression of a variety of cell-specific transcription factors mediating different developmental fate responses. These include up-regulation of D-type cyclins and N-Myc, resulting in cell proliferation (Duman-Scheel et al., 2002; Yoon et al., 2002; Kenney et al., 2003) up-regulation of anti-apoptotic proteins such as B-cell lymphoma 2 (Bcl2) mediating cell survival (Bigelow et al., 2004; Regl et al., 2004), production of vascular endothelial growth factor (VEGF) and angiopoietins regulating angiogenesis (Pola et al., 2001), and transcription of SNAIL, initiating the epithelial-mesenchyme transition in metastasis (Feldmann et al., 2007). It is, therefore, not surprising that dysregulated HH signaling can lead to a variety of cancers.

TUMORS WITH DEREGULATED HH PATHWAY

In the adult the role of HH signaling is limited, and thus re-activation of HH signaling can give rise to cancer formation. Inappropriate activation of HH signaling may contribute to development of skin, brain and gastrointestinal tract tumors. It has been speculated that HH signaling is responsible for 25% of all cancer deaths (Lum and Beachy, 2004).

Nevoid basal cell carcinoma syndrome

The first connection of the HH pathway to cancer was the discovery that the Nevoid basal cell carcinoma syndrome (NBCCS) (also known as Gorlin syndrome) is caused by a mutation in *PTCH1* (Gailani et al., 1992; Hahn et al., 1996b; Johnson et al., 1996). NBCCS is inherited as an autosomal dominant disorder and predisposes both to developmental defects and cancers (Gorlin, 1995). NBCCS patients are predisposed to develop multiple basal cell carcinomas (BCCs) during their lifetime, and have an increased risk to develop other kinds of cancer as well, especially medulloblastomas, ovarian fibromas and fetal rhabdomyomas. The developmental defects include skeletal malformations, spina bifida occulta and jaw keratocysts, and are highly penetrant, thus reflecting the role of HH signaling during development. The mouse model of this syndrome, *Ptch1* heterozygous mice, exhibits many features of this disease, including a high frequency to develop medulloblastoma and rhabdomyosarcoma spontaneously (Goodrich et al., 1997; Hahn et al., 1998), and also BCC-like tumors after UV-irradiation (Aszterbaum et al., 1999). Somatic inactivation of the remaining *PTCH1* allele was demonstrated in BCC from NBCCS patients, showing that it behaves as a classical tumor suppressor gene according to Knudson's two-hit model (Knudson, 1971).

Basal cell carcinomas

BCCs are keratinocyte tumors of the skin and the most common cancer in the western world. It has been found that sporadic BCCs also have either a high frequency of inactivating mutations in *PTCH1* or, to a lesser extent, activating mutations in *SMO* (Gailani et al., 1996; Reifenberger et al., 2005; Xie et al., 1998; Reifenberger et al., 1998), resulting in constitutive activation of the HH pathway. BCCs appear as slow-growing, elevated lesions on the sun-exposed areas of the skin of persons of fair complexion. BCCs rarely metastasize and although they are benign they can cause significant tissue destruction by local invasion (Epstein, 2008).

Brain tumors

Medulloblastoma (MB) is classified as a primitive neuroectodermal tumor of the cerebellum, and is the most common malignant brain tumor in children. Approximately 3-5% of NBCCS patients develop MB, and mutations of *PTCH1* are found in 10-20% of sporadic MBs (Pietsch et al., 1997; Raffel et al., 1997; Xie et al., 1997). Mutations in *SMO* and *SUFU* also occur at a low frequency (Reifenberger et al., 1998; Zurawel et al., 2000; Taylor et al., 2002; Pastorino et al., 2009). Accordingly, mouse models

targeting *Ptch1*, *Sufu* and *Smo* all develop MB spontaneously (Goodrich et al., 1997; Lee et al., 2007; Schuller et al., 2008).

GLI1 was originally identified as a gene amplified 75-fold in a malignant glioblastoma, a highly aggressive CNS tumor. Therefore, GLI1 was suggested to be an oncogene and named after the tumor (Kinzler et al., 1987). However, only a minority of glioblastomas harbours GLI1 amplifications (Bigner et al., 1988; Mao and Hamoudi, 2000). Recent data indicate a role for HH pathway activity in the maintenance of glioblastomas, as glioma cells are responsive to HH ligands secreted from the tumor stroma (Ehteshami et al., 2007; Clement et al., 2007; Becher et al., 2008).

Other tumors with aberrant HH signaling

Several cancers involving overexpression of HH, but lacking identifiable genetic aberrations in HH components have been identified in the past few years. Thus, these tumors are ligand-dependent. HH signaling can be autocrine, meaning that HH is both produced and received by the same cell, or paracrine, in which the tumor cell signals to the surrounding stroma cells, which respond by producing additional growth factors to support tumor growth and survival. The last type is analogous to the epithelial-mesenchymal signaling occurring during normal development. This type of activated HH signaling has been observed in lung, pancreatic, colorectal, prostate, breast and melanoma tumors (Watkins et al., 2003; Yuan et al., 2007; Thayer et al., 2003; Qualtrough et al., 2004; Yauch et al., 2008; Sanchez et al., 2004; Karhadkar et al., 2004; Mukherjee et al., 2006; Stecca et al., 2007). They are all connected to the HH pathway in the sense that they originate from tissues in which HH played an essential patterning role during development.

RHABDOMYOMA AND RHABDOMYOSARCOMA

As mentioned before, the HH pathway plays an important role in myogenesis: the formation and specification of muscle (Brand-Saberi, 2005; Bryson-Richardson and Currie, 2008). Myogenesis occurs after muscle precursor cells have been specified, and is driven by a network of transcriptional and post-transcriptional regulators and growth factors. The myogenic transcription factor cascade includes the PAX genes, myogenic regulatory factors and FOXO1. HH is a regulatory signal for the induction of muscle-specific genes and expansion of muscle progenitor populations during embryogenesis. SHH has been found to regulate the cell fate of adult muscle satellite cells, promoting cell proliferation and preventing differentiation (Koleva et al., 2005; Collins et al., 2005). Although normally turned off in the adult musculature, SHH is reactivated during regeneration of adult skeletal muscle after an injury (Straface et al., 2009).

