The GPCR-like protein Smoothened involved in the Hedgehog pathway:

Signalling properties of variants resulting from somatic mutations

Thesis in pharmacology

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by

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ABBREVIATIONS

Abbreviations	Full name
A_{260}	Absorbance at 260 nm
A_{280}	Absorbance at 280 nm
Arg/Lys	Arginine/Lysine
ATCC	American Type Culture Collection
BCA	Bicinchoninic acid
BCC	Basal cell carcinoma
bp	basepair
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
C_i	Cupitus interruptus
CNS	Central nervous system
COSMIC	Catalouge of somatic mutations in cancer
CRD	Cysteine-rich domain
C_t	Cycle threshold
C-terminal	Carboxy terminal
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
Dhh	Desert hedgehog
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleoside triphosphate
DTT	Dithiothreitol, Cleland`s reagent
293 EcR	Epithelial kidney cells
FRET	Fluorescence resonance energy transfer.
Fu	Fused
FW	Forward
G-protein	Guanine nucleotide binding protein
GAPDH	Glyceraldehyde-3-phosphate-dehydrogenase
GPCR	G-protein-coupled receptor
Hh	Hedgehog
Hip	Hedgehog interacting protein
HRP	Horseradish peroxidase
hSmo	Human Smoothened
Ihh	Indian hedgehog
kb	kilobase
LB	Luria Bertani
mGli	Mouse Gli
mPtch	Mouse Patched
mRNA	Messenger RNA
NBCCS	Nevoid basal cell carcinoma syndrome
NCS	Newborn calf serum
NIH/3T3	Mouse embryonic fibroblast cells
N-terminal	Amino terminal
OD	Optical density

Ptch	Patched
PCR	Polymerase chain reaction
Rev	Reverse
RNA	Ribonucleic acid
RNase	Ribonuclease
rRNA	Ribosomal RNA
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase polymerase chain reaction
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
Shh	Sonic hedgehog
Smo	Smoothened
Sufu	Suppressor of Fused
Tb-p	TATA box binding protein
7TM	Seven transmembrane
UV-light	Ultra violet light
wt	Wild type

1. ABSTRACT

The Hedgehog (Hh) signalling pathway is essential for numerous processes during embryonic development including development of the skin and pancreas. Dysregulation of Hh-signalling during development might be lethal and may lead to cancer in adult cells. This pathway is important in several types of cancers including cancers in the pancreas, brain and skin. The best known examples of excessive Hh-signalling causing cancer are the frequent mutations and dysregulation observed in basal cell carcinoma (BCC), a type of skin cancer. The Hh-pathway is considered as a very promising target for new anti-cancer treatments and increased knowledge of this pathway may lead to new strategies of cancer treatment.

Hedgehog (Hh), Patched (Ptch), Smoothened (Smo), Suppressor of fused (Sufu), fused (Fu) and Gli are signalling proteins in the Hh-pathway. Mutations in genes encoding these members of the Hh signalling pathway may lead to cancer. Smoothened is a seven transmembrane protein which has been studied in this current project and shows several characteristics of being a G-protein-coupled receptor (GPCR). Fourteen somatic mutations in Smo have been identified. Five of these mutations have been analysed herein and one of these mutations was generated as part of the thesis work (K575M-Smo).

First, PCR-based mutagenesis was performed to generate a mutated version of Smo, K575M-Smo. Subsequently the PCR-product containing K575M-Smo was inserted into the pEF.6 vector.

Secondly, K575M-Smo, four other mutants (R484W-, L514F-, S533N- and W535L-Smo) and Smo wt were sub-cloned from the pEF.6 vector into the mammalian expression vector p3xFLAG-CMV-10.

NIH/3T3 mouse fibroblast cells were stably transfected with the following constructs: p3xFLAG-CMV-10 vector (empty vector control), p3xFLAG-CMV-10 R484W-, L514F-, S533N-, W535L-, K575M-Smo and Smo wt, to analyze the signalling properties of the different mutated versions of Smo. From each transfection, six independent monoclonal cell lines potentially harbouring the transfected plasmid were isolated, and different experiments were performed, including real-time reverse transcriptase polymerase chain reaction (RT-PCR), Western blotting and reporter gene assay.

Taken together, the K575M-Smo encoding plasmid was successfully generated in the laboratory. Initial experiments showed a trend of constitutive activity of W535L-Smo. Based on the variable data from real-time RT-PCR experiments, it is not possible to make conclusions about the signalling properties of the various Smo mutations, and further studies are needed.

2. BACKGROUND

2.1 CANCER

Cancer (malignant neoplasm*) is a class of diseases where a group of cells undergo uncontrolled multiplication and may spread within the body. Cancer may affect people at all ages, but the risk of developing cancers increases with age. Nearly all cancers are caused by abnormalities in the genetic material of the cells, but cancer may also occur from environmental factors as smoking and sun-exposure (UV-light) on skin. The major cause of death in the developed nations is cancer, and at least one in five of the population of Europe and North America can expect to die from cancer. Genetic changes that lead to cancer are activation of proto-oncogenes** to oncogenes, and inactivation of tumour suppressor genes. These changes are result of point mutations, gene amplification, loss of heterozygosity or chromosomal translocation [1].

*Neoplasm means "new growth". ** Oncogene is a gene that helps turning a normal cell into a cancer cell due to mutations or increased expression.

2.1.1 Hedgehog signalling in cancer

The Hedgehog (Hh) signalling pathway is required for cell proliferation and differentiation, and plays a central role during embryogenesis and during development of the foetus. For the most part the activity of the pathway is reduced after embryogenesis, but there are examples of pathway activity in some adult tissues, like adult stem cells in the brain, pancreas and skin. Abnormalities like sporadic mutations in the Hh-pathway during development can be lethal and may lead to cancer in adult cells. The best known examples of excessive Hh-signalling causing cancer are the frequent mutations and dysregulation observed in the most common form of skin cancer, basal cell carcinoma (BCC) [2-4].

The Hh signalling pathway was confirmed linked to tumourigenesis in 1996 when a human homologue of *Drosophila* patched (Ptch1), a component in the Hh-pathway, was found to be mutated in nevoid basal cell carcinoma syndrome (NBCCS), also known as Gorlin's syndrome [5, 6]. Gorlin`s syndrome is an autosomal congenital condition characterised by

the presence of multiple BCCs of the skin, abnormalities in facial and skeletal development, and a predisposition to medulloblastoma and rhabdomyosarcoma [5].

Point mutations in genes encoding members of the Hh signalling pathway may lead to dysregulation of the signalling pathway and causes cancer in different tissues like skin, pancreas, brain and breast [7] (Figure 2.1).

Cancer types containing mutations in Hh-pathway molecules include [7]:

- Basal cell carcinoma (BCC)
- Medulloblastoma
- Rhabdomyosarcoma

Cancer types containing dysregulation of Hh-signalling are present in [7]:

- Breast cancer
- Pancreas cancer
- Lung cancer
- Ovary cancer
- Oesophagus cancer
- Stomach cancer
- Liver cancer
- Prostate cancer
- Colon cancer
- Glioma [8]

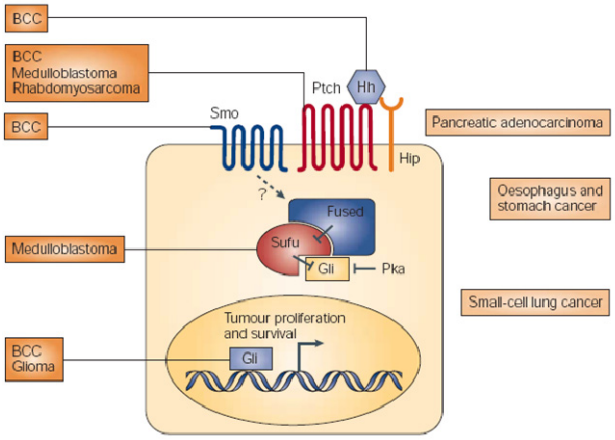


Figure 2.1: Overview of different types of cancer caused by dysregulation of Hh-signalling. Dysregulation of Hh-signalling causes cancer in different tissues (Adopted from Pasca di Magliano and Hebrok, Nat Rev Cancer, 2003 [3]).

Skin cancer is the most common cancer in the Western world and BCCs account for 90% of all skin cancers. Sporadic BCCs are rarely found before the age of 20 years but thereafter the risk of developing BCCs increases with age [9]. The current understanding of the development of BCCs has increased with the understanding of mutations that are known to activate Hh signalling pathways genes related to BCC [10]. In BCCs there are identified mutations in several components of the Hh-pathway (like Hedgehog (Hh), Smoothened (Smo) and Patched (Ptch) genes) resulting in aberrant Hh-signalling [9].

2.2 THE HEDGEHOG SIGNALLING PATHWAY

The Hh-pathway takes its name from the polypeptide ligand activating the pathway, the intercellular signalling protein called Hedgehog, which was first discovered in the two-winged insect *Drosophila melanogaster* [11-13]. The main differences between the Hh-pathway in insects and vertebrates are the increasing numbers of related genes present in vertebrates, and the Hh-pathway in vertebrates is less studied and more complex than in insects [2, 14-17]. In vertebrates (e.g. human and mouse) there are currently identified three Hh proteins (called Sonic (Shh), Indian (Ihh) and Desert (Dhh)), two patched genes (called Ptch1, Ptch2) and three *cubitus interruptus*-like proteins (called Gli1-3) [2, 13, 18, 19]. *Cubitus inerruptus* (Ci) is a gene regulatory protein present in *Drosophila* [4]. Shh is the most potent of the three ligands and most frequently expressed in embryonic and adult tissues [8, 20, 21].

In this thesis, the term Hh will be used to refer to all three Hh isoforms. If there are different effects of the three isoforms, Shh will be considered the reference substance if not otherwise stated. The experiments in this project are performed in mouse fibroblast cells, thus the main focus of this thesis will be the vertebrate Hh signalling pathway, although most of what is known about the downstream signalling of the Hh-pathway comes from genetic studies in flies [4]. Additionaly, Smoothened (Smo), a component in the Hh-pathway, is analysed in this project and will be described in detail in this thesis.

The Hh-pathway is activated when Hh (ligand) binds to its receptor, Patched (Ptch) (Figure 2.2). In the absence of ligand, Ptch normally functions as a tumour suppressor that binds and represses Smo from activating downstream components and transcription of target genes (Figure 2.2; left). Ligand binding to Ptch results in de-repression of Smo which activates the

transcriptions factor Gli, allowing Gli to enter into the nucleus and regulate the expression of its target genes (Figure 2.2; right) [3].

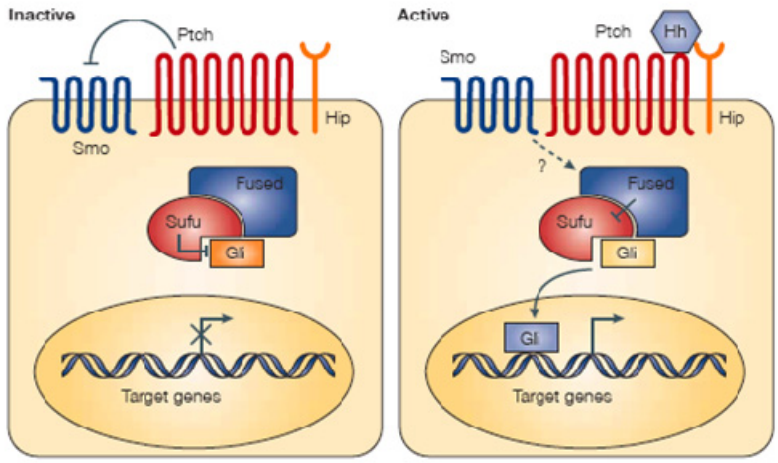


Figure 2.2: Hedgehog signalling pathway. Left figure shows the signalling pathway in the absence of Hh-ligand. Hh-ligand-dependent activation of the pathway is shown in the right panel (Adopted from Pasca di Magliano and Hebrok, Nat Rev Cancer, 2003 [3]).

Hedgehog (Hh; Fig. 2.2) is an unusual secreted protein which undergoes autocatalytic cleavage and covalent lipid modifications [5]. Hh plays an essential role in the Hh signalling pathway involved in cell type specification, pattering and the regulation of cell proliferation and differentiation in almost all tissue types during development [9]. The active form of Shh is unusual in that it is covalently coupled to cholesterol. The cholesterol becomes attached to Shh during the autocatalytic cleavage process [4]. The protein is also modified by the addition of a fatty acid chain, which also is required for its signalling activity. Shh is able to signal at short-range as well as at long-range in multiple context, which is controlled by the lipid modification of the Hh molecule (See section 2.2.1) [22, 23]. Two transmembrane proteins, Patched (Ptch) and Smoothened (Smo), mediate the response to Hh [24].

Patched (Ptch; Fig. 2.2) is a twelve-pass transmembrane protein and binds Hh with high affinity [25, 26]. The two isoforms Ptch 1 and Ptch 2 will be referred to as Ptch in this thesis. In the absence of a Hh signal, Ptch inhibits the activity of Smo (Figure 2.2., left), and this inhibition is repealed when Hh binds to Ptch (Figure 2.2; right) [4]. Ptch represses transcription of Hh target gene and is considered to be a tumour suppressor gene [3, 8, 15]. Mutation in the Ptch gene may lead to excessive Hh-signalling, which may cause cancer [24]. BCC may be a consequence of mutations in the gene encoding Ptch, and have been identified in at least half of all sporadic BCC [27].

Smoothened (Smo; Fig. 2.2) is a seven-pass transmembrane protein and a transducer of Hh-signalling, resulting in activation of downstream components of the Hh-pathway (Figure 2.2; right). Smo shows several characteristics of being a G-protein-coupled receptor (GPCR). However the Smo protein does not bind ligands (read: Hh) [25, 28-30]. Unregulated Smo-signalling may cause cell proliferation, and constitutively active variants of Smo may be oncogenic, and cause cancer, e.g. BCC [3].

Gli (Fig. 2.2): Three vertebrate Gli genes have been identified; Gli1, Gli2 and Gli3 [13, 18, 19]. The Gli proteins are zinc-finger transcription factors and downstream mediators of the Hh response, just like *Cubitus interruptus* (Ci) in *Drosophila* [25, 31, 32]. Gli1-3 exhibit distinct repressor and activator functions depending on cellular context [3, 33]. Gli1 is mainly a transcriptional activator, and is therefore upregulated in response to Hh [25, 34]. Gli2 and Gli3 contain potent repressor domains and are proteolytically cleaved to forms having either activator or repressor function [5, 35]. The proteolytic processing of Gli depends on a large multiprotein complex. The complex contains the serine/threonine kinase Fused (Fu) and an adaptor protein called Suppressor of Fused (Sufu). This complex is located in the cytosol of the vertebrate cells [3, 4, 34]. Ectopic expression of Gli has been shown to cause glioma, a CNS tumour derived from glia cells [8].

Fused (Fu; Fig.2.2): The gene encodes a serine-threonine kinase that functions as a positive regulator of the transcription factors of the Gli-family in the Hh-pathway [5, 36]. Fu is required for the regulation of the Hh-pathway, but the mechanistic role of Fu remains unknown [37].

Suppressor of Fused (Sufu; Fig. 2.2) is a negative regulator of Gli-activity in the Hh-pathway [38-41]. Sufu is thought to be important for the shuttling of the Gli proteins between the cytoplasm and nucleus [9]. Sufu is a tumour-suppressor gene interacting with Fu and Gli [40, 42]. Loss-of-function mutations in Sufu have been identified in some medulloblastoma cells, a tumour in CNS [38].

Hedgehog interacting protein (Hip; Fig. 2.2) is an inhibitor of Hh-signalling only found in vertebrates. Hip encodes a membrane-bound glycoprotein that binds Hh, appearing to be a negative regulator of Hh-signalling as overexpression of Hip in vertebrates reduces the Hh signalling pathway activity [43].

2.2.1 Maturation of Sonic hedgehog protein (Shh)

The Hh-family of secreted proteins are subjected to different autoprocessing and posttranslational modifications in order to be fully activated [9, 44]. Shh is encoded by a single gene and undergoes autocatalytic cleavage as well as covalent lipid modification [5]. The autocatalytic protein is synthesized as a 45 kilodalton (kDa) precursor protein which is divided into two domains by intramolecular cleavage (Figure 2.3).

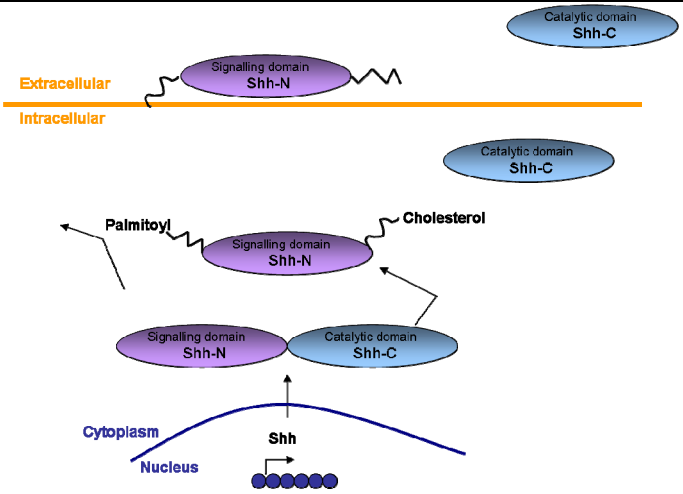


Figure 2.3: Maturation of the Shh protein (Modified figure from Daya-Grosjean *et al*, Cancer Letters, 2005 [9]).

The C-terminal portion of the precursor catalyzes the catalytic process, generating a 19 kDa N-terminal signalling domain (Shh-N) and a 25 kDa C-terminal domain (Shh-C). Shh-C has no known function other than catalyzing the autoproteolytic cleavage. During the autocleavage process, a cholesterol moiety is covalently attached to the last amino acid of Shh-N (Figure 2.3), increasing the hydrophobicity. The hydrophobic cholesterol moiety is thought to bind Shh to cell membranes. The hydrophobicity of the signal domain is further increased by attaching another lipid moiety (palmitoyl) to the highly conserved N-terminal cysteine residue which is dependent on the cholesterol addition [9, 43, 44]. The Shh-N signal domain may either be attached to the plasma membrane of the producing cell or transported to responding cells through different mechanisms. Shh acts in an autocrine manner, affecting the cells in which it is produced, as well as in a paracrine manner, affecting cells positioned next to the producing cells. Shh can also diffuse for long range signalling through extracellular space towards responsive cells by interacting with heparan sulphate proteoglycans, which also allow presentation of Shh to receptors on target cells [7, 9, 15, 45,

46]. Another mechanism for long range signalling of Shh is presented in an article of Zeng *et al* [47]. They showed that the Shh signal peptide multimerises at lipid rafts with the lipid adducts sequestered at the centers, rendering it soluble and able to diffuse for long range signalling [9, 47]. Another protein involved in regulating Shh-signalling is the Hh-interacting protein (Hip), which is a membrane bound glycoprotein that binds Shh and moderates its response. Hip has a binding affinity for Shh comparable to that of Ptch, suggesting the existence of an additional negative feedback mechanism for regulating responses to Shh-signalling [48].

2.2.2 The Hedgehog signalling cascade

The exact details of the Hh signalling pathway are still not fully understood, but the current model proposes that the Hh signalling cascade is initiated by Hh-binding to the Ptch transmembrane protein expressed on the responsive cells. Ptch inhibits the activity of Smo, presumably by preventing translocation of Smo to the primary cilium at the cell surface of vertebrate cells. The Ptch-mediated inhibition is repealed following ligand binding. In the presence of ligand, Smo becomes active and initiates the signalling cascade that results in the activation of the Gli transcription factors (Figure 2.2; right) [3, 7, 9, 22, 49]. The mechanisms by which the activation of Smo is translated into signals that converge on the Gli transcription are not fully understood. It is known that once the Hh-pathway is activated, the interactions between Smo and the cytoplasmic multiprotein complex formed by Fu, Sufu and Gli are dispersed, allowing Gli to enter the nucleus, where it may bind DNA and regulate the expression of its target genes [2, 3, 7, 50]. The genes targeted by Hh have been identified to include Gli1, Ptch1 and Hip. Gli1 serves as a positive feedback for the Hh-pathway, and Ptch1 and Hip results in negative feedback [23]. Genetically, Hh, Smo, and Fu are positively acting components in the pathway, whereas Ptch, Hip and Sufu have a negative role [5].

2.3 G-PROTEIN-COUPLED RECEPTORS

G-protein-coupled receptors (GPCRs) are one of the largest known families of membrane signalling proteins, and are also known as metabotropic receptors or seven-transmembrane (heptahelical) receptors, according to their secondary and tertiary structures. Through

conformational changes, GPCRs transmit extracellular ligand-induced signals to the intracellular environment through activation of one or more heterotrimeric G-proteins that subsequently regulate intracellular enzymes. The GPCRs are involved in a wide variety of physiological functions and are one of the major targets for currently used drugs and new drug development [51, 52]. Among membrane-bound receptors, the GPCR family is certainly the most diverse. The GPCR superfamily consists of more than 1000 members and the receptors are divided into subfamilies based on sequence homology, ligand structure, and receptor function. As mentioned previously Smo resembles a G-protein-coupled receptor, and activating Smo mutations may lead to unregulated activation of the Hh-pathway and a physiological consequence may be cancer formation [7]. Increased knowledge of the GPCR-like protein Smo and mutated variants of Smo will extend our understanding of the physiological and pathological processes regulated by the Hh signalling pathway. The Hh-pathway is considered as a very promising target for new anti-cancer treatments and increased knowledge of Hh/Smo/Gli-mediated signalling may lead to new strategies of cancer treatment.