Rhabdomyoma (RM) and rhabdomyosarcoma (RMS) are mesenchymal tumors showing immature skeletal muscle differentiation. They are thought to arise as a consequence of regulatory disruption of the growth and differentiation of myogenic precursor cells. The cell of origin is suggested to be a satellite (committed muscle progenitor) cell for E-RMS, and a mesenchymal stem cell in A-RMS (Merlino and

Khanna, 2007; Charytonowicz et al., 2009). However, it is likely that human RMS heterogeneity reflects a more complex etiology, depending on the cells of origin (satellite, mesenchymal or other cells), as well as on their susceptibility, combined with the type of genetic events and with host factors. Many of the genes regulating myogenesis are abnormally expressed in RMS, and such alterations play a likely role in RMS onset or malignancy, but it is still unclear whether a differentiation error initiates the malignant state or is caused by it.

Rhabdomyoma

Rhabdomyomas are benign tumors. They can be subdivided into cardiac, fetal (FRM) and adult types (Kapadia and Barr, 2002). Cardiac rhabdomyomas are the most common primary tumor of the heart in infants and children. The most common sites for FRM and adult RM are the head and neck region. The median age for developing FRM is 4 years. The majority of cases occur sporadically, but FRM also appear in the NBCCS syndrome.

Rhabdomyosarcoma

RMS is the most common soft-tissue sarcoma in children under the age of 15, accounting for approximately 5-10% of all paediatric solid malignancies, with an annual incidence of 4-7 cases per million children (Ries et al., 1999). Based on histological criteria they are subdivided into two main variants, embryonal (E-RMS) (Parham and Barr, 2002b), which is the most common type accounting for 70-80% of all RMS, and alveolar (A-RMS) (Parham and Barr, 2002a). A minor variant is pleomorphic RMS, which does not affect children, but mainly adults. The greatest proportion (46%) of E-RMS occurs in children less than 5 years of age, but there is a rare variant occurring in adults. The main location is head and neck, followed by genitourinary regions. Histologically the E-RMS cells exhibit a lower degree of differentiation than RMs, are composed of spindle-shaped primitive mesenchymal cells in various stages of myogenesis, i.e. rhabdomyoblasts, and resemble embryonic muscle. A-RMS is typically composed of small round densely packed cells, arranged around spaces resembling pulmonary alveoli, but which show partial skeletal muscle differentiation. A-RMS commonly arises in the trunk and extremities, and affects all ages with a predominance in adolescents and young adults. Expression profiling has revealed that RMS shows a gene profile more similar to embryonal muscle compared with young or adult muscle (Schaaf et al., 2005). To determine which subtypes these tumors fall into, clinical presentation, histological features and the detection of distinct molecular markers are used.

RMS tumors have a variety of genetic and molecular abnormalities, some of which are useful for accurate prognosis or the identification of biological subtypes. Specific chromosomal translocations are detected in 70-80% of A-RMS by cytogenetic and molecular analysis. Translocation t(2;13)(q35;q14) or t(1;13)(p36;q14), results in the creation of PAX3-FOXO1 or PAX7-FOXO1 fusion genes (Galili et al., 1993; Davis et al., 1994). The expression of these oncogenic transcription factors results in altering control of proliferation, apoptosis and differentiation.

E-RMS tumors have complex karyotypes, which are characteristics of severe genomic instability. LOH in 11p15.5 has long been considered a “hallmark” of E-RMS (Scrabble et al., 1989; Besnard-Guerin et al., 1996), and was recently shown to occur at a frequency of 77% in a wide series of RMS (Davicioni et al., 2009). This LOH region includes the genes for *IGF2*, *H19* and *CDKN1C* (*p57/KIP2*), all subject to parental imprinting. LOH in E-RMS results in loss of the maternal (silenced) allele and duplication of the active paternal allele, resulting in overexpression of IGF2 (Cavenee et al., 1989). Loss of imprinting, coincident with active transcription from both alleles has also been demonstrated, which can contribute to the overexpression IGF2 (Zhan et al., 1994). Other LOH regions were found in considerable proportions in E-RMS, including the 9q22, 10q and 17p genomic regions (Bridge et al., 2000). In both A-RMS and E-RMS amplifications of the 2p24 and 12q13-15 chromosomal regions are frequent (Weber-Hall et al., 1996). The 12q13-15 region contains many growth-related genes such as the *GLI*, *CDK4*, and *MDM2*, whereas the 2p24 region harbours the *MYCN* oncogene, and the latter is more frequently amplified in A-RMS (Xia et al., 2002). Several molecular pathways are altered in RMS, including the IGF signaling system via the IGF1R, the TP53 and RB pathways (Taylor et al., 2000; Iolascon et al., 1996; Rikhof et al., 2009). A higher expression of the oncogenes MYCN, EGFR and the PDGF receptor have been reported (Toffolatti et al., 2002; Ganti et al., 2006; Blandford et al., 2006; Taniguchi et al., 2008).

Most RMS tumors occur sporadically, but it sometimes associates with the NBCCS, Li-Fraumeni (which harbours germ-line TP53 mutations) and Neurofibromatosis type 1, as well as other rare syndromes (Xia et al., 2002).

The main prognostic parameters for RMS are histologic subtype, disease stage and site of onset. The 5-year overall survival for RMS is approximately 73% for E-RMS and 48% for A-RMS (Ognjanovic et al., 2009). Both subtypes are highly malignant, but A-RMS shows a more aggressive behaviour. The main metastatic sites are lung, bone marrow, lymph nodes and bones. Treatment for RMS consists of initial chemotherapy, then surgical resection of residual tumor mass, followed by another round of chemotherapy and/or radiotherapy (Paulino and Okcu, 2008).

Connection to HH signaling

Comparative genomic hybridisation studies have found that E-RMS tumors show loss of heterozygosity at 9q22, which encompasses the locus of *PTCH1*, at a rate of 33% (Bridge et al., 2000) and also loss of the *SUFU* region 10q23 at a rate of 18% (Bridge et al., 2002). Also in pleomorphic RMS loss at 10q23 has been observed in 71% of tumors (Gordon et al., 2003). Frequent gain (49%) of the *GLI1* 12q13-15 region was found in E-RMS (Bridge et al., 2002). The *GLI* gene has also been shown to be amplified in RMS (Roberts et al., 1989), and *GLI1* expression was found in 73% of A-RMS and E-RMS analysed (Ragazzini et al., 2004). One study has analysed the coding region of *PTCH1* in RMS and found LOH in 1/12 tumors, but no mutations were detected (Calzada-Wack et al., 2002).

The mouse model of NBCCS, the *Ptch1* heterozygote, develops spontaneous RMS, resembling the embryonal subtype. The tumor frequencies, depending on genetic

background, range from 5% to 15% (Hahn et al., 1998; Svärd et al., 2009), suggesting the presence of modifier genes in the mouse genome. Genetic removal of Trp53 in *Ptch1*^{+/-} mice increases the incidence of RMS (Lee et al., 2007), and the incidence is probably higher since most mice die early from medulloblastomas. The remaining *Ptch1* wild-type allele in the *Ptch1*^{+/-} RMS tumors is thought to be transcriptionally silenced, possibly by promoter methylation (Uhmman et al., 2005). Also, RMS development is dependent on Igf2 since *Ptch1*^{+/-} mice on an Igf2 null background are resistant to developing RMS (Hahn et al., 2000). Additional Hh pathway genetic models that develop RMS are the *Hhip*^{+/-}, *Sufu*^{+/-};*Ptch1*^{+/-} and *Sufu*^{+/-};*Trp53*^{-/-} (Gerber et al., 2007; Lee et al., 2007; Svärd et al., 2009). Interestingly, the *R26-SmoM2;CAGGS-CreER* mice, expressing an activated allele of SMO, develop RMS with a 100% penetrance (Mao et al., 2006).

However, other mouse models demonstrate that the HH pathway is not the only determinant of RMS development, as also mice with deletions of Trp53, Ink4/Arf, and Fos or activation of HGF/SF, HER-2 or K-RAS develop RMS at high frequencies (Sharp et al., 2002; Fleischmann et al., 2003; Tsumura et al., 2006; Nanni et al., 2003). Animal models have shown that the genetic event mostly required for RMS induction, common to all histotypes, is knockdown of the p53-pathway. The second main genetic alteration consists of activation of an oncogenic growth signaling pathway, such as HGF/SF, RAS, SMO-GLI or the knock-in of PAX3- or PAX7-FOXO1 gene (Sharp et al., 2002; Keller et al., 2004; Langenau et al., 2007).

INHIBITION OF HH SIGNALING – A TREATMENT PERSPECTIVE

The implication of deregulated HH signaling in many different types of cancer suggests that the pathway could be an important target for pharmacological intervention in cancer patients. HH pathway inhibitors (HPIs) are any drug, antibody or protein applied to cells or animals in order to inhibit HH signaling. The first HPIs, cyclopamine and jervine, were isolated from corn lilies as compounds causing teratogenic effects (including cyclopia) in lambs whose mother had ingested this plant (Bryden et al., 1971) and were subsequently shown to inhibit the HH pathway (Cooper et al., 1998) by binding SMO (Chen et al., 2002). Cyclopamine is widely used in the research field to inhibit HH signaling activity, but its efficacy as a pharmacological drug is limited due to its acid lability and poor water solubility. Therefore, several attempts to make derivatives have generated synthetic drugs that are already in phase I/II clinical trials in patients with advanced or metastatic solid tumors (Mas and Ruiz i Altaba, 2010). Novel synthetic SMO inhibitors have also been identified by several pharmaceutical companies and are being tested in clinical trials, with variable success (Low and de Sauvage, 2010).

In theory, all HH pathway-dependent cancers where activation occurs at the level of SMO or upstream should respond to SMO inhibition. However, if the activating events occur downstream of SMO, these inhibitors would be ineffective. Such events are exemplified by the loss-of-functions mutations in SUFU or REN in medulloblastoma or amplifications of GLI genes in gliomas, RMS, medulloblastomas and pericytomas (Di Marcotullio et al., 2004; Pastorino et al., 2009; Kinzler et al., 1987; Roberts et al., 1989;

Dahlen et al., 2004). Crosstalk with other pathways, such as the TGF- β , can also lead to activation of GLI factors (Dennler et al., 2007; Nolan-Stevaux et al., 2009). In these cases targeting the GLI effectors directly would be more beneficial. Our lab has performed a screen for small-molecule GLI inhibitors, and identified two candidates, GANT58 and GANT61 (Lauth et al., 2007). They were found to inhibit GLI-mediated transcription, and reduced tumor cell proliferation *in vitro* and xenograft tumor growth *in vivo*. Recently, another screen identified four novel small molecules, HPI-1 to HPI-4 (Hyman et al., 2009), which act through different mechanisms interfering with GLI1 and/or GLI2 activity.

Another class of HPIs are the HH antagonists, which will be useful in cancers producing HH to promote tumor growth. A monoclonal antibody, 5E1 (Ericson et al., 1996), which reacts with both SHH and IHH, has been shown to effectively down-modulate HH pathway activity in tumor cell cultures (Berman et al., 2003). Another compound, robotnikinin (Stanton et al., 2009), binds SHH and blocks signaling in cell lines, primary keratinocytes and cultured artificial human skin.

In conclusion, the type of activation of the HH pathway in a specific cancer will determine the choice of a particular HPI for therapeutic intervention.

ALTERNATIVE SPLICING

Alternative splicing and transcriptome complexity

Alternative splicing is considered to be a key factor underlying increased cellular and functional complexity in higher eukaryotes (Blencowe, 2006). It is the process whereby a pre-mRNA transcript generated from a single gene may be subjected to several alternative splicing patterns, yielding multiple, distinctly structured mRNAs, many of which may in turn encode distinct proteins. Alternative splicing is controlled both spatially and temporally, resulting in the expression of different splice variants in different tissues, in different cells within the same tissue, or in the same tissue at different stages of development or in response to pathological processes (Ward and Cooper, 2010). It is estimated that 94% of human genes are alternatively spliced and that as many as 50% of disease-causing mutations affect splicing (Lopez-Bigas et al., 2005; Pan et al., 2008; Wang et al., 2008).