2.3.1 Structure and classification of G-protein-coupled receptors

Based on certain conserved amino acid sequences three major families (I, II and III) and several subfamilies of GPCRs have been defined [53]. Family I, the largest group, includes receptors related to rhodospin, comprising most monoamine and neuropeptide receptors. Family II contains receptors related to the secretin/glucagon/calcitonin receptors, and family III is the smallest group related to the metabotropic glutamate receptors [54, 55]. The different families of GPCRs share essentially no sequence similarities, but they all share some common features. GPCRs consist of a single polypeptide chain that have an extracellular amino terminal (N-terminal) domain, a central core domain and an intracellular carboxy terminal (C-terminal) domain. The central core domain consists of seven transmembrane (7TM) α-helices, which are linked by alternating intracellular (i1-i3) and extracllular (e1-e3) loops (Figure 2.4).

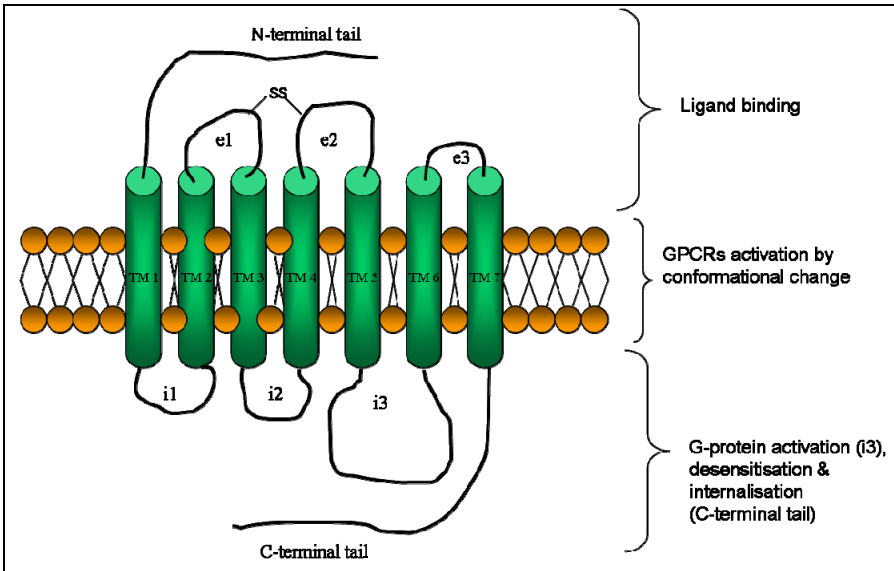


Figure 2.4: General secondary structure of GPCRs. The seven transmembrane spanning domains are linked by alternating intracellular (i1-i3) and extracellular (e1-e3) loops. The N-terminal tail of the receptor is extracellular and the C-terminal tail is intracellular (Modified figure from Hollmann M.W. *et al*, Anesthesiology, 2005 [51]).

In addition to sequence variations, GPCRs differ in their length of the 7TM α-helices, the extracellular N-terminal domain, the intracellular C-terminal domain and their intracellular loops, as well as functions of these domains [51]. Among the extracellular loops e1 has the most steady loop size and the other two extracellular loops (e2 and e3) have more variable sizes. Most of the GPCRs have two cysteine residues in the e1 and e2 forming a disulfide bond important for the packing and stabilisation of the native receptor conformation [56]. The extracellular receptor surface varies substantially among different GPCRs. This area consisting of the N-terminal domain, the extracellular loops and the extracellular portions of the 7TM α-helices is important for ligand binding (Figure 2.4). The intracellular receptor surface consisting of the C-terminal domain and the intracellular loops are involved in G-protein recognition and activation (Figure 2.4) [54]. The i3 is larger than the other loops and interacts with the G-protein. G-proteins are intracellular membrane associated proteins consisting of three subunits, the αβγ-heterotrimer. They are called G-proteins because of the α-subunits interaction with the guanine nucleotides GTP and GDP. Activated G-proteins regulate intracellular enzymes. The C-terminal tail is also involved in controlling desensitisation and internalisation of the receptors (Figure 2.4), due to phosphorylation and palmitoylation sites located in the tail [51]. Desensitisation is loss of functional response from an agonist activated receptor, and internalisation is reduction in surface receptor number, determined by a combination of the effects of endocytosis and recycling [57]. A

conformational change of the 7TM core regions is probably responsible for activation of GPCRs [1, 56, 58].

2.3.2 GPCR constitutive activity

GPCRs may spontaneously isomerise from inactive to active receptor conformation, leading to agonist-independent activation of heterotrimeric G-proteins. When GPCRs are active in absence of ligand they are called constitutively active receptors. Somatic mutations in GPCRs may lead to constitutively active receptors, and mutated GPCR genes may be disease-causing through the expression of inactive or constitutively active receptors. These mutations appear to decrease the energy barrier required for the isomerisation from the inactive to the active state of the receptor, thus causing increased signalling in the absence of ligand. Mutations causing increased ligand independent activity of GPCRs are frequently located in the intracellular part of the seventh transmembrane domain, but other locations for such mutations have also been reported [59-62].

2.3.3 Smoothened as a GPCR-like protein

Smo is related to the GPCR family II. Smo is a therapeutic target for several candidate drugs in the treatment of Hh-related diseases [63]. The Smo GPCR-like protein is essential for transduction of the Hh-signal across the cell membrane.

The Hh signalling pathway differs from the common mechanisms of GPCR activation, because Smo lacks the ability to directly interact with the ligand. Smo uses Ptch as the receptor for the Hh-ligand. Ligand binding to Ptch releases Smo from Ptch inhibition inducing a change in conformation of Smo that may lead to coupling to and activation of G-proteins. The third intracellular loop and the seventh transmembrane domain of Smo are important for coupling to heterotrimeric G-proteins. Mutations in these regions may cause Smo to become constitutively active [2]. One mutation studied in this thesis (W535L-Smo) has been shown to result in constitutively active Smo. The W535L mutation resides in the seventh transmembrane region of the Smo protein (Figure 2.5) [5, 25]. The mechanisms by which the activation of Smo is translated into signals that converge on the Gli transcription factors are not fully understood [50].

Smo has a long extra-cellular N-terminal domain about 250 amino acids long, containing a conserved cysteine-rich domain (CRD). The cysteines in this domain are essential for achieving the correct tertiary structure, and play an important role in Smo-regulation. Occurrence of correct disulphide bridges within the CRD is required to accomplish Smo-activity. The disulphide bridges are also required for the interaction between Smo and Ptch [2, 55, 56].

Traditionally, GPCRs have been thought to act as monomers, but it is now also accepted that GPCRs may exist as multimers. How the GPCR-like protein Smo is activated remains poorly understood, but fluorescence resonance energy transfer (FRET) studies suggest that Smo may exist as a constitutive dimer and that Hh induces a conformational change, leading to increased proximity of the two Smo C-terminal tails. The Smo C-terminal tail is essential in activating Smo. Smo proteins contain multiple conserved clusters of basic residues in their C-terminal tails, including a long stretch of arginine-lysine (Arg/Lys) residues in the middle region. Mutating this long stretch of Arg/Lys residues to alanine (Ala) may result in constitutively active Smo. Smo may employ an Arg/Lys-cluster to regulate its conformation and activity [64]. The K575M-Smo mutation studied in this thesis is located in this lysine-rich stretch of the Smo C-terminal tail. Finally, the activated Smo-dimers would interact with the Fu-Sufu-Gli complex, inducing Sufu phosphorylation by Fu, and activation of the Gli transcription factors [64].

2.4 MUTAGENESIS IN SMOOTHENED

Presently, fourteen somatic mutations are identified in Smo (Table 2.1). Two additional mutations have also been reported. These mutations have not been included in table 2.1, because one of the mutations (A404A-Smo) is silent, and the R168H-Smo mutation described by Yan T. *et al* [65] may not represent a somatic mutation and is not registered in the Catalogue Of Somatic Mutations In Cancer (COSMIC) database. The identified somatic mutations in Smo are located throughout the entire protein. Many of the mutations are typically UV-light induced mutations comprising C→T or CC→TT mutations. Most of the mutations in Smo have been identified in BCCs and the main risk factor for BCC is sun light exposure, especially for those with a lighter skin. The somatic mutations in Smo are also identified in the large intestine and CNS. The tumours containing the mutations are all

carcinomas, except mutation S533N, which is identified in medulloblastoma. According to the model of Hh-signalling, loss-of-function mutations in Ptch will de-repress Smo, leading to constitutive activation of the pathway. The same result would be obtained by gain-of-function mutations in Smo. Such activating mutations have been found in BCC and medulloblastomas [5, 9].

2.5 AIMS OF THE PRESENT STUDY

This study is part of a broader project, aiming at analysing the signalling properties of somatic mutations found in Smo. Specifically, one mutated version of Smo (K575M-Smo) will be generated in the laboratory and the signalling properties of this and four additional mutated version of Smo will be analysed.

The following five mutated variants of Smo will be studied in the current project: R484W-, L514F-, S533N-, W535L- and K575M-Smo (Figure 2.5; Table 2.1). R484W and L514F-Smo are UV-light induced mutations (CC→TT mutations) identified in BCC and located in extracellular loop 3 (e3) [66]. The e3 loop in GPCRs is known to be responsible for ligand binding [54]. However, since Smo does not bind the ligand activating the pathway, the effect of these mutations will be difficult to predict [67]. The e3 in Smo may be responsible for the Ptch interaction and the two mutations situated in this extracellular loop may alter the signalling properties of Smo. Two of the mutations which will be analysed in the laboratory are located in the seventh transmembrane domain; S533N- and W535L-Smo [65]. Reifenberger J *et al*. [67] identified the S533N-Smo mutation in a primitive neuroectodermal tumour (PNET), a G→A mutation at nucleotide 1598 that leads to the exchange from serine to aspargine at codon 533 [66, 67]. The W535L-Smo mutation has been identified in four different studies addressing Smo modifications in sporadic BCCs [66-69]. The W535L-Smo mutation is a UV-light induced mutation (C→T mutation) and known to be constitutively active and oncogenic, resulting in cancer in the skin [5, 70, 71]. W535L-Smo has been shown to result in receptors no longer sensitive to Ptch inhibition and thus constitutively active [66-68, 72]. In the majority of GPCRs, the codon corresponding to codon 535 in Smo encodes a conserved tyrosine residue, the function of which is to keep these receptors in a latent state by the formation of a polar pocket. Disruption of this site by mutation of Smo has been shown to result in constitutive Hh-signalling important in BCC development [65].

Furthermore, the homology with GPCRs indicates that the seventh transmembrane domain and the third intracellular loop of the Smo protein are required in the activation of downstream signalling. The W535L-Smo constitutes a hot spot for Smo protein modifications, and it has been shown to cause constitutive activity [69]. The K575M-Smo mutation, which will be generated in the laboratory, was identified by Sicklick J *et al.* [73] in a cirrhotic liver-tumour in a 67-year-old female. The K575M-Smo mutation is an A→T mutation located in the C-terminal tail [73]. The Smo C-terminal tail is essential in activating Smo. The cytoplasmic tail and the intracellular loops of GPCRs are critical domains for the interaction with G-proteins, a step essential in signal transduction. The homology to GPCRs may suggest that the K575M located in the cytoplasmic tail of the Smo protein may alter Smo protein function. Modifications in this region of the protein may also be important in affecting receptor desensitisation or internalisation, as mentioned previously [65].

In summary, it may be expected that the W535L-Smo mutation shows altered signalling properties. The signalling properties of R484W-, L514F-, S533N- and K575M-Smo are previously not known, and the goal of this thesis is to compare these mutated versions of Smo with Smo wildtype and W535L-Smo, the latter one known to show constitutive activity.

Amino acid-mutation (1-letter)	Amino acid-mutation (3-letter)	Basepair mutation	Mutation ID (COSMIC sanger)	Sequence	Primary tissue	Histology subtype
A68V	Ala68Val	203-204CC>TT	13235	GCTGTTCCCT	Skin	BCC
R199W	Arg199Trp	595C>T	13145	CCCTTGGTTTGGAC	Skin	BCC
A324T	Ala324Thr	970G>A	13147	TACTACACCCTGAT	Large intestine, biliary tract	Adenocarcinoma
T349I	Thr349Ile	1046_1047CC>TT	13236	CACCATTTACCAGCC	Skin	BCC
V404M	Val404Met	1210G>A	13148	CGTGCGGGCTTCATGC	Large intestine	Adenocarcinoma
R484W	**Arg484Trp**	**1452-1453CC>TT**	**13237**	**GCAGCTTTTGGGAC**	**Skin**	**BCC**
L514F	**Leu514Phe**	**1542-1543CC>TT**	**13238**	**CCCGAGTTTTCT**	**Skin**	**BCC**
S533N	**Ser533Asn**	**1598G>A**	**13144**	**GCCATGAACACCT**	**CNS**	**Medulloblastoma**
W535L	**Trp535Leu**	**1604G>T**	**13146**	**ACCTTGGTCTGG**	**Skin, upper aerodigestive tract**	**BCC**
R562Q	Arg562Gln	1685G>A	13152	CCAAAGCAGATCA	Skin	BCC
K575M	**Lys575Met**	**1724A>T**	**20769**	**TTCTCTATGCGGCA**	**Liver**	**Carcinoma**
T640A	Thr640Ala	1918A>G	13150	TTCTGTCGCCCCTGT	Large intestine	Adenocarcinoma
A652V	Ala652Val	1955C>T	13240	GGAACAAGTCAACC	Skin	BCC
P755F	Pro755Phe	2263-2264CC>TT	13239	GGCCTTCGTGGCATGG	Skin	BCC

Table 2.1: The information of the different mutants is taken from the Catalogue Of Somatic Mutations In Cancer (COSMIC) database (http://www.sanger.ac.uk/genetics/CGP/cosmic/). The mutations highlighted in blue and bold have been analysed in this project. The plasmid construct encoding the K575M-Smo (dark blue) was generated as part of the thesis work whereas the mutated constructs in light blue was available.

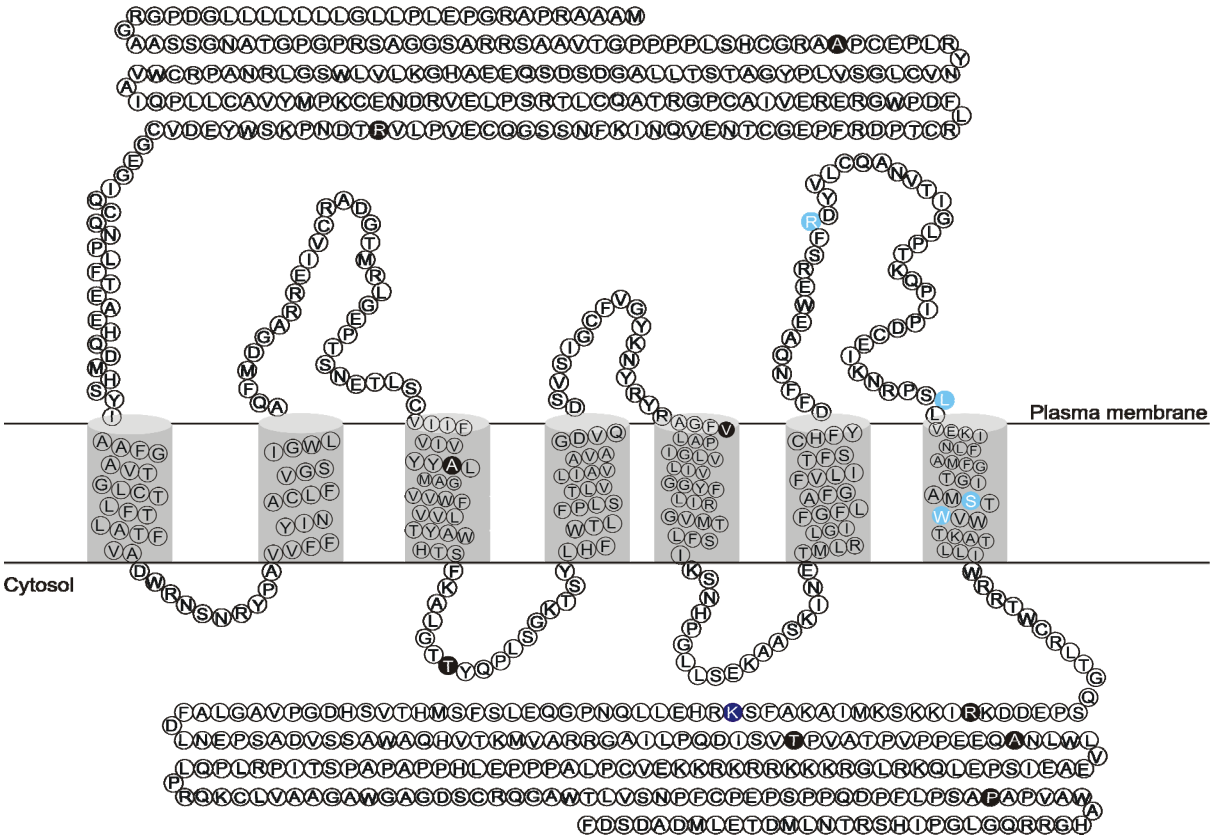


Figure 2.5: Secondary structure of the human Smo-receptor with the identified somatic mutations. The mutations marked in blue have been analysed in this project. The plasmid construct encoding the K575M-Smo marked dark blue was generated as part of the thesis work.

3. METHODS

This chapter describes the methods used in this thesis. Buffers and solutions used in the methods are listed in the appendix (section 8.1 “Materials and recipes”). A general presentation of the methods used in these studies is given in this chapter while more detailed descriptions of the practical approach are presented in the appendix (section 8.2 “Protocols”).

3.1 OVERVIEW METHODS

PCR-based mutagenesis was performed to generate a mutated version of Smoothened (Smo), K575M-Smo. The PCR-product containing the mutated Smo-sequence was digested with specific restriction enzymes and ligated into the equally cut pEF.6 Smo wt plasmid. To amplify the plasmid construct in large scales competent bacteria were transformed and DNA plasmid was isolated and purified. The DNA plasmid were analysed by sequencing. This mutated version of Smo, K575M-Smo, and four other mutants R484W-, L514F-, S533N-, W535L-Smo (which were available in the laboratory), and Smo wt were inserted in the mammalian expression vector p3xFLAG-CMV-10. An EcoRI restriction site was generated in the Smo constructs to accomplish the insertion. These five mutated versions of Smo and Smo wt were digested with EcoRI and XbaI restriction enzymes and ligated in the equally cut p3xFLAG-CMV-10 vector. Competent bacteria were transformed with these plasmids, and plasmid DNA was isolated from the bacteria. Isolated and purified plasmid DNA was analysed by sequencing. The QIAGEN maxiprep plasmid purification kit was used for large-scale plasmid preparations of these six DNA plasmids. NIH/3T3 mouse fibroblasts were stably transfected with the following constructs: p3xFLAG-CMV-10 vector (empty vector control), R484W-, L514F-, S533N-, W535L-, K575M-Smo and Smo wt in the p3xFLAG-CMV-10 vector, to analyze the signalling properties of the different mutated versions of Smo. From each transfection, six independent monoclonal cell lines potentially harbouring the transfected plasmid were isolated, and different experiments were performed, including real-time polymerase chain reaction, Western blotting and gene reporter assay.

Flow chart: methods

MUTAGENESIS REACTION

K575M-Smo (Two PCR-reactions)

↓

1. Digestion

(K575M & pEF.6 Smo wt vector digested with Tth111I & Sph I)

↓

2. Ligation

(K575M + pEF.6.Smo wt vector)

↓

3. Transformation

↓

4. Small-scale plasmid preparation

↓

5. Quantification of DNA

↓

6. DNA sequencing

(Product: K575M-Smo pEF.6 Smo wt plasmid)

↓

SUB-CLONING

↓

1. Generation of restriction site

(Make EcoRI restriction site in R484W-, L514F-, S533N-, W535L-, K575M-Smo and Smo wt)

↓

2. Digestion:

The five mutated versions of Smo and Smo wt and p3xFLAG-CMV-10 vector digested with EcoRI & XbaI

↓

3. Ligation:

R484W-, L514F-, S533N-, W535L-, K575M-Smo and Smo wt + p3xFLAG-CMV-10 vector

↓

4. Transformation

↓

5. Small-scale plasmid preparation

↓

6. Quantification of DNA

↓

7. DNA sequencing

(Products: p3xFLAG CMV-10 R484W-, L514F-, S533N-, W535L-, K575M-Smo and Smo wt)

↓

8. Large-scale plasmid preparation

↓

TRANSFECTION

p3xFLAG CMV 10 R484W-, L514F-, S533N-, W535L-, K575M-Smo and Smo wt + NIH/ 3T3 cells

↓ + G418

Stable transfection of NIH/3T3-cells with the five mutated versions of Smo and Smo wt.

↓

Isolation of six monoclonal cell-lines of each mutant

↓

ANALYSIS OF CELL EXPERIMENTS

Real-time polymerase chain reaction (RT-PCR), Western blotting, and gene reporter assay

3.2 MUTAGENESIS REACTION

A lysine was mutated to a methionine at amino acid residue at position 575 in the Smo wt receptor, resulting in the K575M-Smo-receptor. This mutated version of Smo was generated by using mainly two PCR-reactions, where the first PCR-reaction (PCR-I) occurs in two independent tubes (Figure 3.1). Smo wt was used as template in PCR-I generating PCR-products harbouring the desired mutation. These two PCR-products were used as templates in a new PCR-reaction (PCR-II) generating a longer PCR-product that was cut with specific restriction enzymes, Tth111I and SphI, and ligated in the equally cut pEF.6 Smo wt plasmid. The DNA molecule was cut because these enzymes break the phosphodiester bonds that link one nucleotide to the next. Generally, selection of buffer is essential for selective cutting of the DNA, and the optimal temperature is usually 37˚C. Finally, the digested PCR-product and pEF.6 Smo wt plasmid were ligated by T4 DNA ligase. T4 DNA ligase catalyzed the formation of phosphodiester bonds between the 3`-hydroxyl and the 5`-phosphate groups in nicked DNA.