Splice variants of several key components in HH signaling

The complexity of the HH signaling pathway has increased with the discovery of splice isoforms of several of its key components. The PTCH1 cDNA is characterized by different 5' ends (Johnson et al., 1996; Hahn et al., 1996b; Hahn et al., 1996a) with 4 distinct first exons, which are alternatively spliced to exon 2 (Shimokawa et al., 2007; Shimokawa et al., 2004; Nagao et al., 2005). Additionally, observations of skipping of exon 10 and inclusion of a novel exon 12b have been reported (Smyth et al., 1998; Uchikawa et al., 2006). For PTCH2, splicing variants are observed in the 3' end (Zaphiropoulos et al., 1999; Smyth et al., 1999), or as skipping of the internal exons 9-10 (Rahnama et al., 2004). Internal splicing events have also been reported for GLI2, of

which one variant showed increased expression in BCCs (Tanimura et al., 1998; Tojo et al., 2003; Speek et al., 2006). For GLI1, both mouse and human alternatively expressed isoforms differing in their 5' untranslated region have been reported (Wang and Rothnagel, 2001; Palaniswamy et al., 2010). Interestingly, one of the splicing variants, skipping exon 1A, was highly abundant in BCCs.

Several alternatively spliced transcripts of SUFU with tissue-specific expression have been identified. Skipping of exon 10 or inclusion of an additional exon, exon 8a, which results in premature termination of the protein have been detected. One frequently expressed SUFU variant had a tri-nucleotide insertion at the start of exon 7 (Grimm et al., 2001). Furthermore, an isoform lacking the C-terminal part of SUFU, was described by Stone et al, and found to be highly expressed in testis (Stone et al., 1999). The truncated SUFU uses a unique terminal exon harbouring an early stop codon, exon 10a, which is derived from sequences within intron 10 of full-length SUFU. The biological properties of this isoform are addressed in this thesis.

AIMS

The general aim of this study was to elucidate the importance of the HH signaling pathway in human rhabdomyosarcoma tumorigenesis and the role of GLI factors and splice variants in HH signal transduction.

Specific aims:

1. To investigate the presence of active HH signaling in human rhabdomyoma and rhabdomyosarcoma tumor samples.
2. To evaluate the functional importance of HH signaling pathway in embryonal rhabdomyosarcoma cell lines.
3. To identify splicing variations occurring in the human GLI1 gene and to study the functional properties of the novel GLI1 splice variants in the transduction of the HH signal.
4. To analyse a suppressor of fused carboxy-terminal splice variant and its impact on HH signal transduction.

RESULTS AND CONCLUSIONS

PAPER I

Deregulation of the HH signaling pathway: a possible role of the *PTCH* and *SUFU* genes in human rhabdomyoma and rhabdomyosarcoma development

Several case reports of FRM and RMS have been reported to occur in patients with NBCCS (Gorlin, 1995; Watson et al., 2004; Beddis et al., 1983), but whether HH signaling also plays a role in sporadic rhabdomyoblastic tumor development has not been evaluated.

The aim of this study was to analyse the status of the HH signaling pathway in a FRM and a meningioma that occurred in a patient with NBCCS. Both tumors lacked immunostaining of PTCH. We performed direct sequencing of the coding region of *PTCH*, and found a novel germ-line mutation present in exon 10, resulting in truncation of the PTCH protein. The FRM also harboured a 30bp in frame deletion in exon 18 in the second allele, proving for the first time that FRM is a true component of the NBCCS.

Next, we wanted to evaluate whether deregulation of HH signaling is a general finding in sporadic cases of RM and RMS. We analyzed all tumors for PTCH and GLI mRNA overexpression by in situ hybridization. All 43 tumor samples exhibited a positive signal for PTCH mRNA, and 41 samples were positive for GLI1mRNA. Protein expression of PTCH was however lacking in 12 of the tumors, all of which belonged to the E-RMS and FRM subgroups. Similarly, SUFU immunostaining was lacking in 17 tumors. No consistent expression of SHH could be identified in RM and RMS, suggesting that autocrine stimulation is not an important event in RM/RMS. Our findings indicate that the HH signaling pathway is indeed activated in the whole spectrum of sporadic rhabdomyoblastic tumors.

The fact that 12/46 tumors lacked PTCH protein expression, although PTCH mRNA was present, could suggest that inactivating mutations in PTCH had occurred, resulting in a non-functional protein. A first approach of addressing this is to look at genomic instability. Loss of heterozygosity can be identified in cancers by evaluating the presence of heterozygosity at a genetic locus in a human's germline DNA, and the absence of heterozygosity at that locus in the cancer cells. This is informative only when the two parents contributed different alleles. In this study, we used polymorphic microsatellite markers located near and within the tumor suppressor genes *PTCH* and *SUFU* loci, and screened paired normal and tumor tissue samples of both RM and RMS. LOH at the *SUFU* locus was observed in two of four tumors, and LOH at the *PTCH* locus in four of nine tumors. One example is shown in figure 2. Interestingly, the two tumors with loss of *SUFU* had simultaneous LOH at the *PTCH* locus indicating that haploinsufficiency for both tumor suppressor genes *PTCH* and *SUFU* could cooperate to promote rhabdomyoblastic tumor development.

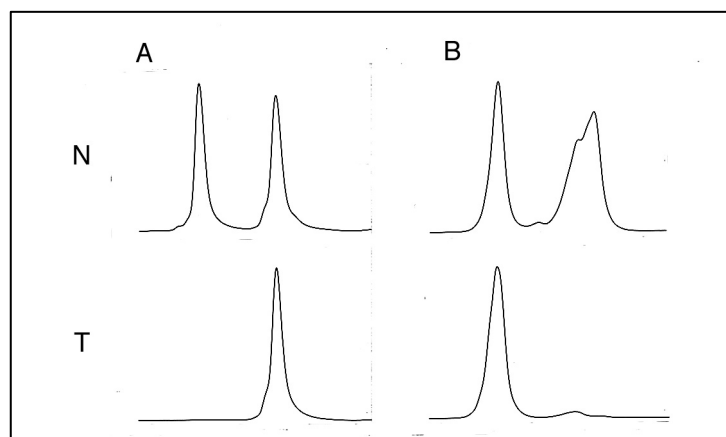


Figure 2. LOH of A, *PTCH* and B, *SUFU* in an E-RMS tumor; N=normal tissue, T= tumor tissue.

In conclusion, deregulation of the HH pathway is likely to be of pathogenic importance for rhabdomyoblastic tumors, in particular the FRM and E-RMS subgroups. This is the first report firmly linking deregulation of HH pathway to rhabdomyoblastic tumor development.

(Note: In this paper *PTCH1* is referred to as *PTCH*, according to the 2006 nomenclature.)