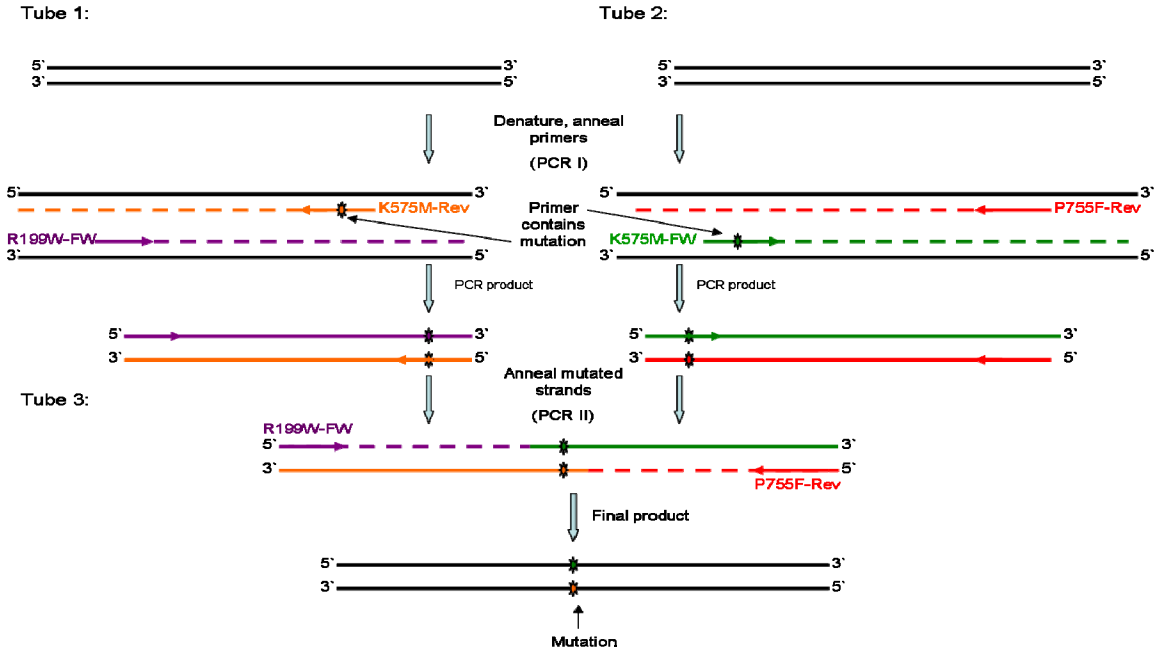


Figure 3.1: K575M-Smo mutagenesis reaction I and II. The K575M-Smo was generated by two polymerase chain reactions (PCR-I & PCR-II). The first PCR-reaction (PCR-I) was performed in two separate tubes (1 and 2), and gave two products with the desired mutation using pEF.6 Smo wt as template and two sets of primer pairs (Primer pair 1; R199W-Forward (FW) and K575M-Reverse (Rev), primer pair 2; K575M-FW and P755F-Rev). The mutagenesis primers K575M-FW and K575M-Rev contain a "mismatch" which gave the desired mutation in the generated PCR-I products. In PCR-II, the two products from PCR-I served as templates, and the flanking primers from PCR-I, R199W-FW and P755F-Rev, were used to initiate the DNA synthesis.The polymerase chain reaction gave linear double-stranded DNA containing mutations.

PCR is an extremely sensitive method to amplify a specific DNA sequence *in vitro.* The method is based on the use of thermostable DNA polymerase to copy a DNA template in repeated cycles of replication, giving an exponential increase in the DNA-product. The method requires two specific primers, a thermostable polymerase enzyme and a dNTP mix, containing equal amount of dATP, dGTP, dCTP and dTTP.

In an automated thermal cycler (PCR-machine) DNA was amplified by first heating the sample to 94˚C in order to separate the two strands, and the DNA was melted into single stranded DNA. Secondly, the temperature was lowered below the melting point of the primers allowing the primers to anneal to their corresponding DNA sequence. Finally, the temperature was increased to the optimum of the polymerase, usually 68-72˚C, allowing extension of the primers replicating the DNA (DNA replication). Typically, 20-30 cycles of reaction is required for effective DNA amplification, and each cycle doubles the amount of DNA. Within a few cycles the predominant product in the reaction is a DNA fragment corresponding to the sequence between the two primers. The thermostable Vent DNA polymerase enzyme that survives prolonged exposure to temperatures as high as 95˚C was used in this thesis. The Vent polymerase stays active after the denaturation steps in the PCR procedure and has proofreading activity. At temperatures this high normal DNA polymerase would have denatured together with the DNA.

Table 3.1: Primer used in mutagenesis reactions, sub-cloning and DNA-sequencing (FW, forward primer; Rev, reverse primer).

Primer	**Sequence (5`-3`)**
A68W-FW	5`-GGC CGG GCT GTT CCC TGC GAG CC-3`
R199W-FW	5`-GCC CTT GGT TTG GAC AGA CAA CC-3`
R199W-Rev	5`-GGT TGT CTG TCC AAA CCA AGG GC-3`
T349I-FW	5`-CAC CAT TTA CCA GCC TCT CTC GGG-3`
S533N-Rev	5`-GAC CCA GGT GTT CAT GGC GAT GC-3`
K575M-FW	5`-CTT CTC TAT GCG GCA CGA GC-3`
K575M-Rev	5`-GCT CGT GCC GCA TAG AGA AG-3`
P755F-Rev	5`-CAT GCC ACG AAG GCC GGT GCA CT-3`
pEF.6 29U31	5`-CTG TGT TCA CTA GAA TTC TCA AAC AGA CAC C-3`
pEF.6 202L18	5`-GTG ATA CTT GTG GGC CAG-3`

(See protocol description 8.2.1).

3.2.1 Agarose gel electrophoresis

Agarose gel electrophoresis was performed to identify and separate DNA fragments of different sizes. Agarose dissolves upon heating to ≤ 90˚C and forms a gel by polymerisation upon cooling. Small DNA fragments of 0.1-2 kilobases may be separated by 1.0-1.3 % agarose gels, whilst larger fragments 2-10 kb are separated by 0.6-1.0% agarose gels. The DNA molecule`s negatively charged phosphate groups make the DNA molecules migrate through the agarose gel towards the cathode in an electrical field. To determine the size of the separated DNA molecules, the DNA bands were compared to DNA marker fragments of known sizes run on the same gel. The gel was preloaded with ethidium bromide, which binds between the basepairs of the DNA double helix, and makes the fragments visible under UV-light.

Agarose gel electrophoresis was used to identify and isolate the PCR-products, and to separate and purify restriction products for ligation reaction.

(See protocol description 8.2.2).

3.2.2 Extraction of DNA from agarose gels

Isolation and purification of DNA fragments from agarose gels was performed using QIAquick Gel Extraction kit (QIAGEN). First, the agarose gel was solubilised in the presence of a chaotropic salt ($NaClO_4$) which breaks the hydrogen bonds in the agarose. The solution was then filtered through a silica membrane, which binds DNA at high salt concentration. The silica membranes with bound DNA were washed with the Buffer PE, an ethanol containing solution, to remove contaminants. Any residual Buffer PE was removed by an additional centrifugation step. The pure DNA was eluted with EB Buffer, a buffer with low salt concentration.

(See protocol description 8.2.3).

3.3 PLASMID PROPAGATION

Eukaryotic DNA plasmids transformed into bacteria are replicated independently of the host genome. Thus transformed bacteria can be used to produce plasmids copies at large scale, which in turn can be used in transfection of cell lines (*i.e*. NIH/3T3).

Strains of *Escherichia coli* (*E. coli*) were used for transformation. These bacteria have been pre-treated with various chemicals to become competent for DNA transformation. "TOP-10 Chemically Competent *E.coli*" from Invitrogen was used in this thesis. The transformed bacteria were grown on selective medium plates (Luria Bertani (LB) medium with agar containing antibiotic ampicillin 80 µg/ml). The pEF.6 Smo wt vector and p3xFLAG-CMV-10 vector contains an ampicillin resistance encoding gene, and only the bacteria transformed with vectors would survive in the presence of ampicillin. The plasmid DNA was isolated using either small-scale (Wizard® Plus SV Miniprep Kit, Promega) or large-scale (HiSpeed Plasmid Maxi Kit, QIAGEN) plasmid preparations. Both kits were used for amplification of these constructs:

- p3xFLAG-CMV-10 Smoothened wild type
- p3xFLAG-CMV-10 R484W-Smo
- p3xFLAG-CMV-10 L514F-Smo
- p3xFLAG-CMV-10 S533N-Smo
- p3xFLAG-CMV-10 W535L-Smo
- p3xFLAG-CMV-10 K575M-Smo
- pEF.6 K575M-Smo

3.3.1 Vectors

The pEF.6 vector

K575M-Smo encoding cDNA was inserted into the pEF.6 vector (pEF.6 Smo wt; Figure 3.2). The other Smo-mutation constructs were available in the laboratory, already inserted to the pEF.6 vector. The pEF.6 vector was a kind gift from Professor Richard Marais laboratory, Institute of Cancer Research, London UK.

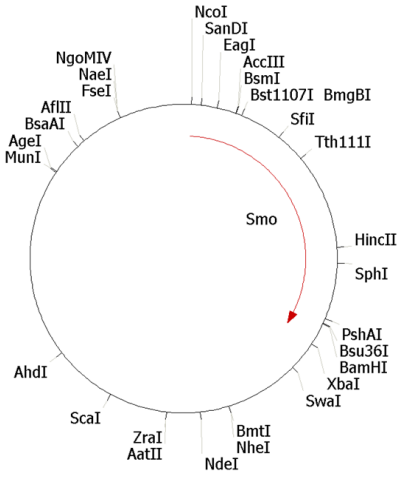
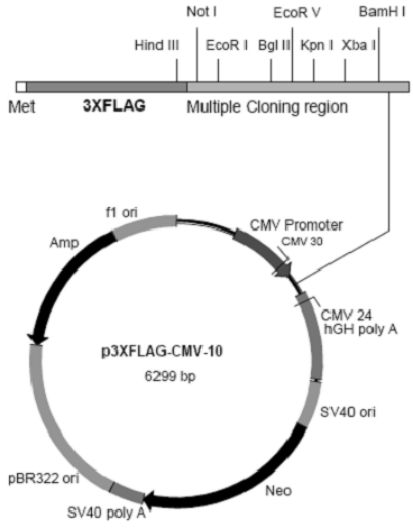


Figure 3.2: pEF.6 Smo wt

The p3xFLAG-CMV-10 vector

Complimentary DNA (cDNA) encoding R484W-, L514F-, S533N-, W535L-, K575M-Smo and Smo wt has been inserted into the p3xFLAG-CMV-10 vector (Sigma) (Figure 3.3), after generating restriction sites enabling direct ligation. The p3xFLAG-CMV-10 vector is a 6.3 kb vector derived from pCMV5. The vector encodes three adjacent FLAG-epitopes that results in increased detection sensitivity using anti-FLAG M2 antibody. It also contains the aminoglycoside phosphotransferase II gene (Neo), which confers resistance to aminoglycosides such as G-418. This allows for selection of stably transfected cells.



p3XFLAG-CMV-10 Features

Feature	Map Position
CMV promoter	166-916
CMV 30 sequencing primer	825-854
Translational initiation	928-930
3XFLAG sequence	931-996
Multiple cloning region	994–1056
hGH poly A	1061-1680
CMV 24 sequencing primer	1118-1141
SV40 ori	1699-2037
Neo	2073-2864
SV40 poly A	3511-3609
pBR322 ori	4528-4647
Ampicillin resistance	4824-5684
f_1 ori	5847-6299

Figure 3.3: p3xFLAG-CMV-10 expression vector (Adopted from Sigma-Aldrich, Inc. Product Information).

3.3.2 Transformation

The DNA was forced into the "TOP10" competent *E.coli* bacteria (Invitrogen) by incubating the cells and the DNA together on ice, placing them briefly at 42^{o}C (heat shock), and then putting them back on ice according to the protpcol description 8.2.4. This presumably caused the bacteria to take in the DNA. The cells were then plated out on ampicillin containing LB agar plates, selecting the bacteria cells with incorporated plasmid containing ampicillin-resistent gene [74].

(See protocol description 8.2.4)

3.3.3 Small-scale plasmid preparation

The Wizard Plus SV Minipreps DNA Purification System kit (Promega) was used for small-scale isolation and purification of plasmid DNA from transfected *E. coli* hosts. This system provides a simple and rapid isolation of plasmid DNA using a solid-phase silica-impregnated filter membrane. The procedure includes bacterial cell disruption and lysis using a solution containing NaOH and SDS. The chromosomal DNA, cell membrane components and denatured proteins precipitate upon neutralisation and increasing salt concentration. Plasmid DNA remains intact in the solution, but endonucleases and proteins are degraded when adding an alkaline protease solution. A silica-based membrane binds the plasmid DNA when running the plasmid lysate through using a microcentrifuge. The binding is accomplished due to interaction between the positively charged groups at silica-membrane and the negatively charged backbone of DNA molecules. The membrane is washed with an ethanol containing buffer with high salt concentration to remove residual contaminants. Finally, the plasmid DNA is eluted in nuclease-free water. Purified plasmids were sent for DNA-sequencing (protocol description 8.2.9).

(See protocol description 8.2.5).

3.3.4 Quantification of DNA

Spectrophotometrical quantification of plasmid DNA was used to determine nucleic acid concentrations. Quantification was performed by measuring optical absorption at λ = 230 nm, 260 nm and 280 nm (OD_{230}, OD_{260} and OD_{280}). These wavelengths were selected based on the UV-light absorption of nucleotides (200-300 nm), amino acids, urea and phenol (190-280 nm). All nucleotides have a λ_{max} near 260 nm which is specific for the purine and pyrimidine bases thus absorption at this particular wavelength can give an estimate of the DNA concentration in a sample. Measuring absorption at 230 nm and 280 nm indicate possible impurities (*e.g.* urea, proteins and phenols, but not RNA). Contamination by RNA can not be detected by UV analysis as both RNA and DNA have absorption maxima at about 260 nm. The ratio OD_{260}/ OD_{280} and OD_{260}/ OD_{230} estimates DNA purity, where values between 1.8-1.9 and 1.8-2.2, respectively, are acceptable. The total amount of DNA can be calculated using the formula:

$$\textbf{[DNA] (µg/µl)} = \textbf{OD}_{260} \textbf{ x dilution/20}$$

The spectrophotometer Ultrospec 2100 UV pro UV/Visible from Amersham Pharmacia Biotec was used when quantifying DNA.

(See protocol description 8.2.6).

3.4 SUB-CLONING

The term sub-cloning is used for the process of moving a DNA-insert from one vector to another. The method involves restriction enzyme digestion, agarose gel electrophoresis, isolation of the DNA-fragments and finally ligation. The method was used to move the desired coding region of wild type and the five mutated versions of Smo from the pEF.6 vector to the p3xFLAG-CMV-10 vector.

Before inserting the mutants in the desired vector, an EcoRI restriction site had to be generated adjacent to the ATG start codon of the various Smo constructs. This was done with PCR-reaction as described in section 3.2 (protocol description 8.2.1 step 2), where the forward primer contains a mismatch resulting in an EcoRI restriction site in the PCR-product. Subsequently the vectors and inserts were digested with EcoRI and XbaI restriction enzymes and separated by gel electrophoresis. Finally, the purified digestion products were ligated using T4 DNA ligase.

(See protocol description 8.2.7).

3.4.1 Large-scale plasmid preparation

To produce larger amounts of purified plasmid-DNA, large-scale plasmid preparation was set up using the QIAGEN Hispeed Plasmid Maxi Kit. First, bacteria culture was prepared and incubated in a shaking incubator overnight, and then centifugated the next day, precipitating the bacteria. Secondly, the bacteria containing desired plasmid DNA were resuspend in presence of RNase A (buffer P1), and then lysed using a NaOH and SDS containing solution (Buffer P2). SDS solubilises phospholipids and proteins, NaOH denatures chromosomal and plasmid DNA, while RNase A digests liberated RNA. Addition of acidic potassium acetate (Buffer P3) neutralizes the lysis and causes potassium dodecyl sulphate (KDS) to precipitate. The cell residue becomes trapped in salt-detergent complexes,

while plasmid DNA remains in the solution because of its smaller size. The lysate was cleared through a QIAfilter Cartridge and loaded on a QIAGEN anion-exchange resin. The resin consists of silica beads with a defined particle size and a hydrophilized surface coated with diethylaminoethyl (DEAE). Under low salt and pH conditions, DNA is bound to the resin through interaction between their negatively charged phosphate backbone and the positively charged groups of DEAE. A medium-salt buffer (Buffer QC) removes any remaining contaminants, such as RNA traces and proteins. The plasmid DNA was efficiently eluated with high-salt buffer (Buffer QF) and the purified plasmid DNA was used in transfection and other experimental procedures.

(See protocol description 8.2.8).

3.5 CELL TREATMENTS

3.5.1 NIH/3T3-cells and 293 EcR Shh cells

The following methods were all carried out with the mouse embryonic fibroblast cell line, NIH/3T3-cells obtained from American Type Culture Collection (ATCC) (Figure 3.4). NIH/3T3-cells adhere to plastic surfaces and grow in newborn calf serum (NCS). Under optimal growth conditions (37°C, 5% CO_2 in air), the cells double about every 36 hours. The cells were subcultured twice per week at 80% confluency or less. (Complete growth medium: DMEM (4.5 g glucose/L) supplemented with 5% NCS. For the stably transfected cells 0.4 mg/ml G-418 was added to the growth medium).

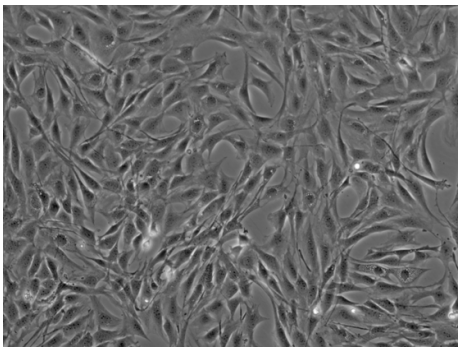


Figure 3.4: NIH/3T3-cells growing in culture (Olympus IX81, 40x).

293 EcR Shh cells are epithelial kidney cells also obtained from ATCC. 293 EcR Shh cells are transformed with one arm of the adenovirus 5 DNA created in 1998 by stably transfecting HEK293 cells with the Ecdysone-Inducible Mammalian Expression System (Invitrogen). 293 EcR Shh cells carry a construct for the expression of murine Sonic hedgehog (Shh) under control of an Ecdysone-inducible promoter. The cells were used for production of biologically active murine Shh secreted in the medium. Ponasterone A is an

analogue of Ecdysone and was used to induce production of Shh. Under optimal growth conditions (37°C, 5% CO_2 in air), the cells double about every 23 hours. (Complete growth medium: DMEM (4.5 g glucose/L) supplemented with 10% FBS, fetal bovine serum. Antibiotics used: 0.4 mg/ml G-418 and 0.4 mg/ml Zeocin).

3.5.2 Thawing cells

The NIH/3T3-cells and EcR 293 Shh cells were stored frozen in the liquid nitrogen tissue culture library tank. These cells were thawed according to protocol 8.2.10.

(See protocol description 8.2.10)

3.5.3 Splitting cells

NIH/3T3 cell cultures grow as a monolayer attached to the bottom of the plastic culture dish. The cells were cultured until they were 70-80% confluent before splitting at a ratio of 1:3 to 1:12. At higher densities, the cells may form a very dense monolayer, which is difficult to split. At 70-80% confluency the NIH/3T3-cells were treated with trypsin-EDTA. Trypsin is a proteolytic enzyme and EDTA is a calcium chelator. The cells were detached from the culture dish surface when adding trypsin-EDTA, and the cells were subcultured onto new dishes after centrifugation and resuspension.

293 EcR Shh cells showed a tendency to grow in clusters, and were cultured until 50-70% confluence. The cells were subcultured at a ratio of 1:4 to 1:10, and medium were changed two to three times weekly. 293 EcR Shh cells were used to generate conditioned medium used to stimulate the Hedgehog-signal pathway in NIH/3T3-cells. Conditioned medium was prepared by plating $1x10^6$ cells in 100 mm dishes. The next day the growth medium was changed and the cells were cultured in the presence of Ponasterone A (5 μM) for 24h to induce expression of Shh. Ponasterone A is an analog of Ecdysone, an insect steroid that regulates the metamorphosis of *Drosophila melanogaster*.

(See protocol description 8.2.11)

3.5.3.1 Counting cells

Cell counting was performed when setting up experiments like RT-PCR, gene reporter assay, and when generating conditioned media. The cells were counted when it was desirable to achieve confluencity at the same point of time. Countess Automated Cell Counter from Invitrogen was used according to protocol description 8.2.11.1.

(See protocol description 8.2.11.1)

3.5.4 Freezing cells

When cells freeze, their plasmamembranes disrupt and vital parts are damaged due to ice crystal formation. The use of cryoprotective medium containing 15% DMSO prevents cell rupture. A slow cooling and freezing process, reducing the sample temperature approximately 1˚C per hour, increases survival of the cells, but the process should not be too slow due to the toxicity of DMSO present in the cryoprotective medium. Freezing cells was performed according to protocol 8.2.12.

(See protocol description 8.2.12).

3.6 CELL BASED EXPERIMENTS

3.6.1 Transfecting cells

Transfecion is a method used for introduction of biomolecules like DNA, RNA or proteins into eukaryotic cells. This can be achieved by different techniques, but for NIH/3T3-cells the method employing Lipofectamine 2000 is widely used and works well. This is a cationic lipid based method giving transfection rates of up to 60%. Lipofectamine 2000 is a 3:1 (w/w) liposome formulation of positively charged liposomes, which interact with the negatively charged phosphate groups of the nucleic acid and forms a complex. These complexes enable hydrophilic DNA to cross the hydrophobic plasma membrane of the cells by endocytosis. Inside the cell the plasmids can be replicated and transcribed episomally. Transfection of cells was performed according to protocol 8.2.13.

(See protocol description 8.2.13).

3.6.2 Generation of monoclonal cell lines

Monoclonal cell lines were generated according to protocol 8.2.14.

(See protocol description 8.2.14)

3.6.3 Transfection for reporter gene assay (transient transfection)

Luciferase-assay was used to address the signalling properties of the mutated and wt versions of Smo expressed in monoclonal cell lines. The different cell lines were seeded in separate columns in a 96-well plate, and subsequently transfected with the Gli-BS-Luc- and Renilla-Luciferase constructs according to protocol 8.2.15. Fore more details about the Luciferase-assay method see section 3.7.1.

(See protocol description 8.2.15).

3.6.4 Stimulation and harvesting of cells for gene reporter assay

To analyse activation of Gli-reporter in transfected NIH/3T3-cells, the half of the cells were serum-starved in 0.5% NCS-DMEM, and the other half were treated with Shh-conditioned medium produced by the 293 EcR Shh cells. The Gli-reporter in stimulated cells should be more activated than in the starved cells, because Shh increases the Hedgehog signalling pathway activity, and thereby Gli transcription factor activity. See section 3.7.1 for more information about the Luciferase-assay method.

(See protocol description 8.2.16).

3.7 ANALYSIS OF CELL EXPERIMENTS

3.7.1 Gene reporter assay: Luciferase assay

Methodological principle

Luciferase-assay is a genetic reporter system used to analyse eukaryotic gene expression and cellular physiology. The assay contains firefly luciferase protein which catalyzes luciferin oxidation and generate light in the reaction. In this study we have used the firefly luciferase

protein as a report gene analysing the Gli1 activity in stably transfected NIH/3T3-cells. In this method there are two individual reporter enzymes within a single cell, where the experimental reporter is correlated with the effect of specific experimental conditions. The activity of the control reporter provides an internal control and serves as the baseline response. Experimental variability caused by differences in cell viability or transfection efficency is, in theory, minimised when normalising the activity of the experimental reporter to that of the internal control [75].

Before running Luciferase-assay, transfection of the NIH/3T3-cells was preformed. The constructs to be transfected are the Gli BS-Luc (Gli-binding sequence-luciferase) experimental reporter construct, and Renilla-luciferase construct (internal control). The Gli BS-Luc construct expresses Luciferase under control of the Gli1-dependent promoter. Increased Gli1 activity is a result of activated hedgehog signalling pathway. The cells were lysed with Passive Lysis Buffer prior to reagent addition. The Gli BS-Luc reporter activity was measured first by adding Luciferase Assay Reagent II (LAR II) to generate a luminescent signal lasting at least one minute. After quantifying this signal, this reaction was quenched, and the Renilla luciferase reaction was initiated simultaneously by adding Stop & Glo reagent to the same sample. Dual-Luciferase® Reporter 1000 Assay System (Promega) was used according to the protocol description 8.2.17.

(See protocol description 8.2.17).

3.7.2 Gene expression analysis by quantitative polymerase chain reaction

Methodological principle

Quantitative analysis of mRNA expression was performed using real-time reverse transcription polymerase chain reaction (real-time RT-PCR), which is a highly sensitive technique used for mRNA detection and quantification [76]. Total RNA was isolated from NIH/3T3-cells, purified and transcribed into complementary DNA (cDNA) by SuperScript III reverse transcriptase. DNA polymerase and specific primers amplifies the new-formed cDNA, and the PCR-products were measured continuously during the amplification step. Subsequently, calculation of the original level of mRNA in the sample was performed.

In this study, the mRNA expression levels of human Smo (hSmo), mouse Gli1 (mGli1) and mouse Ptch (mPtch) in stably transfected NIH/3T3-cells was measured by real time RT-PCR.

3.7.2.1 Isolation of RNA

A critical step when isolating nucleic acids is to separate nucleic acids from proteins. This is accomplished due to their different chemical properties. Nucleic acids are hydrophilic compared to the more hydrophobic proteins due to the highly charged phosphate backbone. RNeasy Mini Kit from QIAGEN was used to isolate total RNA. The protocol contains a DNase treatment step, where all RNA samples were treated with deoxyribonuclease (DNase). The DNase digests every strand of DNA present. To avoid introducing exogenous ribonuclease (RNase) to the samples, gloves were worn at all times and changed frequently. All reagents and equipment used were RNase free and decontaminated with RNase-OFF routinely.

(See protocol description 8.2.18).

3.7.2.2 Determination of RNA purity, quality and concentration

The RNA quality was assessed both by spectrophotometry and gel electrophoresis. The concentration and purity of RNA in the samples was determined by measuring the absorbance at 260 nm (A_{260}). The ratio between the absorbance at 260 nm and 280 nm (A_{280}) gives an estimate of the RNA purity concerning protein contaminations. The NanoDrop ND-1000 (NanoDrop Technologies) spectrophotometer was used, which is a full-spectrum (220-750 nm) spectrophotometer measuring sample volumes down to 1 µl with high accuracy and reproducibility. Two µl samples were positioned on the electrodes. The electrodes were wiped clean between every measurement when measuring multiple samples. RNase-free water was used as a blank.

To verify the RNA quality, 1 µg of the total RNA preparations were fixed in formamide and subjected to agarose gel electrophoresis (1% agarose gel preloaded with ethidium bromide) to separate the 28S and 18S rRNA fragments. RNA moves towards the positive pole in an electrical field because it is negatively charged at neutral pH. The 18S and 28S bands of RNA in the gel are stained with ethidium bromide. The bands are visualized under ultra violet (UV) light, and are visible when the RNA is of high quality (Figure 3.5).

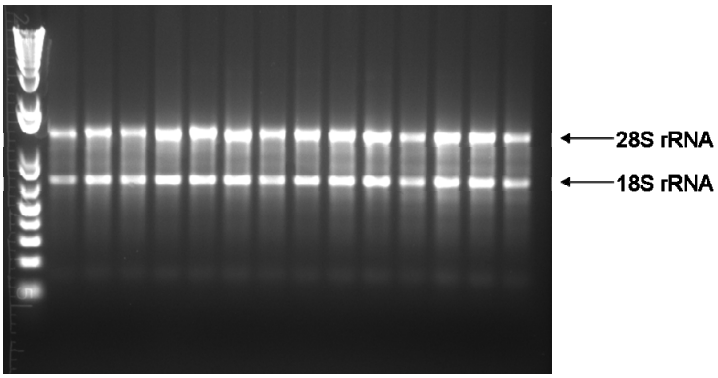


Figure 3.5: Agarose (1%) gel electrophoresis of a representative selection of total RNA samples. The RNA integrity was evaluated by inspection of the 28 S and 18 S rRNA bands The RNA was of high quality and showed only minor degradation of the total RNA samples.

(See protocol description 8.2.19).

3.7.2.3 First strand cDNA synthesis

Single stranded mRNA is too unstable to serve as a template for PCR therefore single stranded mRNA was transcribed into cDNA (complementary DNA). This was accomplished by the use of the SuperScript III (SSIII) reverse transcriptase (RT) (Invitrogen) which is a RNA-dependent DNA polymerase. Based on spectrophotometric concentration determinations 2.5 µg total RNA was mixed together with oligo dT primers, a dNTP mix (equal amount of dATP, dGTP, dCTP and dTTP, all Invitrogen), and RNase-free water to a volume of 13 µl. The mixture was heated to 65ºC allowing the primers to anneal. SSIII, RNaseOut, First-Strand Buffer and DTT were added to a total volume of 20 µl. To synthesise cDNA the following temperature-program was used:

25 ˚C 5 minutes
50 ˚C 60 minutes
70 ˚C 15 minutes (Inactivate the reaction)
4 ˚C End

Reactions were also carried out without reverse transcriptase (-RT) as a negative control. These control samples were used to determine whether there were traces of genomic DNA in the prepared RNA samples. In these reactions SuperScript III RT and RnaseOut was replaced by DNase-free water.

(See protocol description 8.2.20).

3.7.2.4 RT-PCR

Quantitative real-time RT-PCR (qPCR/RT-PCR) was used to amplify and simultaneously quantify a specific sequence in a DNA molecule (amplicon). Quantification was accomplished by registration of a reporter conjugated to the amplicon that accumulates at the same rate. Both SYBRgreen and Taqman probe was used in this study as labelling reagents, and allow detection of PCR-products via generation of a fluorescent signal. In this study, SYBRgreen technology was used to quantify the genes of interest including hSmo, mGli and mPtch and the housekeeping control gene TATA box binding-protein (Tb-p) (see below). The primer and probe technology (Taqman probe) was used for quantification of the housekeeping control gene glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) (see below).

SYBRgreen is a dye that binds to the minor groove of the nascent double-stranded DNA during elongation. The resulting DNA-dye-complex sends a fluorescent signal during the polymerisation step. The main drawback of this method is that SYBRgreen binds to any double-stranded DNA present which may lead to an overestimation of the target concentration. Specific primer pairs secure high specificity when using the SYBRgreen technology. The specificity of the RT-PCR products was verified by melting-point analysis [76].

Primer and probe-based RT-PCR distinguishes between specific and non-specific amplification. The TaqMan probe is a short single stranded oligonucleotide sequence that is complementary to one of the DNA strands in the amplicon. A fluorophore-reporter is attached to the 5`-end of the probe and a quencher to the 3`-end. While the probe is intact, the closeness of the quencher will reduce the fluorescent signal sent out by the reporter due to fluoroscence resonance energy transfer (FRET). The reporter transfers the energy to the quencher, which releases the energy as light of a higher wavelength which is not being detected. The DNA polymerase displays a 5`-3`nuclease activity, and will therefore hydrolyse an oligonucleotide bound to its target sequence. Based on this the reporter is separated from the quencher during the synthesising process and FRET will no longer occur when the reporter and quencher are separated. This leads to an increase in fluorescence proportional to the accumulation of amplicon which the real-time quantitative RT-PCR machine will detect [76].

The cycle threshold (C_t) value is defined as the cycle number at which the fluorescence is higher than the baseline fluorescence. The higher the starting copy number of the nucleic acid target, the sooner this is reached [77].

A standard curve with increasing concentrations of RNA is run in parallel to ensure a representative transcription of mRNA into cDNA for reliable quantification. A pooled reference sample is made from the RNA-samples, which is coamplified with the experimental sample during cDNA-synthesis. A standard curve for quantification of amplification of the amplicon was generated. The standard curve is an important control of reverse transcription of mRNA into cDNA.

Two housekeeping genes were used to normalise the expression of the template for sample-to-sample variations in RT-PCR efficiency and for errors in sample quantification. Ideally, the housekeeping genes should not be affected by the experimental conditions, and should have minimal variations in expression among different tissues of an organism, at all stages of development. In this study, mRNA for glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and TATA box binding-protein (Tb-p) were used as internal standards.

Figure 3.6 shows an example of measured hSmo expression level in one RT-PCR experiment analysed relative to both GAPDH (Figure 3.6 A) and Tb-p (Figure 3.6 B). Both normalisation genes gave approximately the same pattern of hSmo expression level in the various monoclonal cell lines.

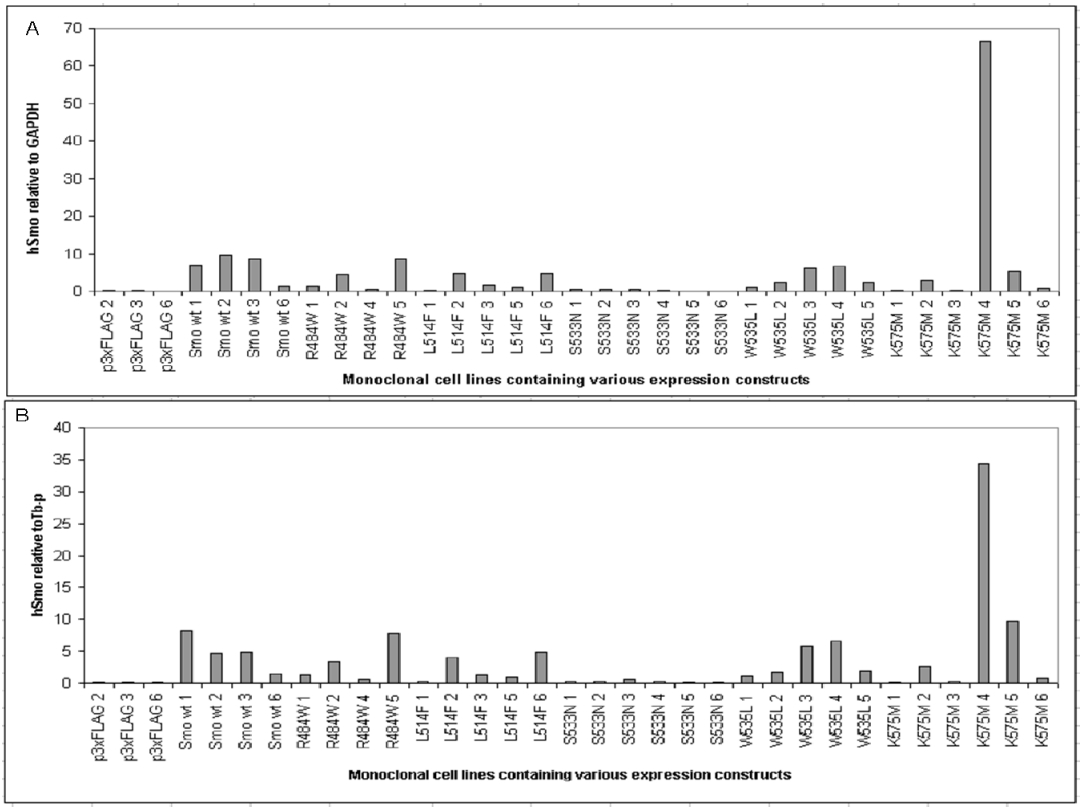


Figure 3.6: Human Smo mRNA expression level relative to GAPDH (A) and Tb-p (B).

The real time RT-PCR assays (15µl) were run in a 384-well temperature block operated by an ABI Prism 7900HT Sequence Detection System (Applied Biosystems).

(See protocol description 8.2.21).

3.7.2.5 Primer and probe design

The primers and probes used in this study were available in the laboratory (Table 3.2 and 3.3). To get an effective amplification of the target sequence and reliable quantification, it is very important to design specific primers and probes. Using specific primers and probes avoids unreliable signals from primer dimers, primer-probe dimers and replication of other genes due to unspecific bindings.

Receptor/Gene	Gene ID (PubMed)	Primer	Sequence (5`-3`)
hSmo	6608	Forward #499 Reverse #500	5`-GGA CTA TGT GCT ATG TCA GG-3` 5`-AGG TTG ATC TTC TCC ACC AG-3`
mGli1	14632	Forward #507 Reverse #508	5`-CTG TCG GAA GTC CTA TTC AC-3` 5`-ACG TAT GGC TTC TCA TTG GA-3`
mPtch	19206	Forward #503 Reverse #504	5`-CTG TAA CAA CTA TAC GAG CCT G-3` 5`-AGG ACC ATG ACA ATG ATC CC-3`

Table 3.2: Oligonucleotide sequences of the primers used for real-time RT-PCR.

Normalization gene	Gene ID (PubMed)	Primer/ Probe	Sequence (5`-3`)	Repor-ter dye	Quencher dye
GAPDH	10190788	Forward Reverse Probe	5`-CCA AGG TCA TCC ATG ACA ACT T-3` 5`-AGG GGC CAT CCA CAG TCT T-3` 5`-CTC ATG ACC ACA GTC CAT GCC ATC ACT-3`	Yakima Yellow	Dark Quencher
Tb-p	51948367	Forward #454 Reverse #455	5`-ATCTTGGCTGTAAACTTGAC-3` 5`-GGATTGTTCTTCACTCTTGG-3`		

Table 3.3: Oligonucleotide sequences of the primers and probes for normalization genes used in real time RT-PCR.

3.7.2.6 Calculations

Analysis of quantitative RT-PCR data

An amplification plot was generated from every sample by using Sequence Detector Software (SDS version 2.2, Applied Biosystems). The amplification plot shows the increase in the reporter dye fluorescence with each PCR cycle. C_t values from each amplification cycle were automatically calculated, and it represents the PCR cycle number at which the fluorescence is first detected above the baseline. The C_t values were then exported into Microsoft Excel for further calculations.

Standard curves for each set of primer/probe set were made by plotting C_t values versus log initial amount of RNA for the different standard dilutions, giving a straight line (Equation 1). The slope describes the efficiency of the PCR. If the PCR amplification is optimal, the slope will be -3.3, as 3.3 cycles are required to generate a 10-fold increase in product amount.

$$C_t = a (\log Q) + b \qquad (1)$$

C_t is the threshold cycle, a is the slope, Q is the initial copy number and b is the intercept on the y-axis.

Equation 1 can easily be rearranged to:

$$\log Q = \frac{C_t - b}{a} \qquad (2)$$

The initial amount of target RNA can be calculated using this equation. The slope a and intercept b is calculated from the standard curve and the obtained C_t values for the different samples.

The expression of hSmo, mGli1 and mPtch mRNA were normalized to the expression level of the reference gene GAPDH across all samples and some values were also normalized to the expression level of the Tb-p reference gene. Each sample was run in triplicates, and the mean values of the triplicates were used to calculate the ratio between the different genes and the normalization gene. The data were transferred into Microsoft Excel and GraphPad Prism5 Software (Prism) for graphical presentation. The data in GraphPad were expressed as mean ± SEM from n experiments, and the data in Excel were expressed as mean ± SD from n experiments.

3.7.3 Protein concentration determination

Methodological principle

BCA (bicinchoninic acid) Assay protein quantitaion kit (BCAssay: protein quantitation kit, Uptima) was used to determinate the protein concentration in cell lysate samples, and this is a colorimetric assay based on the Biuret reaction. Proteins reduce Cu^{2+} in alkaline solutions to Cu^{+}, and reduced Cu^{+} interacts with two molecules of bicinchoninic molecules and forms a water-soluble purple coloured complex with an absorption maximum of 562 nm (figure 3.7). The absorption is proportional to the initial protein concentration. The protein concentrations were recorded using a microplate reader-Multiscan EX (Thermo Electron Corporation) or an EnVision 2104 Multilabel Reader (Perkin Elmer precisely).

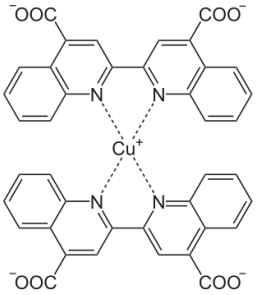


Figure 3.7: Bicinchoninic acid interacting with Cu^{+}.

(See protocol description 8.2.22).

3.7.4 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Methodological principle

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is an electrophoresis method used to separate proteins of various sizes in an electric field. Polyacrylamide is a polymer of acrylamide monomers that forms a dense porous gel when it polymerises. SDS is a strong anionic detergent which has a hydrophobic tail that interacts

strongly with polypeptide chains, giving them a negative charge. SDS also disrupts the bonds that make up the three dimensional conformation of proteins. β-mercaptoethanol is a reducing agent and was added to disrupt disulfide bridges in the polypeptide. Disulfide bridges can influence the migration of the proteins. The samples were boiled at 95˚C for 5 minutes to facilitate the denaturation process. The denaturated polypeptides obtained a linear structure covered with negatively charged SDS. The protein migration through the polyacrylamide gel are only depended on the size of the proteins, because SDS-denatured polypeptides have an approximately uniform mass:charge ratio. The negatively charged polypeptides migrate towards the positive pole.

(See protocol description 8.2.23).

3.7.5 Western blotting

Methodological principle

After electrophoresis the proteins in the gel were subjected to electroblotting. The proteins were negatively charged by the bound SDS, and were readily transferred to a PVDF membrane (or nitrocellulose membrane when using Odyssey Infrared Imaging System, LI-COR Biosciences, see below) in an electrical field. For this procedure, an electric current was applied to the gel so that the separated proteins moved through the gel and onto the membrane while maintaining their relative position as they were separated by the SDS-PAGE. All sites on the membrane which do not contain transferred proteins from the gel were then “blocked” so that antibodies used for detection of the target protein did not bind non-specifically to the membrane. Non-specific binding of antibodies to membrane causes a high background signal. “Blocking” of the membrane was achieved by placing the membrane in a non-fat dry milk protein solution dilute in phosphate buffer saline (PBS)/0.5% Tween 20.

After blocking, a primary antibody was added in an appropriate dilution to detect a specific protein (antigen) blotted on the membrane. The antibody-dilution was incubated with the membrane overnight. The next day, the membrane is rinsed and subsequently incubated with a secondary antibody. Horseradish peroxidises (HRP)-linked secondary antibody was added to PVDF membranes, and Infrared (IR)-Dye linked secondary antibody was added to nitrocellulose membrane when using Odyssey. The secondary antibody binds to the Fc

portion of primary antibodies. In order to detect the specific protein-antibody interactions when using HRP-linked secondary antibody, a chemiluminescence substrate containing luminol was added to the conjugate. Upon reaction with the conjugate, HRP catalyses the breakdown of luminol thus emitting light and resulting in a visible band where the primary antibody has bound to the protein, when detected with an EpiChemi II Darkroom-camera (UVP Laboratory Products). The IRDye-linked secondary antibody did not need to be activated when using Odyssey. The Odyssey Infrared Imaging System detected the specific protein-antibody interactions by measuring the infrared light from the IRDye-linked secondary antibody. The intensity of the signal correlated with the abundance of the antigen on the blotting membrane.

(See protocol description 8.2.24).

4. RESULTS

This thesis is part of an ongoing project investigating the importance of somatic mutations in Smo on Smo-mediated signalling. Smo is part of the Hh signalling pathway, and currently fourteen somatic mutations have been identified in Smo and are registered in the Catalogue Of Somatic Mutations in Cancer (COSMIC) database. In this thesis five of the fourteen mutations were studied, in addition to Smo wild type (wt) and the p3xFLAG-CMV-10 empty vector (control). One of the mutants, K575M-Smo, was generated in the laboratory as part of this thesis work.