PAPER II

Reduction of human embryonal rhabdomyosarcoma tumor growth by inhibition of the hedgehog signaling pathway

As a continuation of the previous study we wanted to evaluate whether *PTCH1* mutations are frequent in human RM and RMS, so a set of FRM and E-RMS tumors were selected. All the tumor cases were overexpressing *PTCH1* and *GLI1* mRNA, but some of them lacked *PTCH1* protein expression. We also included 6 E-RMS cell lines in this study. We screened the coding region of *PTCH1* for mutations using direct sequencing, but could not detect any mutations.

However, we were still interested to evaluate if the upregulated HH activity seen in sporadic E-RMS had any functional importance. For this purpose we analysed the E-RMS cell lines, and also one Ewing sarcoma (EWS) cell line. All E-RMS cell lines expressed HH signaling components and showed an upregulated mRNA expression of at least one out of three target genes.

To investigate the significance of this upregulated HH signaling activity, we treated the cells with two different HH signal inhibitors, cyclopamine, which blocks SMO activity

and GANT61, which inhibits GLI1 and GLI2. Treatment led to reduced growth and widespread cell death. A proliferation assay showed reduced cellular proliferation, with GANT61 giving a more pronounced effect in all cell lines, but not in the EWS cells. To ensure that the reduced growth was a HH-specific event, we analysed target gene expression after treatment with the inhibitors. Both inhibitors reduced GLI1 and HHIP target gene expression, again with a greater effect shown by GANT61. Furthermore, GANT61 induced apoptosis whereas cyclopamine only gave rise non-specific cell death. This prompted us to analyse whether the effects seen with cyclopamine were off-target effects. Knockdown of SMO had no influence on proliferation, and this is therefore indicating that a SMO-independent HH signal pathway is operating in these cell lines.

As GANT61 proved to be an efficient drug *in vitro*, we also wanted to establish whether it could affect tumor growth *in vivo*. For this purpose we used the chicken chorio-allantoic membrane (CAM) assay. The chicken CAM is a densely vascularised, extraembryonic tissue, on which tumors rapidly develop, and the assay allows fast testing of anticancer drugs. Pretreatment of tumor cells with different doses of GANT61 led to reduced tumor growth of E-RMS cell lines, but not of EWS cells, on the chicken CAMs.

Interestingly, among the GLI factors, the GLI3 was expressed at the highest levels in E-RMS cells. We also analysed protein levels of GLI3 and this revealed that the GLI3 activator form predominated. However, GANT61 could not repress the weak activation capacity of GLI3. We performed a proliferation assay after siRNA knockdown of the three GLI factors to evaluate their significance in E-RMS growth. This revealed that GLI1 and GLI3 ablation led to reduced proliferation, whereas GLI2 had no such effects.

In conclusion, we found that GLI1 is the most important GLI factor for E-RMS tumor growth, and GANT61 could constitute a good therapeutic option for these tumors.

PAPER III

Novel human glioma-associated oncogene 1 (GLI1) splice variants reveal distinct mechanisms in the terminal transduction of the hedgehog signal

Several studies have identified alternative spliced transcripts in/of members of the HH signaling pathway. Splice variants of PTCH1, PTCH2 and GLI2 have previously been described. In this study, we performed *in silico* screening and identified a novel human GLI1 splice variant, GLI1 Δ N, which is generated by the skipping of exons 2 and 3, resulting in a N-terminal truncated protein.

Analysis of the expression levels of GLI1 Δ N and full-length GLI1 (GLI1FL) in adult human tissues revealed similar levels of both isoforms. However, in tumor cell lines a generally lower and more variable expression pattern was observed. In RMS cell lines the ratio of GLI1FL/GLI1 Δ N was low, whereas in lung cancer cell lines this ratio was very high. This may indicate that expression of GLI1 isoforms can be controlled by

tissue-specific factors representing an additional mechanism of regulation of HH signaling.

Since GLI1 is a target gene of HH signaling and its expression is upregulated by HH activity, we examined whether HH signal activation differentially controls expression of GLI1FL and GLI1ΔN. Both forms were equally upregulated, implying that HH signaling does not alter the expression ratios of GLI isoforms, and that GLI1ΔN is also a target of HH signaling.

As GLI1FL is a transcription factor, we wanted to evaluate whether GLI1ΔN also have the same potential to activate transcription as GLI1FL. Analysis of the activation capacity of endogenous target genes revealed that GLI1ΔN could indeed act as a transcription factor. However, GLI1FL was generally more potent in increasing transcriptional responses.

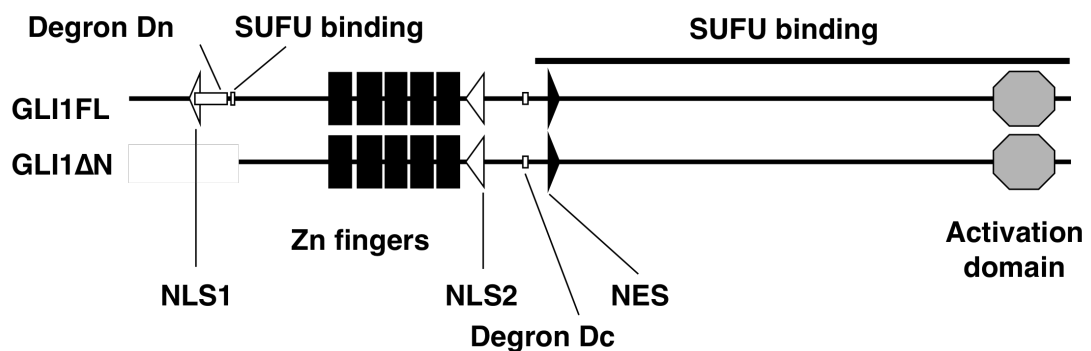


Figure 3. Schematic representation of the predicted protein domains for GLI1FL and GLI1ΔN.

Zinc finger domains are indicated by black boxes. Nuclear localization signals (NLS) and nuclear export signal (NES) are represented by white and black triangles, respectively. Octagons show activation domains. The N-terminal and the C-terminal domains for SUFU binding as well as the Degron domains (white boxes) are also shown.