4.1 MUTAGENESIS, K575M-SMO, IN SMO WT

The K575M-Smo was generated by two polymerase chain reactions (PCR) as described in the Methods chapter. Two products with the desired mutation were generated in the first set of reactions (PCR-I), using pEF.6 Smo wt as template and two sets of primer pairs (A R199W-Forward (FW) and K575M-Reverse (Rev), B K575M-FW and P755F-Rev; Table 3.1). The PCR-product sizes determined by agarose gel electrophoresis corresponded to the theoretical fragment sizes of 1151 bp and 558 bp, respectively (Figure 4.1).

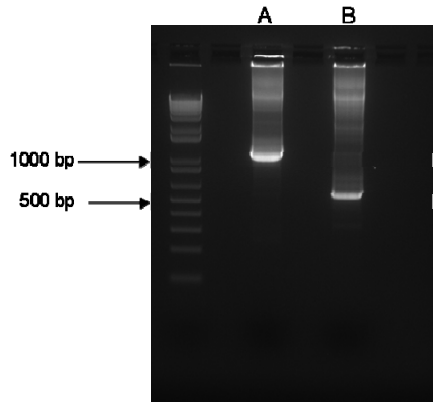


Figure 4.1: Agarose (1%) gel of Smo PCR-products from PCR-I. pEF.6 Smo wt served as template in both PCR-I reactions with the primer pairs R199W-FW and K575M-Rev (lane A, expected product size 1151 bp) and K575M-FW and P755F-Rev (lane B, expected product size 558 bp). The 1 kb Plus DNA ladder was used as molecular size marker (left lane).

In the second set of PCR-reactions (PCR-II) the two products from PCR-I served as templates, and the flanking primers from PCR-I (R199W-FW and P755F-Rev) were used to initiate DNA synthesis. The theoretical size of this product was 1689bp. The polymerase chain reaction gave linear double-stranded DNA containing the K575M Smo-mutation. This product was not separated on agarose gel, but was purified directly with QIAquick Gel Extraction Kit and subsequently digested with the restriction enzymes Tth111I and SphI.

4.1.1 Restriction enzyme digestion and ligation of K575M-Smo and pEF.6 vector

The PCR-II-product containing the mutant K575M-Smo mutation and pEF.6 Smo wt plasmid were digested with the restriction enzymes Tth111I and SphI. Digestion of the PCR-II-product, K575M-Smo, should theoretically generate DNA fragments of 359 bp, 520 bp, and 840 bp. The pEF.6 Smo wt vector was digested into fragments of sizes 840 bp (Smo wt) and 6337 bp (pEF.6 vector; Figure 4.2). The fragment of 840 bp from K575M-Smo and the pEF.6 vector fragment of 6337 bp were isolated from the gel and purified.

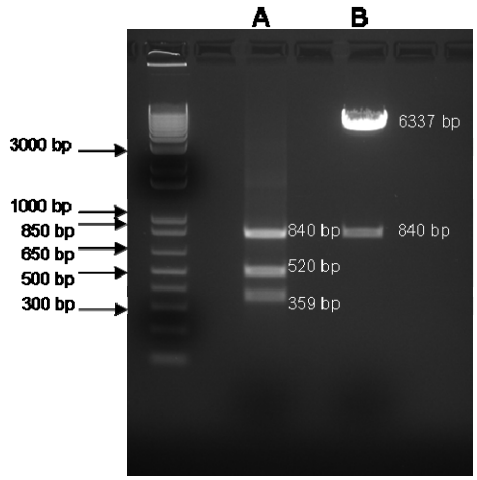


Figure 4.2: Agarose (1%) gel electrophoresis of restriction enzyme fragments of the PCR-II-product K575M-Smo and pEF.6 Smo wt vector digested with Tth111I and SphI. The 840 bp product from K575M-Smo (lane A), and the 6337 bp product of the pEF.6 Smo wt plasmid (lane B) were isolated from the gel and purified. The 1 kb Plus DNA ladder was used as molecular size marker (left lane).

After purification, digested K575M-Smo was ligated into the equally cut pEF.6 vector by T4 DNA ligase (Figure 4.3). The resulting plasmid was transformed into "TOP10" competent *E. coli* bacteria.

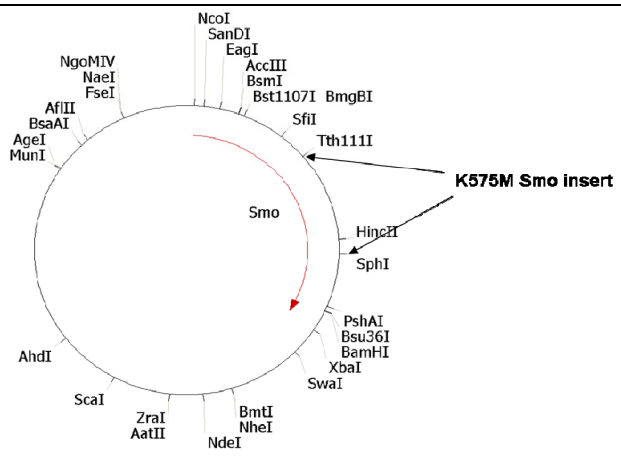


Figure 4.3: pEF.6 vector. The PCR-II-product of the K575M-Smo mutation was inserted into the pEF.6 vector in the Tth111I and SphI restriction sites as indicated. The pEF.6 vector was a kind gift from Professor Richard Marais, Institute of Cancer Research, London UK.

4.1.2 The sequence of pEF.6 K575M-Smo wt was verified by DNA-sequencing and agarose gel electrophoresis

The pEF.6 vector contained an ampicillin resistance encoding gene, therefore only the bacteria that were transformed with these vectors survived in the presence of ampicillin in LB agar plates. Ten colonies of bacteria were selected and grown in culture tubes but only nine grew in the presence of ampicillin. The plasmid DNA in the bacteria was isolated in small scale. After isolation, the nine plasmid DNA presumably containing the pEF.6 K575M-Smo plasmid were sent for DNA-sequencing and analysed by agarose gel electrophoresis (Figure 4.4). The plasmids were uncut and circular, and could therefore not directly be compared with the linear DNA-fragments of the molecular size marker applied in the left lane. Plink (polylinker, a pEF.6 empty vector) was run as control and was of smaller fragment than pEF.6 K575M-Smo plasmids, which indicate that the plasmids contained the expected inserts. The pEF.6 Smo wt was also run as control, and was of approximately same size as the pEF.6 K575M-Smo plasmids, indicating that the plasmids contained the expected inserts. The R199W-FW and T640A-Rev primers were used for sequencing, giving a nucleotide sequence of approximately 1300 bp in the middle of the Smo wt cDNA sequence containing the mutation K575M. The DNA sequences from the sequencing-lab were analysed (not shown in this thesis), and five of the nine sequenced constructs (Figure 4.4 lane 2, 3, 4, 7 and 9) showed the intended K575M-Smo mutation and no additional PCR-introduced mutations. One of the five plasmids (Figure 4.4 lane 2) containing the K575M mutation was chosen for sub-cloning and for HiSpeed Plasmid Maxi preparation.

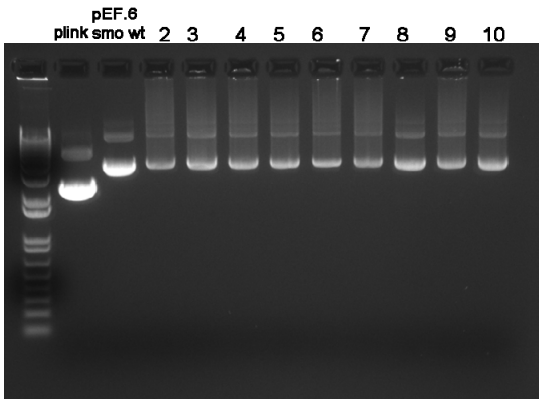


Figure 4.4: Agarose gel (0.8%) showing nine pEF.6 plasmids presumably containing the K575M Smo. Plink, pEF.6 Smo wt and the nine isolated pEF.6 K575M-Smo plasmids (lane 2-10) were run on a 0.8% agarose gel. Plink and pEF.6 Smo wt were run as controls. The term "plink" stands for pEF.6 polylinker, a pEF.6 empty vector, and should therefore be smaller than pEF.6 K575M-Smo plasmids, containing a mutated insert. The 1 kb Plus DNA ladder was used as molecular size marker.

4.2 SUB-CLONING: Insertion of R484W-, L514F-, S533N-, W535L-, K575M-Smo and Smo wt into the p3xFLAG-CMV-10 vector

To accomplish the sub-cloning, an EcoRI restriction site was generated in the wt and five mutated Smo. The EcoRI restriction site was generated adjacent to the ATG start codon of the various Smo constructs, using primer pEF.6 29U31 and pEF.6 202L18. The primer pEF.6 29U31 contains a "mismatch" that introduces an EcoRI restriction site in the various Smo constructs. The products were analysed on 1% agarose gel, and the observed product size was as expected approximately 2500 bp (calculated 2517 bp) (Figure 4.5). These products were isolated and purified from the agarose gel. The five Smo mutants, wt and the empty vector p3xFLAG-CMV-10 (Figure 4.7) were digested with EcoRI (10 units/µl) and XbaI (20 units/µl), and subsequently separated on a 1% agarose gel. The fragment sizes of the Smo mutants and p3xFLAG-CMV-10 vector were approximately 2400 and 6300 bp as calculated (2422 and 6262 bp), respectively (Figure 4.6). These fragments were isolated and purified. The digested Smo wt and mutated versions were ligated into equally cut p3xFLAG-CMV-10 vector by T4 DNA ligase. A control experiment of the p3xFLAG-CMV-10 vector was performed by restriction digestion (Section 4.2.1), verifying that the vector actually was an intact vector (Figure 4.8).

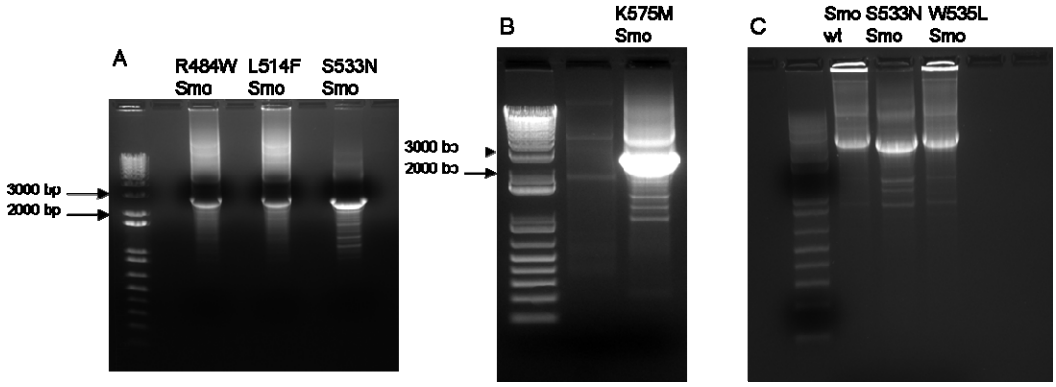


Figure 4.5: Agarose gel (1%) showing PCR-products with a generated EcoRI restriction site. The EcoRI restriction site was generated using primer pEF.6 29U31 and pEF.6 202L18. The primer pEF.6 29U31 contains a "mismatch" that introduces an EcoRI site adjacent to the ATG start codon of the various Smo constructs. The fragment sizes of the products are of size approximately 2500bp. The 1 kb Plus DNA ladder (left lane on A-C) was used as a molecular size marker. A: pEF.6 R484W-, L514F-, and S533N-Smo products. B: pEF.6 K575M-Smo. C: pEF.6 Smo wt, pEF.6 S533N-Smo and pEF.6 W535L-Smo.

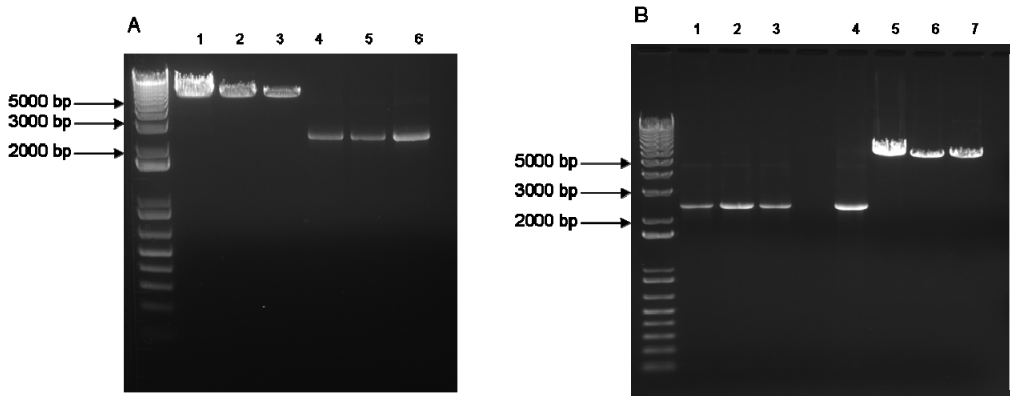
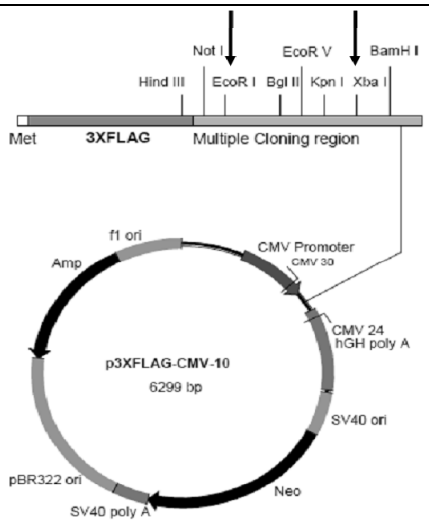


Figure 4.6: Agarose gel (1%) showing Smo mutants in p3xFLAG-CMV-10 vector digested with the restriction enzymes EcoRI and XbaI. The sizes of the digested p3xFLAG-CMV-10 vectors (A1-3 and B5-7) were 6262bp, and the digested p3xFLAG-CMV-10 Smo mutants (A4-6 and B1-4) were 2422 bp. The 1 kb Plus DNA ladder was used as a molecular size marker (left lane on A-B). A: The p3xFLAG-CMV-10 vector (lane 1) was digested with EcoRI and XbaI, isolated and purified for subsequent ligation. Lane 2 displays p3xFLAG-CMV-10 vector only digested with XbaI, and lane 3 displays the vector only digested with EcoRI. Lane 4, p3xFLAG-CMV-10 R484W-Smo; lane 5, p3xFLAG-CMV-10 L514F-Smo; and lane 6, p3xFLAG-CMV-10 S533N-Smo. Each p3xFLAG-CMV-10 Smo mutant were digested with EcoRI and XbaI. B: p3xFLAG-CMV-10 vector (lane 5-7). Lane 5; p3xFLAG-CMV-10 vector digested with EcoRI and XbaI. Lane 6; vector digested with only EcoRI, and lane 7; digested with XbaI. Lane 1, p3xFLAG-CMV-10 Smo wt; lane 2,; p3xFLAG-CMV-10 S533N-Smo; lane 3, p3xFLAG-CMV-10 W535L-Smo; and lane 4, p3xFLAG-CMV-10 K575M-Smo.



p3XFLAG-CMV-10 Features

Feature	Map Position
CMV promoter	166-916
CMV 30 sequencing primer	825-854
Translational initiation	928-930
3XFLAG sequence	931-996
Multiple cloning region	994–1056
hGH poly A	1061-1680
CMV 24 sequencing primer	1118-1141
SV40 ori	1699-2037
Neo	2073-2864
SV40 poly A	3511-3609
pBR322 ori	4528-4647
Ampicillin resistance	4824-5684
f1 ori	5847-6299

Figure 4.7: p3xFLAG-CMV-10 vector from Sigma-Aldrich. The five fragments containing mutated versions of Smo and Smo wt were ligated into the p3xFLAG-CMV-10 vector in the EcoRI and XbaI restriction sites of the vector.

4.2.1 Verification of the p3xFLAG-CMV-10 vector

The p3xFLAG-CMV-10 empty vector was digested with the restriction enzymes SmaI and NcoI as a control experiment, showing that the vector was intact. Treatment with NcoI should give four fragments with theoretical sizes of 233, 703, 1004 and 4359 bp, respectively, and SmaI-treatment should give two fragments with theoretical sizes of 1983 and 4316 bp, respectively, if the vector were intact. The fragment sizes determined by agarose gel electrophoresis corresponded well with the theoretical sizes (Figure 4.8). The p3xFLAG-CMV-10 vector in lane 1 was uncut and circular, and could therefore not directly be compared with the linear DNA-fragments of the molecular size marker applied in the left lane (Figure 4.8).

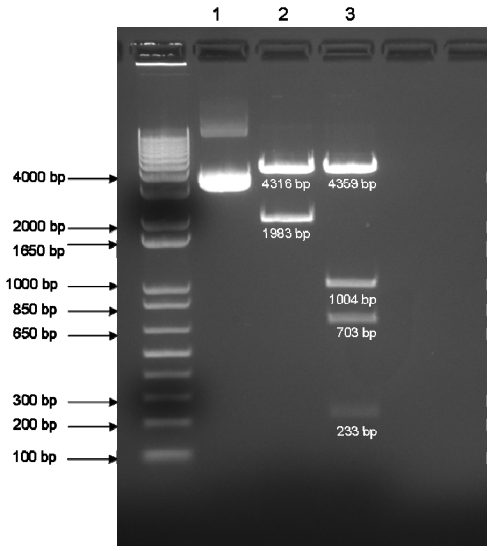


Figure 4.8: Agarose gel (1%) showing the p3xFLAG-CMV-10 empty vector verified with restriction digestion. The 1 kb Plus DNA ladder was used as a molecular size marker (left lane). Lane 1, p3xFLAG-CMV-10 uncut control. Lane 2, p3xFLAG-CMV-10 digested with SmaI. Lane 3, p3xFLAG-CMV-10 digested with NcoI.

4.2.2 Quality control of p3xFLAG CMV-10 R484W-, L514F-, S533N-, W535L-, K575M-Smo and Smo wt

"TOP10" competent *E. coli* bacteria were transformed with one of the five p3xFLAG-CMV-10 Smo mutants or Smo wt. The transformed bacteria were plated on LB-agar plates with ampicillin. Five colonies of each mutant were picked and cultured. The plasmid DNA in these bacteria-cultures was isolated in small scale, subsequently sent to DNA sequencing and were analysed on 1% agarose gels (Figure 4.9). The isolated plasmids of the various mutations of p3xFLAG-CMV-10 Smo had larger sizes than the p3xFLAG-CMV-10 empty vector control, indicating that the plasmids contained the expected inserts (Figure 4.9). The constructs in lane 1-5 (Figure 4.9 A and B) in addition to the control vector (p3x) are

circular plasmids, and not linear DNA-fragments, and should therefore not be compared with the linearised 1 kb Plus DNA ladder molecular size marker applied in the left lane (Figure 4.9 A and B).

Six primers were used for sequencing of the isolated plasmid inserts, A68V-FW, R199W-Rev, T349I-FW, S533N-Rev, K575M-FW and P755F-Rev (Table 3.1). The DNA sequences from the sequencing-lab (not shown) were analysed and one plasmid with the verified correct sequence were chosen for HiSpeed Plasmid Maxi preparation. To verify the insert length of the isolated plasmid DNA (from maxi-prep), restriction enzyme analysis was performed (Section 4.2.3, figure 4.10).

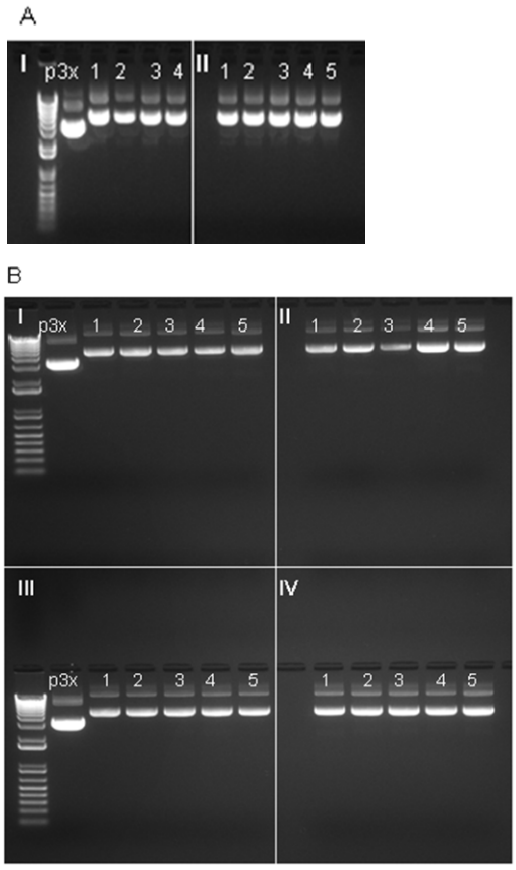


Figure 4.9: Agarose gel (1%) showing the five isolated plasmids for each of the p3xFLAG-CMV-10 Smo mutations. The p3xFLAG-CMV-10 vector was run as control (p3x). A I;lane 1-4: Four isolated plasmids of p3xFLAG-CMV-10 R484W-Smo. A II: Five small-scale plasmids of p3xFLAG-CMV-10 L514F-Smo plasmid. B I: Five small-scale plasmids of p3xFLAG-CMV-10 Smo wt. B II: Five small-scale plasmids of p3xFLAG-CMV-10 W535L-Smo. B III: Five small-scale plasmids of p3xFLAG-CMV-10 S533N-Smo. B IV: Five small-scale plasmids of p3xFLAG-CMV-10 K575M-Smo.

4.2.3 Restriction enzyme analysis of p3xFLAG-CMV-10 R484W-, L514F-, S533N-, W535L-, K575M-Smo, Smo wt and pEF.6 K575M-Smo

After HiSpeed Plasmid Maxi preparation of p3xFLAG-CMV-10 R484W-, L514F-, S533N-, W535L-, K575M-Smo and Smo wt plasmids, the plasmids were digested with Tth111I and subsequently analysed on 1% agarose gel (Figure 4.10). The expected product sizes from the

Tth111I restriction enzyme digestion were 2745 and 5939 bp, respectively (Figure 4.10, lane 2-7). Tth111I restriction enzyme digestion of the p3xFLAG-CMV-10 vector was expected to give one fragment with the size of 6296 bp (Figure 4.10, lane 1). The pEF.6 K575M-Smo has only one Tth111I restriction site and Tth111I digestion gave the expected product of size 7174 bp (Figure 4.10, lane 8). These plasmids were used for transfection of NIH/3T3-cells.

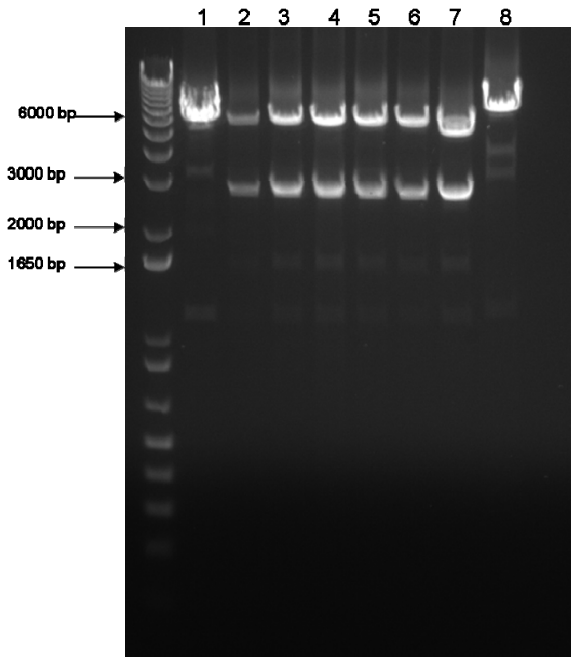


Figure 4.10: Agarose gel (1%) showing fragments from Tth111I restriction enzyme digestion of Smo-containing plasmids.
Lane 1: p3xFLAG-CMV-10 empty vector.
Lane 2: p3xFLAG-CMV-10 Smo wt.
Lane 3: p3xFLAG-CMV-10 R484W Smo.
Lane 4: p3xFLAG-CMV-10 L514F Smo.
Lane 5: p3xFLAG-CMV-10 S533N Smo.
Lane 6: p3xFLAG-CMV-10 W535L Smo.
Lane 7: p3xFLAG-CMV-10 K575M Smo.
Lane 8: pEF.6 K575M Smo.
The 1 kb Plus DNA ladder was used as molecular size marker (left lane).

4.3 CELL BASED EXPERIMENTS

NIH/3T3-cells were transfected with expression constructs according to the protocol described in the Methods chapter. Stably transfected cell lines were generated by culturing transfected cells in the presence of G-418 (Figure 4.11). Six individual clones of each transfection were isolated resulting in forty two potentially monoclonal cell lines (Table 4.1). The cell lines were analysed by reporter gene assay, real time RT-PCR and Western blotting. Reporter gene assay and real-time RT-PCR were used to characterise the monoclonal cell lines and determine the expression levels of the transfected constructs. The number of analysed monoclonal cell lines in real-time RT-PCR is shown in table 4.1. Western blotting was used to demonstrate the expression of Smo proteins from the transfected plasmids in stably transfected NIH/3T3-cell lines.

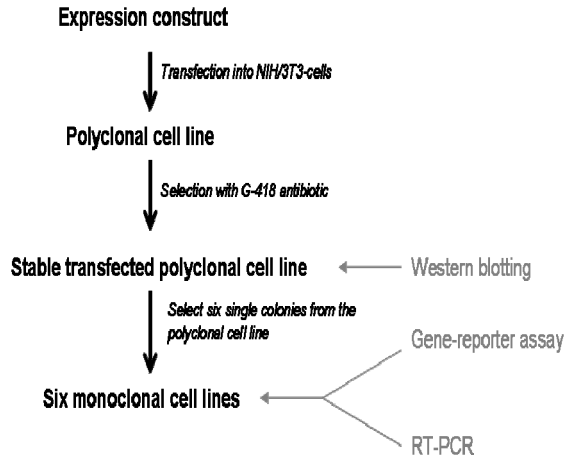


Figure 4.11: Flow-chart showing how monoclonal cell lines were generated.

Expression constructs inserted to p3xFLAG-CMV-10 vector	Total monoclonal cell lines analysed in RT-PCR
Empty vector	3
Smo wt	4
R484W Smo	4
L514F Smo	6
S533N Smo	6
W535L Smo	4
K575M Smo	6

Table 4.1: Monoclonal cell lines for RT-PCR analysis.

4.3.1 Gli1 activity in monoclonal cell lines (Luciferase assay)

Reporter gene assay (Luciferase assay) was used to analyse Gli1 activity in the monoclonal cell lines. The monoclonal cell lines were transfected with two independent plasmids: 1) Gli BS-Luc (Gli Binding Sequence-Luciferase) plasmid encoding firefly-Luciferase under the control of a Gli1 dependent promoter (experimental reporter), 2) the pGL4.74 vector encoding Renilla-Luciferase that served as an internal control. Activated Hh signalling pathway increased Gli1 activity which resulted in increased expression of firefly-Luciferase. Expression of Luciferase was easily monitored by EnVision 2104 Multilabel Reader (PerkinElmer).

Three different experimental approaches were used. In the first experiment the monoclonal cell lines were cultured in complete growth medium, and basal Gli1 activity in the various monoclonal cell lines was analysed (Figure 4.12). The three cell lines containing p3xFLAG-CMV-10 empty vector showed different levels of Gli1 activity (Figure 4.12 A), where cell line p3xFLAG 6 gave the lowest Gli1 activity. The response in p3xFLAG-CMV-10 empty vector transfected cells represents Gli1 activation mediated through endogenously expressed components of the Shh/Gli signalling pathway in NIH/3T3-cells. The cells transfected with R484W- and L514F-Smo showed a trend of lower gene expression levels compared to the other transfected cells containing Smo mutants (except for L514F-Smo number 5). The different clones of each Smo expression construct showed variable activation of Gli1, e.g.

p3xFLAG-CMV-10 S533N-Smo 1-6. In figure 4.12 B the result shown in panel A have been normalised to the p3xFLAG-CMV-10 vector cell line number 6 (p3xFLAG 6). It is difficult to identify a pattern between the expression constructs from only one experiment (Figure 4.12 A and B).

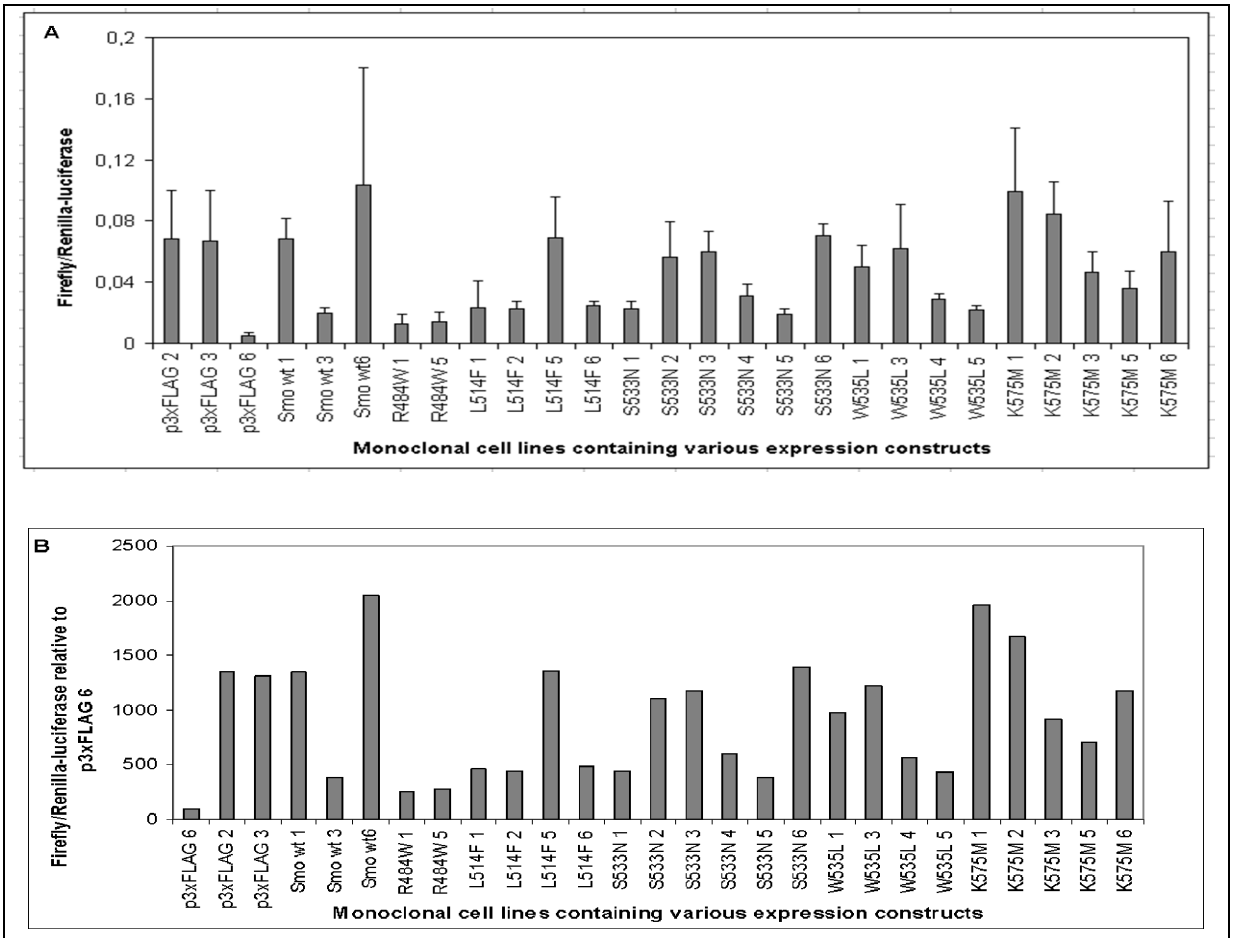


Figure 4.12: Gli1 activity expressed as Firefly/Renilla-luciferase ratio in 27 monoclonal cell lines. Stably transfected NIH/3T3-cells expressing wt or mutated Smo were transiently cotransfected with Gli BS-Luc and Renilla-Luciferase reporter constructs. Firefly-luciferase activity in cell lysates was normalised to Renilla luciferase activity. A: Results are expressed as the mean ± standard deviation (SD) of quadruplicates from one experiment (n=1). B: The results in A were normalised to p3xFLAG-CMV-10 vector cell line number 6 (p3xFLAG 6), which was given the value 100.

In a second experiment the cells were cultured in complete growth medium until confluence and then maintained for 18 h in reduced serum medium (Figure 4.13, black columns). In a third experimental design the cells were cultured in complete growth medium until confluence and then treated with 50:50 (V:V) Shh conditioned medium: 0.5% NCS-DMEM (Figure 4.13, grey columns) for 18 h. The Gli1 activity was determined for each experimental approach. There was no obvious effect of the Shh conditioned medium (Figure

4.13). Based on this single experiment, it is not possible to draw conclusions about the signalling properties of the various mutated versions of Smo.

RT-PCR experiments were performed in parallel with the Luciferase-assay. Based on initial experiments we decided to run RT-PCR to verify the monoclonal cell lines. The reporter gene assay experiment was therefore only performed once.

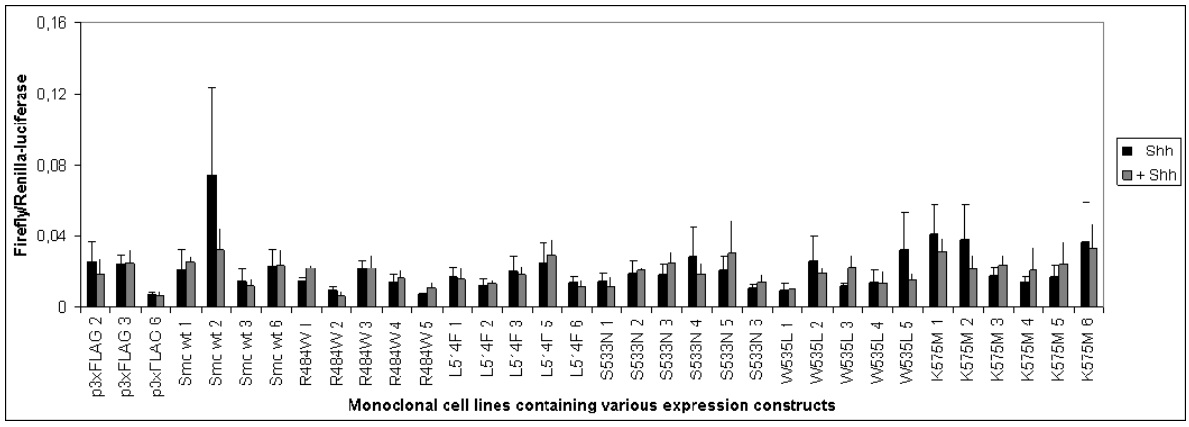


Figure 4.13: Gli1 activation expressed as Firefly/Renilla-luciferase ratio in 34 monoclonal cell lines in presence or absence of Shh. Stably transfected NIH/3T3-cells expressing wt or mutated Smo were transiently cotransfected with Gli BS-Luc and Renilla-Luciferase reporter constructs. Upon confluency the NIH/3T3-cells were serum starved (0.5% NCS DMEM) (black columns), or treated with Shh-conditioned medium (produced from EcR293 Shh cells) (grey columns). Gli activity in the cell lines were measured using Luciferase assay kit. The luciferase activity in cell lysates was normalised to Renilla luciferase activity. Results are expressed as the mean ± standard deviation (SD) of quadruplicates from one experiment (n=1).

4.3.2 Real time RT-PCR

Real-time RT-PCR was used for relative quantification of the expression levels of human Smo (hSmo), mouse Gli1 (mGli1) and mouse Ptch (mPtch) mRNA in each monoclonal cell line.

4.3.2.1 Analysis of pcDNA 3.1 S533N-, W535L-Smo and Smo wt expression and signalling.

RT-PCR was used to address the signalling properties of Smo wild type and two mutated versions of Smo (S533N- and W535L-Smo) inserted in the pcDNA 3.1 vector. These constructs and pcDNA 3.1 empty vector (control) were transfected into separately NIH/3T3-cells.

Four experiments were performed measuring the mRNA expression level of hSmo, mGli1 and mPtch in NIH/3T3-cells containing the various constructs. The different gene expression levels in the transfected NIH/3T3-cells were compared to each other (Figure 4.14). The white columns show gene expression levels in cells maintained in low serum medium (0.5% NCS-DMEM), and the black columns show gene expression levels in cells maintained in complete growth medium (5% NCS-DMEM). The pcDNA 3.1 W535L-Smo showed a higher expression level of hSmo compared to pcDNA 3.1 Smo wt and S533N-Smo, but the difference was not significant (Figure 4.14 A). NIH/3T3-cells transfected with empty vector showed minimal hSmo expression (Figure 4.14 A). The pcDNA 3.1 W535L-Smo gave a higher expression level of mGli1 compared to the other constructs (Figure 4.14 B). The expression level of mPtch did not differ between the different constructs (Figure 4.14 C).

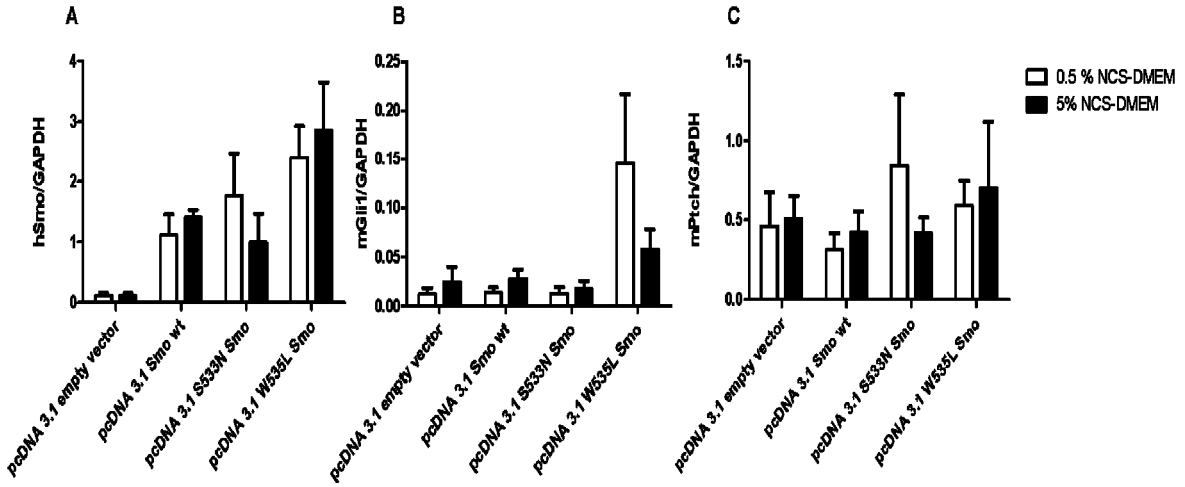


Figure 4.14: Messenger RNA expression of hSmo (A), mGli1 (B) and mPtch (C) in NIH/3T3-cells quantified by real-time RT-PCR. Total RNA was isolated from stably transfected NIH/3T3-cells with the various constructs in pcDNA 3.1. The white columns show gene expression levels in cells maintained in low serum medium (0.5% NCS-DMEM), and the black columns show gene expression levels in cells maintained in complete growth medium (5% NCS-DMEM). The mRNA expression levels are presented relative to the expression of the reference gene GAPDH in each sample. The data are expressed as mean ± SEM of triplicates from four independent experiments (n=4).

As a positive control for the RT-PCR analysis, NIH/3T3-cells transfected with pcDNA 3.1 empty vector were treated with Shh-conditioned medium (Figure 4.15). Minimal hSmo expression was observed in the NIH/3T3-cells (Figure 4.15 A). Figure 4.15 B and C show that mGli1 and mPtch expression levels are increased when the NIH/3T3-cells were treated with Shh-conditioned medium. In the cells transfected with empty vector the Hh signalling pathway became activated through endogenously expressed pathway members.

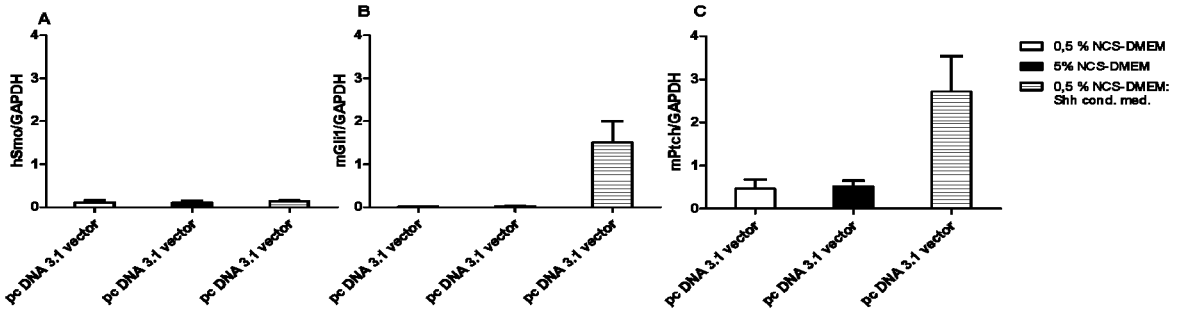


Figure 4.15: Messenger RNA expression of hSmo (A), mGli1 (B) and mPtch (C) in NIH/3T3-cells transfected with pcDNA 3.1 empty vector quantified by real-time RT-PCR. Total RNA was isolated from NIH/3T3-cells stably transfected with the pcDNA3.1 empty vector. The white columns show gene expression levels in cells maintained in low serum medium (0.5% NCS-DMEM), and the black columns show gene expression levels in cells maintained in complete growth medium (5% NCS-DMEM). The stribed columns show gene expression levels in cells treated with 50:50 (V:V) Shh conditioned medium:0.5% NCS-DMEM. The mRNA expression levels are presented relative to the expression of the reference gene GAPDH in each sample. The data are expressed as mean ± SEM, n=4.

4.3.2.2 Verification of monoclonal cell lines

Each confluent monoclonal cell line (Table 4.1) was prepared for analysis as described in the Methods chapter (section 3.7.2). As previously mentioned, real-time RT-PCR was used to detect and relatively quantify the relative expression levels of hSmo, mGli1, mPtch mRNA in each monoclonal cell line. Calculation of the original mRNA in the sample was performed as described in section 3.7.2.6, and presented in figure 4.16-4.26. The data were analysed in three ways:

1. The hSmo (Figure 4.16) mGli1 (Figure 4.17) and mPtch (Figure 4.18) mRNA expression levels in the monoclonal cell lines are presented in separate graphs. Figure 4.16 shows that the three monoclonal cell lines transfected with p3xFLAG-CMV-10 empty vector expressed minimal hSmo mRNA indicating that the primers were specific for human Smo. NIH/3T3-cells transfected with R484W-, L514F- and S533N-Smo shows relatively low hSmo mRNA expression levels compared to cells transfected with W535L-, K575M-Smo and Smo wt. The data show high variability. W535L 4 shows high expression level of mGli1 mRNA compared to the other constructs in figure 4.17. The monoclonale cell lines containing the various expression constructs showed variable degree of mPtch mRNA expression level in figure 4.18.