SUFU can inhibit GLI1 activity by binding both to its N-terminal and C-terminal domains (Figure 3) and sequesters GLI in the cytoplasm. Since GLI1ΔN lacks the N-terminal SUFU binding domain, it may therefore be less inhibited by SUFU and, thus, could be a more active variant. To test this, we compared the GLI1 isoform activity and localization pattern in the presence of SUFU. Indeed, we found that SUFU had weaker repression effects on GLI1ΔN compared to GLI1FL. Also, GLI1FL exhibited a stronger cytoplasmic retention by SUFU, which is supporting the interaction of SUFU to the N-terminal of GLI1.

GLI1 localization and activity are also regulated by the dual-specificity tyrosine phosphorylation-regulated kinase 1 (Dyrk1) by retaining GLI in nucleus (Mao et al., 2002). We analysed the effect of Dyrk1 on the transcriptional activity of GLI1ΔN, and

found that Dyrk1 enhanced activity of GLI1FL but not GLI1 Δ N. Furthermore, localization studies showed GLI1FL to be preferentially accumulated in the nucleus. Our results clearly indicate that the Dyrk1 kinase differently regulates the activity of the GLI1 isoforms.

Collectively, the identification and functional characterization of GLI1 isoforms reveal a novel and complex mechanism of HH signal transduction.

PAPER IV

A suppressor of fused carboxy terminal variant and its impact on hedgehog signal transduction

Several SUFU splicing variants have been identified, as previously mentioned. We determined the relative abundance of these SUFU variants, but found that the expression levels were generally very low. However, for the previously reported C-terminally deleted isoform, SUFU- Δ C, comparable levels as of the full-length SUFU (SUFU-FL) were seen in some tissues and cell lines. Interestingly, in several rhabdomyosarcoma (RMS) cell lines the SUFU- Δ C isoform was expressed at equal mRNA levels as SUFU-FL (Figure 4).

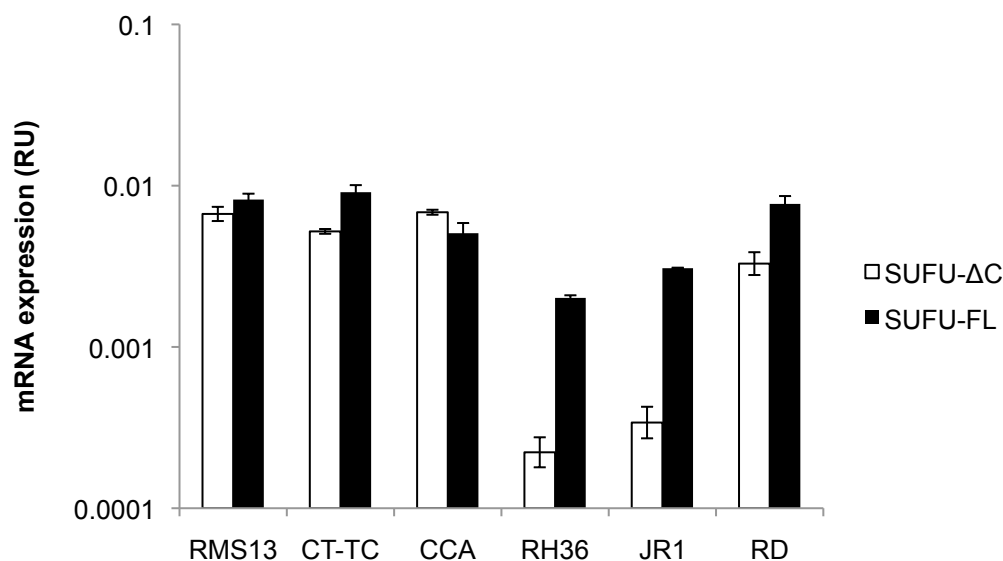


Figure 4. Expression of SUFU-FL and SUFU- Δ C in RMS cell lines.

However, when examining the endogenous protein expression in the RMS cell lines RMS13 and CCA, only very low levels of SUFU- Δ C could be detected. We then evaluated the stability of SUFU- Δ C by two different methods. Heterologous expression of SUFU- Δ C in bacteria revealed that SUFU- Δ C could only be detected in the unsoluble (aggregate) fraction and not in the soluble fraction in contrast to SUFU-FL, indicating that the isoforms are not folded similarly in this setting. Treatment with a specific proteasome inhibitor significantly increased the detectable amounts of SUFU-

ΔC , specifically in the pellet fraction, in comparison to SUFU-FL. Taken together, both assays indicate that the SUFU- ΔC protein is unstable, and is rapidly degraded in the proteasome.

The overexpression of SUFU- ΔC generated two protein bands, corresponding to exogenous and endogenous SUFU- ΔC respectively. It appeared that exogenous SUFU- ΔC upregulates the endogenous SUFU- ΔC levels, which also seemed to be the case for SUFU-FL. Thus, exogenous SUFU proteins might stabilize the corresponding endogenous SUFU variants. To further analyze this stabilizing capacity, co-transfection of SUFU- ΔC and SUFU-FL was performed. In the cotransfected samples the protein expression levels of both exogenous and endogenous SUFU- ΔC and SUFU-FL were further increased, compared to the individually transfected samples.

SUFU act as a negative regulator of HH signaling, and overexpression of SUFU in cell lines inhibits GLI-dependent transcription. We evaluated the repressive function of SUFU- ΔC on GLI activity by reporter assays, and found that SUFU- ΔC could not act as an equally efficient repressor as SUFU-FL in inhibiting GLI1-mediated transcriptional activity. However, when instead of the GLI1FL a GLI variant lacking the N-terminal region (GLI1 ΔN) variant, which encompasses a SUFU binding site, was used comparable repressive effects were noted. Furthermore, both SUFU-FL and SUFU- ΔC were equally capable of inhibiting GLI2 transcriptional activity.

HH signal activation leads to dissociation of SUFU-GLI complex, allowing GLI to enter the nucleus and activate transcription. To determine if HH activation may affect the SUFU repressive effects on GLI, we used Ptch1^{-/-} mouse embryonic fibroblasts, which are characterized by a constitutively active HH signaling pathway. SUFU-FL effectively repressed GLI1FL in this setting, however SUFU- ΔC was more effective than SUFU-FL in repressing the GLI1 ΔN variant.

Thus, our results are suggesting the presence of novel regulatory mechanisms in the HH signaling pathway, which are elicited by the distinct impact of the alternative spliced SUFU proteins on the GLI factors.