2. Mouse Gli1 mRNA expression level is expressed relative to hSmo mRNA expression level within each cell line. This was done with every cell line, but only one graph is shown as an example (Figure 4.19). Expression levels of mGli1 mRNA relative to hSmo mRNA from six monoclonal cell lines containing p3xFLAG-CMV-10 L514F-Smo are shown in figure 4.19. The expression levels of mGli1 and hSmo mRNA is approximately equal in L514F 1, 4 and 5.
3. The expression levels of hSmo, mGli1 and mPtch mRNAs were analysed in every one of the monoclonal cell lines, and the data from the various cell lines are presented in separate graphs (Figures 4.20- 4.26). The data presented in figure 4.20-4.26 show high variability. Figure 4.20 A-C show that the three cell lines containing p3xFLAG-CMV-10 empty vector had minimal expression of hSmo mRNA. The p3xFLAG 3 showed the highest mGli1 mRNA expression level. Figure 4.21 A-D show expression levels of hSmo, mGli1 and mPtch mRNAs in four monoclonal cell lines containing p3xFLAG-CMV-10 Smo wt. Smo wt 1 showed the highest hSmo mRNA expression level compared to the other cell lines, but had very low mGli1 expression level. Smo wt 2 had approximately equal hSmo mRNA and mGli1 mRNA expression levels. Figure 4.22 A-D show expression levels of the three genes in four monoclonal cell lines containing p3xFLAG-CMV-10 R484W-Smo. R484W 2 showed highest hSmo mRNA expression level compared to the other cell lines, but mGli1 mRNA expression level was low. Figure 4.23 show expression levels of the three genes in six monoclonal cell lines containing p3xFLAG-CMV-10 L514F-Smo. L514F 2 had the highest expression level of hSmo mRNA and mGli1 mRNA compared to the other cell lines. Figure 4.24 shows expression levels of hSmo, mGli1 and mPtch mRNAs in six monoclonal cell lines containing p3xFLAG-CMV-10 S533N-Smo. S533N 4 contained highest hSmo mRNA expression level andthe mGli1 expression level was relatively low. Figure 4.25 shows expression levels of hSmo, mGli1 and mPtch mRNAs in four monoclonal cell lines containing p3xFLAG-CMV-10 W535L-Smo. W535L 3 and 4 contained highest level of hSmo mRNA compared to the other cell lines. W535L 4 contained higher mGli1 expression level compared to W535L 3. Figure 4.26 shows expression levels of hSmo, mGli1 and mPtch mRNAs in six monoclonal cell lines containing p3xFLAG-CMV-10 K575M-Smo. K575M 4 contained very high hSmo mRNA expression level compared to the other cell lines, but mGli1 mRNA expression level was low.

1.

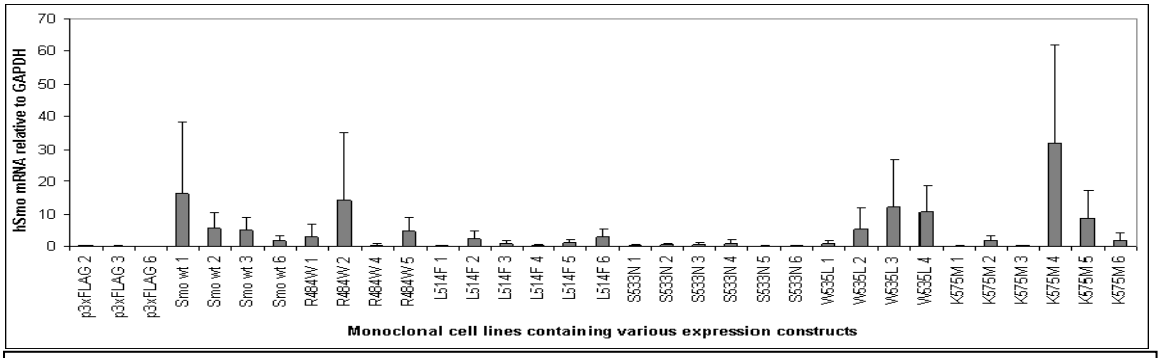


Figure 4.16: Messenger RNA expression of hSmo in various monoclonal cell lines. The values obtained are normalized to the reference gene GAPDH. Data are expressed as the mean ± standard deviation (SD) of triplicates from three experiments (n=3).

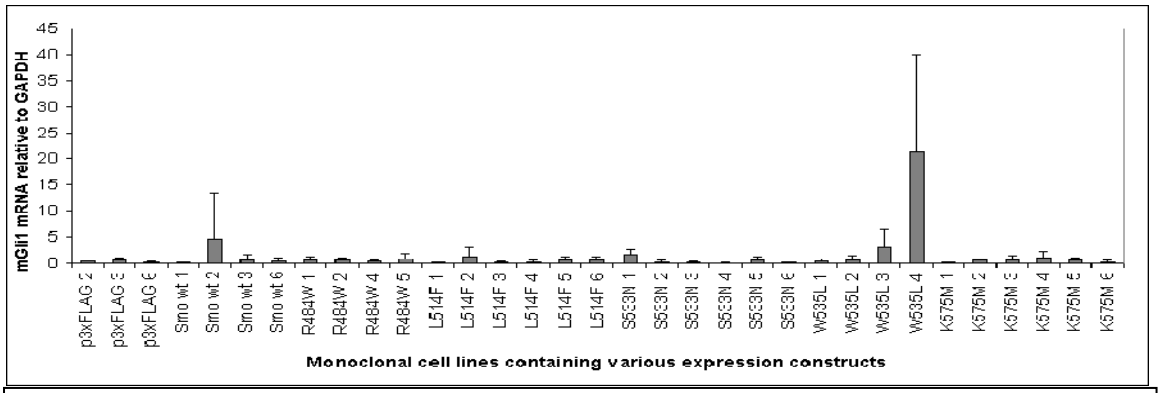


Figure 4.17: Messenger RNA expression of mGli1 in various monoclonal cell lines. The values obtained are normalized to the reference gene GAPDH. Data are expressed as the mean ± standard deviation (SD) of triplicates from three experiments (n=4).

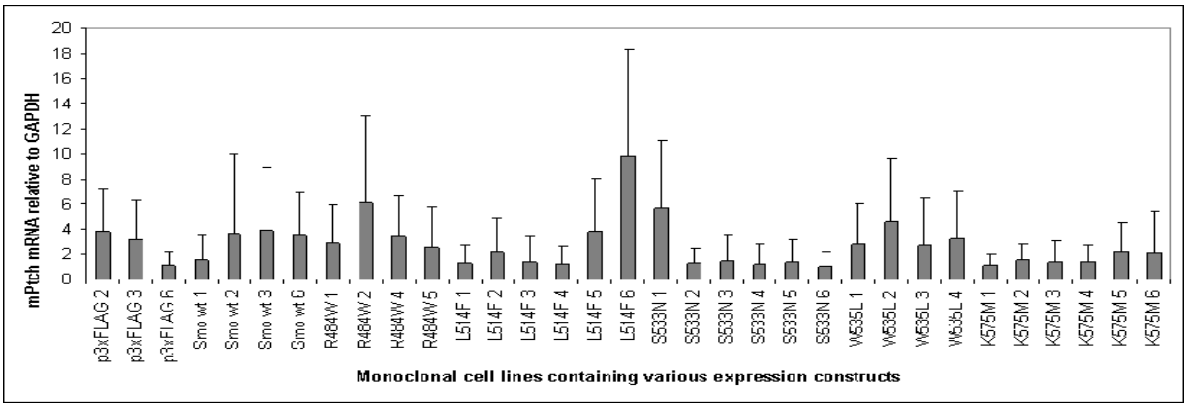


Figure 4.18: Messenger RNA expression of mPtch in various monoclonal cell lines. The values obtained are normalized to the reference gene GAPDH. Data are expressed as the mean ± standard deviation (SD) of triplicates from three experiments (n=4).

2.

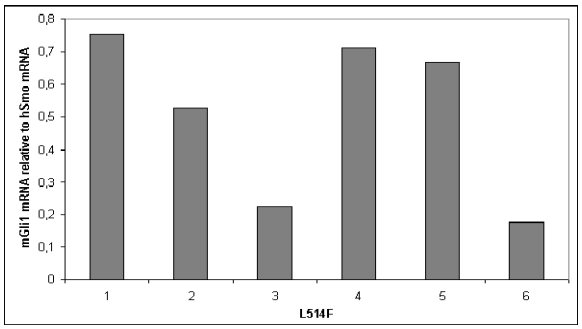


Figure 4.19: Expression level of mGli1 mRNA relative to hSmo mRNA in six monoclonal cell lines containing p3xFLAG-CMV-10 L514F-Smo.

3.

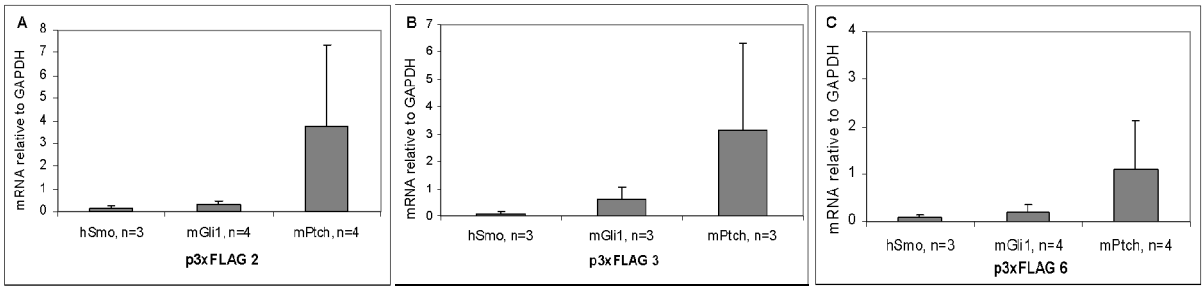


Figure 4.20: Expression levels of hSmo, mGli1 and mPtch mRNAs in three monoclonal cell lines containing p3xFLAG-CMV-10 empty vector. The values obtained are normalized to the reference gene GAPDH. Data are expressed as the mean ± standard deviation (SD) of triplicates from three experiments (n=3) for hSmo, and four experiment for mGli1 and mPtch (n=4). Cell lines: p3xFLAG 2 (A), p3xFLAG 3 (B) and p3xFLAG 6 (C).

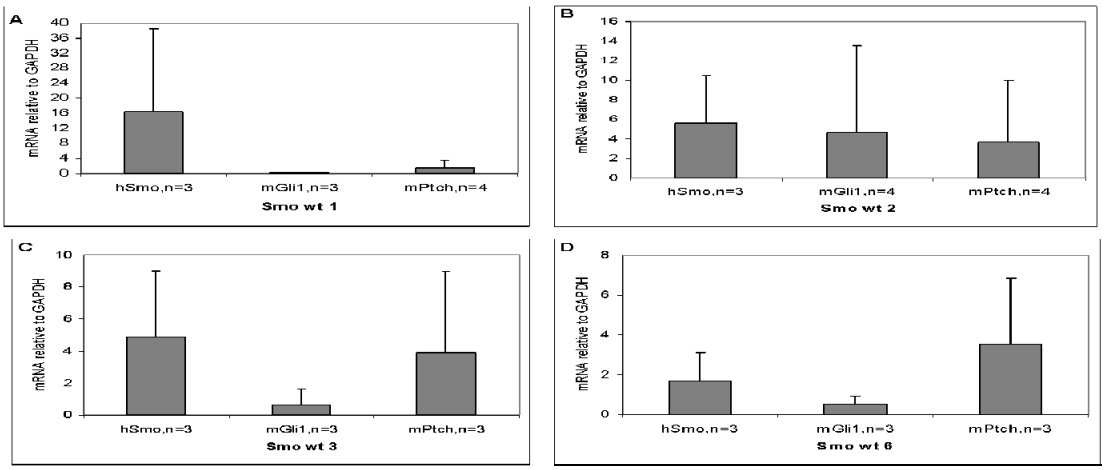


Figure 4.21: Expression levels of hSmo, mGli1 and mPtch mRNAs in four monoclonal cell lines containing p3xFLAG-CMV-10 Smo wt. The values obtained are normalized to the reference gene GAPDH. Data are expressed as the mean ± standard deviation (SD) of triplicates from three experiments (n=3) for hSmo, and four experiment for mGli1 and mPtch (n=4). Cell lines: Smo wt 1(A), Smo wt 2 (B), Smo wt 3 (C) and Smo wt 6 (D).

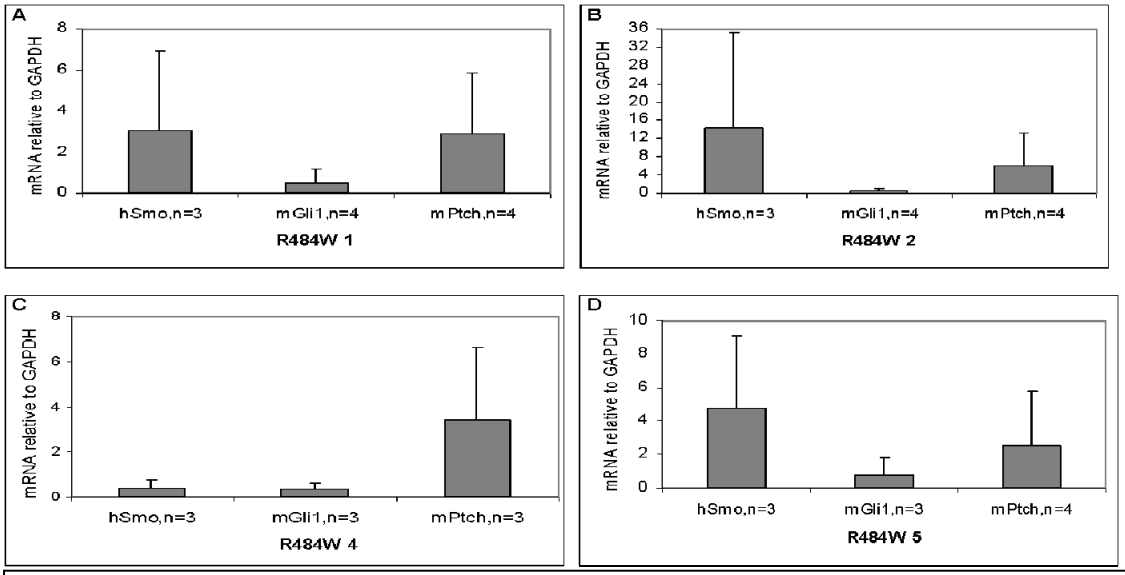


Figure 4.22: Expression levels of hSmo, mGli1 and mPtch mRNAs in four monoclonal cell lines containing p3xFLAG-CMV-10 R484W Smo. The values obtained are normalized to the reference gene GAPDH. Data are expressed as the mean ± standard deviation (SD) of triplicates from three experiments (n=3) for hSmo, and four experiment for mGli1 and mPtch (n=4). Cell lines: R484W 1 (A), R484W 2 (B), R484W 4 (C) and R484W 5 (D).

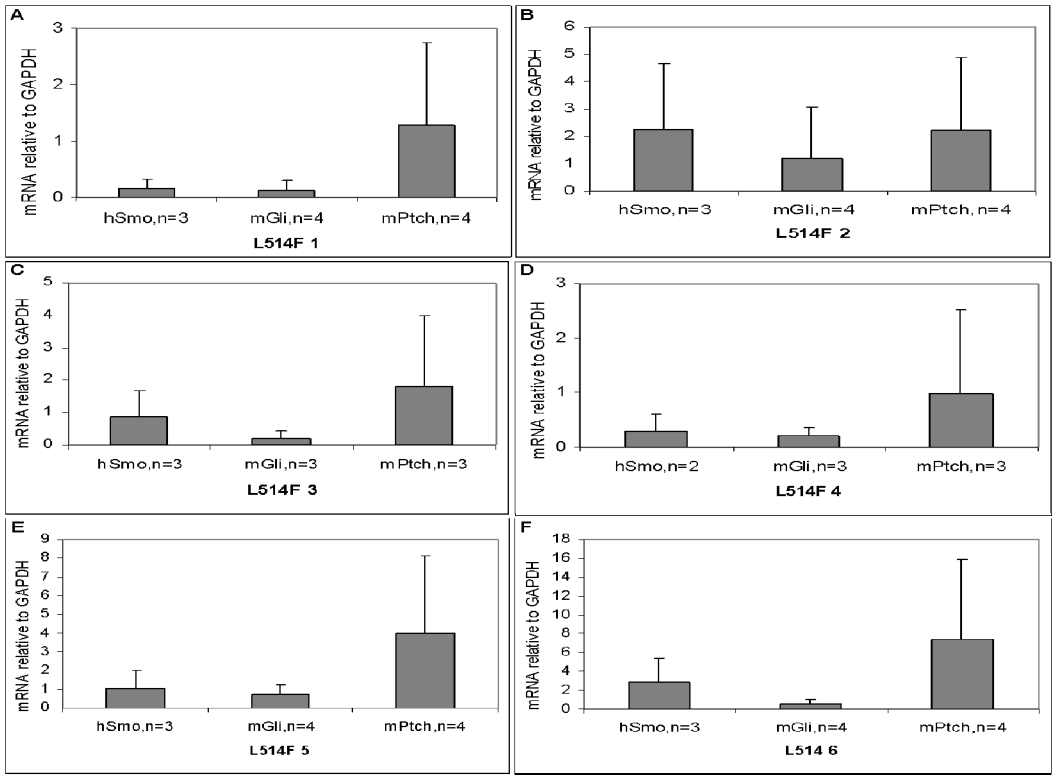


Figure 4.23: Expression levels of hSmo, mGli1 and mPtch mRNAs in six monoclonal cell lines containing p3xFLAG-CMV-10 L514F Smo. The values obtained are normalized to the reference gene GAPDH. Data are expressed as the mean ± standard deviation (SD) of triplicates from three experiments (n=3) for hSmo, and four experiment for mGli1 and mPtch (n=4). A-F: Gene expression levels in cell line number L514F 1-6, respectively.

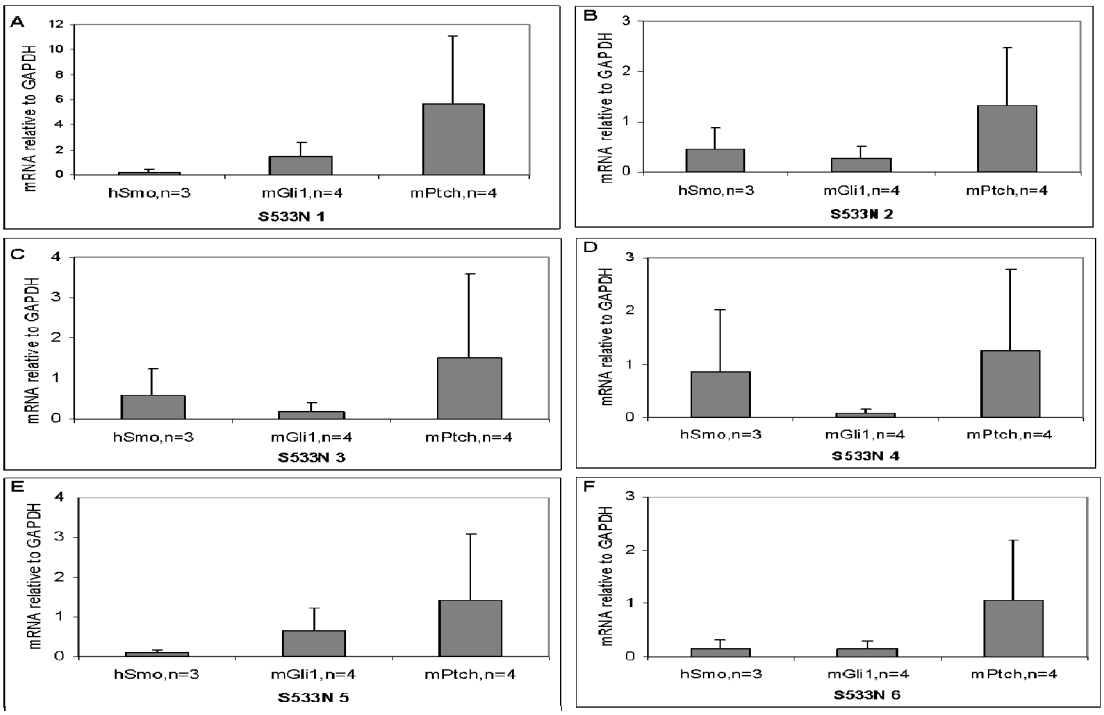


Figure 4.24: Expression levels of hSmo, mGli1 and mPtch mRNAs in six monoclonal cell lines containing p3xFLAG-CMV-10 S533N Smo. The values obtained are normalized to the reference gene GAPDH. Data are expressed as the mean ± standard deviation (SD) of triplicates from three experiments (n=3) for hSmo, and four experiment for mGli1 and mPtch (n=4). A-F: Gene expression levels in cell line number S533N 1-6, respectively.