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

The HH pathway-related genetic mouse models and the NBCCS patients, as well as the analysis of sporadic RMS/RM in humans, clearly suggest the involvement of an aberrantly activated HH pathway in the etiology of RM and RMS. We were the first research group to analyze the significance of the HH pathway in sporadic RMS, and showed that deregulation of the HH pathway plays a role in the development of RMS tumors. Also other researchers have now shown overexpression of HH-related components in sporadic RMS (Oue et al., 2010; Zibat et al., 2010). Recently, Zibat et al confirmed our findings that HH pathway is specifically activated in the E-RMS subgroup.

A probable scenario is that deregulation of HH signaling, for example through genetic elimination of *PTCH*, in combination with other genetic lesions initiates RM/RMS tumor development. Most tumors keep their dependence on HH activity, but the mechanism could also be SMO-independent and thus GLI1 activity would be maintained through other channels. Several additional pathways, such as TGF- β can cross-talk with HH and directly modify GLI1 activity.

Although LOH of *PTCH* was found to be a relatively common feature in the RMS tumors, accompanied by loss of PTCH protein expression, we failed to detect any mutations. One possible explanation for this could be that epigenetic mechanisms would lead to the silencing of PTCH expression in RMS. Actually, treatment regimens addressing this have already been tried in murine RMS. A combination treatment with an inhibitor of DNA methyltransferase 1 (5-aza-2'deoxyctidine) and an inhibitor of histone deacetylase (valproic acid) prevented the onset of RMS in the *Ptch1* mouse model, suggesting that epigenetic therapy might be beneficial in RMS, even though late stage tumors did not respond very well (Ecke et al., 2009).

Although standard treatments of RMS have improved survival, still many patients suffer from side-effects, and may benefit from more specifically designed therapies. Intervention with the HH pathway could be such an alternative.

Treatment with cyclopamine on *Ptch1*^{+/-} -derived RMS has already been tried. Primary RMS cells from the *Ptch*^{+/-} mice were successfully inhibited by cyclopamine. However, when *in vivo* established tumors were treated with cyclopamine no effect on tumor growth could be seen. This might be due to that tumors have acquired additional genetic lesions during tumor progression, rendering them independent of the initial HH pathway dependency. Alternatively, Smo-independent mechanisms for maintaining Gli activity may have developed during tumor progression. The latter seems to be the case for the E-RMS cell lines we have analysed. Our approach using GLI1 as a target, exemplified by treatment with GANT61, suggests that interfering with GLI1 activity could be a good therapeutic option for pediatric E-RMS. Of course, this needs further evaluation in a more clinical setting. Blocking the GLI factors could also be an advantageous approach in other tumors with a ligand-independent activated HH signaling pathway.

Alternative splicing is a crucial mechanism for generating protein diversity. Normal genetic variation affects splicing, and can alter either the total output of a gene or the ratio of alternatively spliced variants. Alternative splicing is also implicated in pathophysiological processes, and alterations in splicing can cause disease directly, modify the severity of the disease phenotype or be linked with disease susceptibility. Mutations that alter cis-acting splicing elements or activation of signaling pathways that can affect the activity of splicing regulatory factors or modify the balance between them can also change the proportions of mRNA splicing isoforms. This can lead to deregulation of crucial cellular processes such as proliferation, differentiation, death, motility and invasion, all of which contribute to cancer formation. A full understanding of alternative splicing regulation in cancer will require additional discoveries in this area.

Splice variants of several key components of the HH pathway exist, as previously mentioned. Interestingly, genome-wide RNAi screening indicated that splicing has a significant role in HH signaling regulation, since a large number of splicing and RNA-regulatory proteins were found to function as positive regulators of HH signaling (Nybakken et al., 2005).

In a recent publication Lo et al detected a GLI1 variant that has lost exon 3 and partly exon 4 (Lo et al., 2009). This isoform was not detected in normal tissues but was highly expressed in malignant glioblastoma. This reflects a differential signal outcome regulated by a change in isoform expression in the tumor, resulting in a growth advantage. In the case of the GLI1 Δ N isoform we have identified, expression was comparable to GLI1FL in normal tissues and variable in tumor cell lines, and GLI1 Δ N was generally less potent than GLI1FL in its activating capacity. This could be of importance for fine-tuning the HH pathway activity, for example during development, but could also be used by some tumors to modify the signaling outputs of the HH pathway.

We found GLI1 to be the most important GLI factor for E-RMS tumor growth. Novel regulatory mechanisms caused by splicing of SUFU revealed that SUFU-FL has generally a higher capacity than SUFU- Δ C in inhibiting GLI1 activity. During conditions of activated HH signaling, SUFU- Δ C was more effective in repressing GLI1 Δ N than SUFU-FL. Thus, it is possible that the regulation of GLI1 is context-dependent, and subject to alternative splicing. The regulation of GLI1 is central for the cells, GLI1 activity is normally inhibited, but in tumor cells the activity of GLI1 is upregulated. So far several ways to control GLI1 activation are known (Figure 5), and many more will undoubtedly be unfolded in the future.

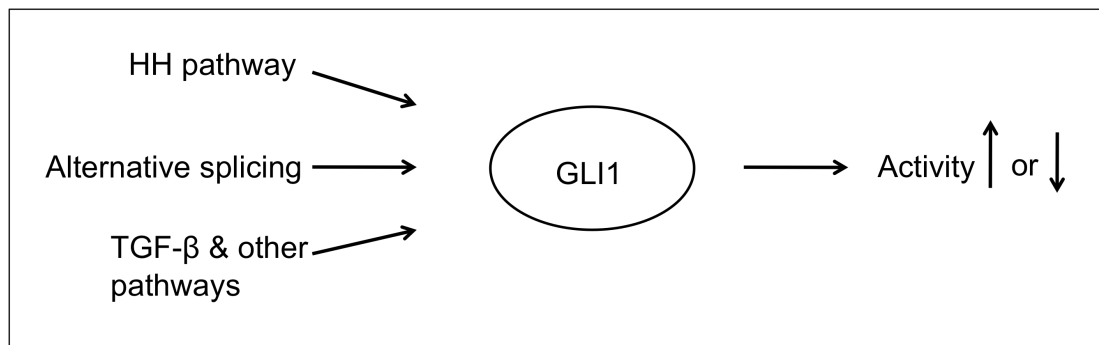


Figure 5. A model of differential GLI1 regulation, leading to increased or reduced activity.

In conclusion, the impact of alternative splicing is emerging as an important element that modulates HH signaling outcomes, both during development and tumorigenesis, and it will be interesting to follow the progress of this field in the future.

SAMMANFATTNING PÅ SVENSKA

Under utvecklingen från ett embryo till en vuxen individ bildas en mängd olika celltyper som i sin tur bygger upp fungerande vävnader och organ, en process som kräver ytterst strikt reglering. Kommunikationen mellan olika celler är avgörande för att de ska veta vilken typ av vävnad/organ de ska bilda. Denna kommunikation kan ske genom att en cell producerar och utsöndrar signalämnen som kan binda till receptorer på andra cellers yta, och därmed orsaka förändringar i mottagarcellerna. Ett sådant signalämne som är viktigt under embryoutvecklingen är Hedgehog (HH). I cellen som mottar HH signalen sätts en komplicerad signalkedja igång, som i slutänden reglerar celldelning, beslut om cellens specificering eller celldöd. Det har visats att felreglering av HH signalvägen, till exempel på grund av mutationer i gener som kodar för proteiner i denna signalkedja, kan leda till cancer och bland annat ligger bakom utvecklingen av den vanligaste hudtumörsjukdomen basalcellscancer.

Receptorn för HH heter patched (PTCH). När HH binder till PTCH medför detta att ett annat membranbundet protein, smoothened (SMO) kan skicka en signal vidare in i cellen. Denna signal leder i slutänden till att GLI-proteinet frigörs från en negativ reglering av ett annat protein, suppressor of fused (SUFU), och kan transporteras till cellkärnan. Både PTCH och SUFU har till uppgift att hindra celldelning i frånvaro av HH, och är således tumörsuppressorgener. Inne i cellkärnan aktiverar GLI en process kallad transkription, då det genetiska materialet kopieras, och därefter omarbetas (translateras) för att bilda ett specifikt protein.

En tumörsjukdom som uppstår i muskler kallas rhabdomyosarkom, och är den vanligast förekommande mjukdelstumören hos barn. Den uppkommer antingen från normala muskelceller eller ifrån förstadier till muskelceller som "blivit kvar" från fosterstadiet. Behandling av rhabdomyosarkom är intensiv cytostatikabehandling, oftast följd av operation och ibland även lokal strålbehandling. Tumörer som uppkommer från de omogna förstadijecellerna är betydligt mer känsliga för cytostatika än tumörer som uppkommer från mogna muskelceller. Det har även betydelse var i kroppen tumören sitter, det är lättare att operera en tumör som sitter ytligt på skulderblad, underben eller liknande till skillnad från en som uppstår i urinblåsa, eller längs ryggraden. Ur behandlingssynpunkt är det alltså viktigt att veta vilken typ av tumör det är, samt var den sitter. Överlevnaden för barn med rhabdomyosarkom har förbättrats markant det senaste decenniet, och omkring 75% av barnen med rhabdomyosarkom blir friska.

Syftet med denna avhandling var att undersöka vilken roll HH signalkedjan spelar i uppkomsten av rhabdomyosarkom, och om tumörerna fortsätter att vara beroende av HH signalering i ett senare skede av tumörtillväxten. Vidare har målet varit att studera olika alternativa genvarianter i HH signalvägen, och deras roll i regleringen av signalkedjan.

I arbete I har vi visat att sporadiska, d.v.s. ej ärvda, rhabdomyosarkom har en överaktiv HH signalkedja. Vi har även visat att dessa patienter ofta har tappat en kopia av tumörsuppressorgenerna *PTCH* och *SUFU*, vilket kan bidra till uppkomsten av dessa tumörer.

I arbete II ville vi undersöka om HH signalkedjan fortsätter att spela en betydande roll för rhabdomyosarkomens tillväxt. Vi använde små molekyler, som har visat sig effektiva i att förhindra att signalkedjan aktiveras. När vi behandlade tumörceller i kultur med dessa små molekyler slutade de att växa eller dog.

Alternativ splitsning är en process som gör att olika varianter av proteiner kan produceras från en enda gen. En gen består av kodande (exon) och icke kodande (intron) delar. Den genetiska informationen översätts först till en RNA-sekvens, som i sin tur är underlaget för proteinsekvensen. Till en början finns både exon och intron med i denna RNA-sekvens, och innan translationen till protein sker kommer intron sekvenser att klippas bort i en process som kallas splitsning. På så vis sammanfogas exonsekvenserna till en sammanhängande RNA-sträng, som kallas för ett moget budbärar-RNA (mRNA). Antalet exon som ingår i ett mRNA kan variera och därmed ge upphov till alternativa mRNA varianter. En konsekvens av den alternativa splitsningen är att en och samma gen kan ge upphov till flera proteiner, ibland med vitt skilda funktioner. Det har visat sig att detta fenomen är vanligare än man tidigare trott; hos människan uppskattar man nu att de flesta generna är alternativt splitsade. I tumörer har många normala processer satts ur spel, och ett annorlunda splitsningsmönster kan uppstå.

I arbete III beskriver vi vår upptäckt av en ny variant av en av GLI-faktorerna, benämnd GLI1ΔN, som är alternativt splitsad och saknar en del av proteinets början. Vi har visat att den generellt har en sämre förmåga att aktivera signalvägen än full-längds-GLI1, utom i vissa situationer då den till och med var bättre.

I arbete IV har vi studerat en sedan tidigare känd variant av SUFU, som saknar den sista biten av proteinet. Funktionen av denna variant är ej känd, men vi har nu visat att den är mindre stabil än full-längds-proteinet, och kan ha en modulerande effekt på regleringen av GLI1, och även av uttrycket av sig själv.

Sammanfattningsvis kan man säga att regleringen av HH signaleringsvägen är mycket komplex, och en mängd olika regleringssteg har visat sig viktiga för normal funktion. Arbetet i denna avhandling har resulterat i en ökad förståelse för några av dessa regleringssteg, och även visat att HH signalvägen spelar en betydande roll för tillväxten av embryonala rhabdomyosarkom. Denna kunskap kommer förhoppningsvis att kunna leda till effektivare behandling av embryonala rhabdomyosarkom med läkemedel som specifikt blockerar GLI faktorerna.

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