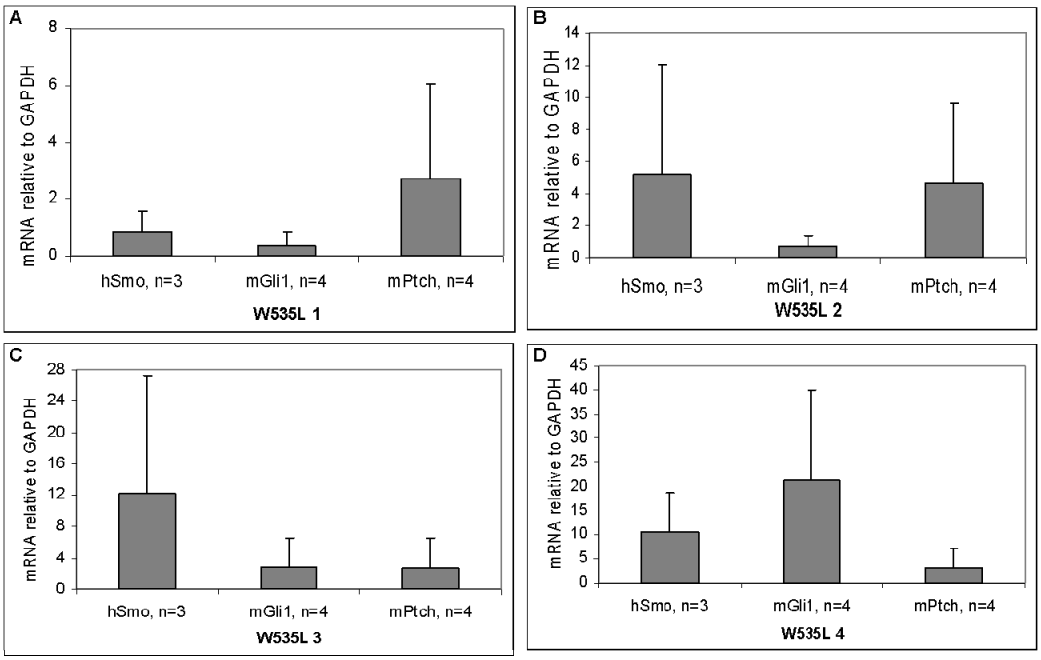


Figure 4.25: Expression levels of hSmo, mGli1 and mPtch mRNAs in four monoclonal cell lines containing p3xFLAG-CMV-10 W535L Smo. The values obtained are normalized to the reference gene GAPDH. Data are expressed as the mean ± standard deviation (SD) of triplicates from three experiments (n=3) for hSmo, and four experiment for mGli1 and mPtch (n=4). A-D: gene expression levels in cell line number W535L 1-4, respectively.

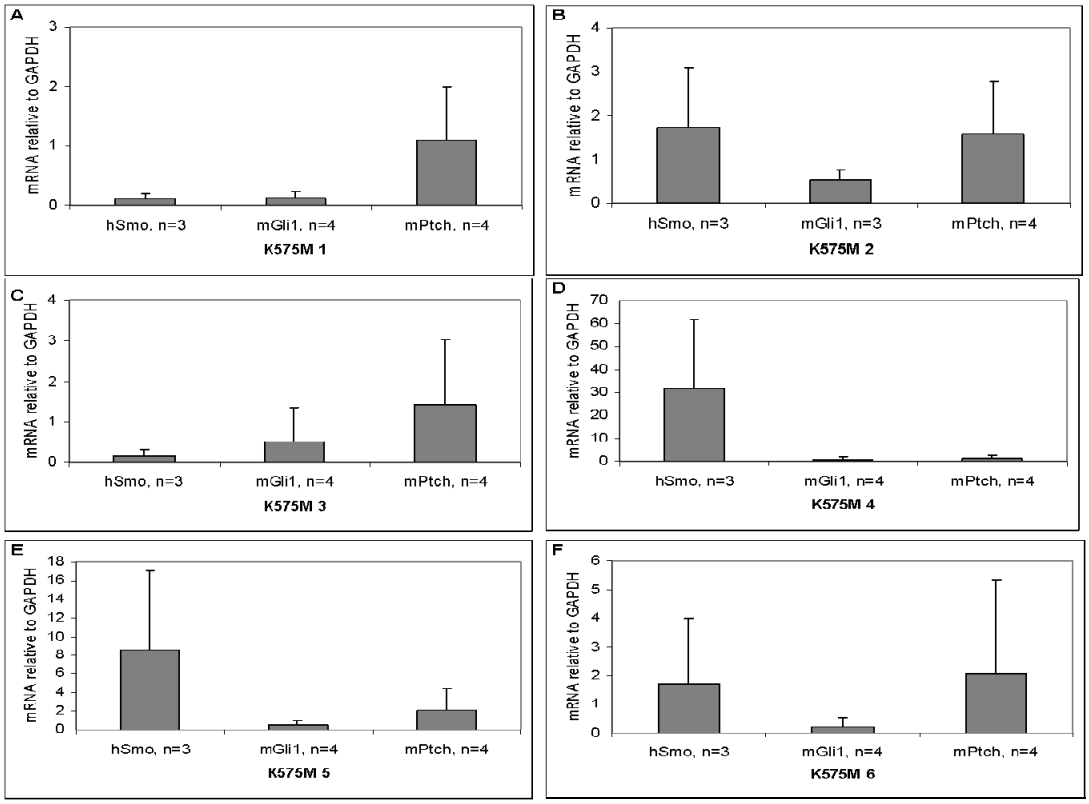


Figure 4.26: Expression levels of hSmo, mGli1 and mPtch mRNAs in six monoclonal cell lines containing p3xFLAG-CMV-10 L514F Smo. The values obtained are normalized to the reference gene GAPDH. Data are expressed as the mean ± standard deviation (SD) of triplicates from three experiments (n=3) for hSmo, and four experiment for mGli1 and mPtch (n=4). A-F: Gene expression levels in cell line number K575M 1-6, respectively.

4.3.3 Western blotting

Samples from stably transfected polyclonal cell lines were prepared for Western blot as described in the Methods chapter (section 3.7.3 – 3.7.5). Figure 4.27 shows proteins at approximate sizes of 75 and 100 kDa detected by Western blotting in lysates of NIH/3T3-cells transfected with various constructs. The expected protein size of the Smo was 86.4 kDa. The anti-FLAG M2 antibody was supposed to bind Flag-tagged Smo expressed in NIH/3T3-cells transfected with various Smo expression constructs. The same bands were also seen in the samples transfected with empty vector, indicating that the anti-FLAG M2 antibody binds non-specifically.

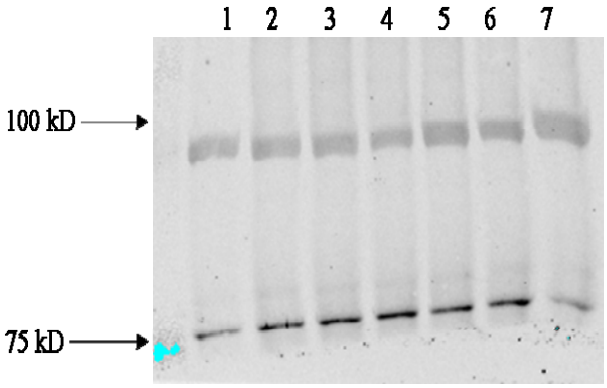


Figure 4.27: Western blot showing proteins from NIH/3T3-cells transfected with various constructs. The protein samples were run on 6% acrylamide gel and subsequently transferred to a nitrocellulose membrane when using Odyssey Infrared Imaging System. Primary antibody used was monoclonal anti-FLAG M2 from Sigma (dilution 1:1000). Secondary antibody used was donkey anti-mouse IR Dye from Odyssey. The Precision Plus Protein Dual Color Standards (Bio-Rad) was used as a protein marker.
Lane 1: p3xFLAG-CMV-10 empty vector. Lane 2: p3xFLAG-CMV-10 Smo wt. Lane 3: p3xFLAG-CMV-10 R484W-Smo. Lane 4: p3xFLAG-CMV-10 L514F-Smo. Lane 5: p3xFLAG-CMV-10 S533N-Smo. Lane 6: p3xFLAG-CMV-10 W535L-Smo. Lane 7: p3xFLAG-CMV-10 K575M-Smo.

5. DISCUSSION

5.1 METHODOLOGICAL CONSIDERATIONS

When evaluating the results presented in this thesis, it is important to bear in mind that the methods used to obtain these results have certain limitations.

5.1.1 Traditional PCR and agarose gel electrophoresis

In this study traditional RT-PCR was used to generate the K575M-encoding sequence from the Smo wt sequence and to generate an EcoRI restriction site in the six following constructs: pEF.6 R484W-, L514F-, S533N-, W535L- , K575M-Smo and Smo wt. Agarose gel electrophoresis was used to identify and isolate the PCR-products, and to separate and purify restriction fragments for ligation reactions, and for quality control of the various plasmid constructs.

Traditional PCR involves several reaction components that may influence the final amplicon products like template, primers, reaction buffer, dNTP mix and thermostable DNA polymerase. Sometimes we obtained little or no PCR-products (not shown in this thesis) after running them on agarose gels. Non-visible bands can be caused by poor PCR primer design, DNA template containing inhibitors (reagents such as DMSO, SDS and formamide), too low template concentration, too high annealing temperature or too low primer concentration. When performing traditional PCR you should be aware of these factors. In some agarose gel analysis unexpected bands were observed (Figure 4.5 B), which may be caused by primer-dimer formation, non-specific annealing of primers to template or contaminated DNA from exogenous sources [78].

5.1.2 Analysis of cell experiments

The cell-based experiments were performed on transfected NIH/3T3-cells. The methods used to obtain results from these cells have certain limitations and the final results may be influenced by many factors. One of the most important factors is the transfection efficiency, which may be affected by many parameters including the cell culture conditions and the

quality of the used vector. Too low or too high cell densities at the time of transfection may result in poor uptake of the transfection complexes and insufficient expression of the transfected genes. Poor plasmid DNA quality may also affect the transfection efficiency negatively, and may be a result of poor plasmid purification or endotoxin contamination during the purification process. Endotoxins are cell membrane components of Gram-negative bacteria (e.g. E. coli) that are released during the lysis step of plasmid preparation. Transfection efficency are, however, never 100%, and as a consequence, some cells remain without the desired plasmid, and thus do not express the protein it encodes.

Primarily, the real-time RT-PCR method is considered in this section, because it is the main method used among the cell-based experiments in this project.

5.1.2.1 Gene-reporter assay

Running luciferase-assay includes rough treatment of the cells. At first, the cells were transfected with different expression vectors, and subsequently stabilised with G-418 antibiotics. By establishing stable cell lines, experimental variation due to differences in transfection efficiencies may be avoided [79]. These stably transfected cells were co-transfected (transient transfection) with reporter gene constructs; one internal and one experimental construct. As mentioned above there are many parameters affecting transfection efficiencies and transfecting the cells twice increases the risk of achieving variable results.

5.1.2.2 Real-time RT-PCR

In this study real-time RT-PCR was used to determine the mRNA levels of different genes in the Hh signalling pathway. All values were normalised to glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), and some experiments were also evaluated to the normalisation gene TATA box binding-protein (Tb-p). Real-time RT-PCR is a sensitive method of comparing the expression of selected genes between different samples. The specificity and sensitivity make it possible to detect small differences in mRNA expression. The method has a large dynamic range and potential for high throughput as well as accurate quantification [80]. Real-time RT-PCR is based on the assumption that there is a quantitative relationship between the amount of template present at the beginning of the reaction and amount of PCR-

product at any given cycle. The quantitation relies on the C_t values determined during the detectable exponential phase of the PCR, and on the standard curve [81]. The standard curve was used to calculate original mRNA amount based on estimated amplification efficiency.

Real-time RT-PCR analysis of cellular mRNA expression involves many technical steps that may influence the final outcome, including the preparation of cells, isolation of RNA, first strand cDNA synthesis and finally the polymerase chain reaction [80]. The quality and density of cells in the sample, the isolation of pure RNA and accuracy in the cDNA synthesis and the use of primers and type of housekeeping gene during PCR are important for the reliability of the final result. A small amount of contamination during these processes may affect the analysis significantly.

Isolation of RNA: RNA, isolated from the cells, is extremely delicate once removed from its cellular environment. RNA isolation and purification is more complicated than of DNA which is more stable, and a template suitable for inclusion in an RT-PCR assay must be of highest quality if quantitative results are wanted [76]. After performing RNA isolation, the RNA should be undegraded and free of protein and DNA (DNAse treated). The evaluation of RNA integrity by inspection of the 28 S and 18 S rRNA bands was accomplished by the use of agarose gel electrophoresis. The sample purity as well as concentration was also assessed spectrophotometrically.

Normalisation: To control for error in real-time RT-PCR, it is important to make use of a correct normalisation strategy. Normalisation remains one of real-time RT-PCRs most difficult problems. Several strategies have been proposed for normalising real-time RT-PCR data [80]. One accepted method for minimising the mentioned errors and correcting for sample-to-sample variation is to amplify, simultaneously with the RNA-samples, a cellular RNA that serves as an internal reference against which other RNA values may be normalised. The ideal internal standard should be expressed at a constant level among different tissues of an organism, at all stages of development, and should be unaffected by the experimental treatment. Additionaly, an internal control should also be expressed at roughly the same level as the RNA under study [81].

The mRNA encoding GAPDH is frequently used as an endogenous control for quantitative RT-PCR analysis. However, there is evidence suggesting that its use as an internal standard is inappropriate [82, 83]. The GAPDH expression level seems to vary between different

individuals and during the cell cycle [81]. As mentioned the values obtained in the current project were all normalised to GAPDH, whereas some experiments were also normalised to the normalisation gene Tb-p. Human Smo expression level in one RT-PCR experiment was analysed relative to both GAPDH and Tb-p and both normalisation genes gave approximately the same pattern of hSmo expression level in the various monoclonal cell lines. Therefore, all data from RT-PCR showed in the Results section are only normalized to GAPDH.

Specific primers: SYBR Green and Taqman probe were used to detect and quantify PCR-products in real-time PCR reactions. The disadvantage of using SYBR Green to detect and quantify PCR-products is that it may bind to any double-stranded DNA in the reaction, including primer-dimers and other non-specific reaction products, which results in an overestimation of the target concentration. To avoid such disadvantages this method requires specific primers. To confirm that the measured gene expression levels were not affected by primer dimerisation, primers were tested by melting point analysis. Probe based (Taqman) PCR is a more reliable quantification method since a single fluorophore is only released from quenching when a specific amplified molecule is synthesized, avoiding detection of unspecific products.

5.1.2.3 Western blotting

Primarily, Western blotting was used to detect Smo-protein expression in polyclonal NIH/3T3-cells. Western blotting involves many technical steps that may affect the final outcome. Sources of error that can contribute to the reliability of the results are the quality of the cells, protein sample processing of the cells, protein quantification, loading of proteins on the gel, transfer of proteins to membrane and attachment and quality (specificity) of primary and secondary antibodies to the membranes. The bands on the membranes are evaluated visually in relation to the other bands on the same membrane and the protein standard. In essence this involves detecting the sizes of the proteins and the intensity of the bands. The appearance of the bands, their intensity and background noise may influence calculations of band density. These factors make it difficult to determine accurately the relative band densities and accounts for large variability in density measurements.

5.2 INTERPRETATION OF THE RESULTS

5.2.1 Generation of K575M-Smo encoding plasmid

The K575M-Smo encoding plasmid was successfully generated in the laboratory. The K575M-encoding sequence was generated from the Smo wt sequence in a PCR-based mutagenesis approach. It is well known that the polymerase enzymes used for PCR may make mistakes and insert another nucleotide than the one present in the template. In this thesis, the high fidelity Vent DNA polymerase was used for all PCR reactions to reduce the problem with PCR-introduced unwanted mutations. The fidelity of Vent is 5-15 times higher than what has been observed for Taq DNA polymerase and potential problems with PCR-induced mutations should be minimal [84, 85]. The generated K575M-Smo encoding construct was sequenced to verify that no unwanted mutations had been introduced during the PCR reactions. Alignment of the sequenced construct and the "original" Smo wt sequence showed that the PCR reactions had been completed without introducing unwanted mismatches.

Using high fidelity polymerases for this kind of PCR-based mutagenesis protocols may cause some additional challenges. The high fidelity polymerase may also "correct" the mismatches introduced by the primers in the first set of PCR reactions. Therefore, several potential K575M-Smo constructs were sequenced. Five of the nine sequenced constructs showed the intended K575M-Smo mutation and no additional PCR-introduced mutations. This PCR-based mutagenesis protocol using the Vent DNA polymerase proved to be reliable and efficient to generate point mutations. One of the K575M-Smo plasmids with the verified correct sequence was used for sub-cloning and subsequent cell based experiments to address the signalling properties of this mutated version of Smo.

5.2.2 Signalling properties of R484W-, L514F-, S533N-, W535L-, and K575M-Smo

Real-time RT-PCR was used for relative quantification of hSmo, mGli1 and mPtch mRNA expression levels in the established monoclonal cell lines. Primer specific for human Smo was used to determine if the cells contained the transfected Smo. It was desirable to identify cell lines expressing the transfected human Smo at relatively equal levels. Mouse Gli1 and

Ptch expression levels were measured to determine the signalling properties of the mutated versions of Smo. Increased expression levels of mGli1 and mPtch in cell lines transfected with a Smo-mutant compared to cell lines transfected with Smo wt might indicate constitutive activity of the mutated version of Smo.

5.2.2.1 Analysis of the pcDNA3.1 S533N-, W535L-Smo and Smo wt expression and signalling

The objective of these experiments was to address the signalling properties of S533N- and W535L-Smo by comparing expression levels of mGli1 and mPtch mRNA in S533N-, W535L-Smo and Smo wt cell lines. NIH/3T3-cells transfected with empty vector showed minimal hSmo mRNA expression as expected since the primers are specific for human Smo. The minimal signal might be caused from unspecific priming. The S533N-Smo showed no increase in mGli1 or mPtch mRNA levels compared with Smo wt. However, the W535L-Smo construct showed increased mGli1 mRNA expression, but no changes in mPtch mRNA. On the other hand, Shh conditioned medium increased both mGli1 and mPtch mRNA indicating that the point of pathway activation may influence gene regulation. The genes targeted by Shh include Gli1, Ptch and Hip, thus Shh stimulation should result in increased levels of Gli1 and Ptch. If the W535L-Smo is constitutive active in this system (transfected into NIH/3T3-cells), increased mPtch mRNA could be expected. Possible explanations for the observed failure of the W535L-Smo to increase the level of mPtch mRNA might be:

- When the Hh signalling pathway is activated downstream of Ptch, not all the pathway components usually activated by ligand become activated and consequently no mPtch mRNA increase is observed for the W535L-Smo induced activation of the pathway.

- Ptch-silencing through DNA promoter methylation. DNA methylation occurs by covalent addition of a methyl group to the DNA, and these methyl groups project into the major groove of DNA and effectively inhibit transcription [86]. Ptch-silencing through DNA promoter methylation has been shown in medulloblastoma and acute myeloid leukemia [87-90]. Proper demethylation of the promoter may require ligand induced activation of the Hh signalling pathway.

However, another explanation might be that the W535L-Smo shows no constitutive activity in the system used in this thesis work. W535L-Smo are expected to show constitutive activity based on reports from Low W.C. et al [70] and Xie J. *et al* [68]. Low W.C. *et al.* [70] showed that W535L-Smo was constitutive active analysing Shh-light cells with gene reporter assay. The Shh-light cells contained a stably integrated Gli-dependent firefly luciferase reporter and a Renilla luciferase reporter control and were infected with a retrovirus containing Smo wt and W535L-Smo cDNAs. Xie J. *et al.* investigated the oncogenic potential of the W535L-Smo missense mutation in BCCs, transfecting rat embryonic fibroblast REF52 cells with E1A together with wild-type or W535L-Smo and assessed for focal cells overgrowth. Focal cells overgrowth indicate oncogenic potential, and this was identified in REF52 cells with E1A and W535L-Smo [68]. Previously reports have not identified oncogenic properties for W535L-Smo in the system we have used in this thesis work.

Based on these results we decided to make tagged Smo expression constructs into a new vector, the p3xFLAG-CMV-10 vector, aiming at increasing the detection possibilities using this vector. The vector encodes three adjacent FLAG-epitopes that will be expressed as a fusion protein with Smo. Use of this expression vector provides several advantages:

- Separation of transfected and tagged Smo from endogenous Smo.
- Antibiotic selection, as the vector contains the G-418 (geneticin) resistance gene.
- Detection of expression levels using an antibody specific against p3xFLAG-tag (Western blotting), and by specific primers in quantitative PCR.

5.2.2.2 Verification of monoclonal cell lines: Analysis of the p3xFLAG-CMV-10 R484W-, L514F-, S533N-, W535L-, K575M-Smo and Smo wt expression and signalling.

The five mutated versions of Smo and Smo wt as well as p3xFLAG-CMV-10 empty vector transfected cell lines were analysed by a gene reporter, RT-PCR and Western blotting assays. In the cells transfected with empty vector the Hh signalling pathway may become activated through endogenously expressed pathway members.

Due to time limitations it was decided to determine the Smo mRNA expression in the monoclonal cell lines performing real-time RT-PCR based on the initial experiments with the pcDNA3.1 Smo cell lines. Furthermore, real-time RT-PCR was preferred versus the gene reporter assays due to the need for an additional set of transfections in this assay and the

uncertainty of transfection efficiency. High cotransfection efficiency is difficult to obtain, due to the many parameters that may affect the transfection including the cell densities, DNA concentrations, transfection reagent and incubation time. Based on the single gene reporter assay performed, it is not possible to conclude about the signalling properties of the various mutated versions of Smo. However, the cells transfected with R484W- and L514F-Smo showed a trend towards lower gene reporter activity compared to the other mutants of Smo (except for L514F 5), indicating that the R484W- and L514F-Smo mutations may result in impaired rather than increased pathway activation. Considering the gene reporter assay addressing Gli activation in 34 monoclonal cell lines treated with or without Shh conditioned medium, there were no stimulatory effect of this medium, as the Gli1 activity from the various cell lines was approximately equal (Figure 4.13). The Shh level in the conditioned medium may be low due to plating of too few EcR293 Shh cells. This experiment was performed only once, and it is therefore not possible to conclude about the signalling properties of the various mutated versions of Smo from this experiment.

Using real-time RT-PCR, the mRNA expression levels of hSmo, mGli1 and mPtch were analysed in the Smo monoclonal cell lines, aiming at identifying cell lines expressing the transfected human Smo. Constitutively active Smo should increase the Hh signalling pathway activity and result in increased mGli1 and mPtch mRNA expression levels. When analysing the relationship between mGli1 and hSmo mRNA expression within the various cell lines, the expression levels of each gene should also be analysed as explained below (See the hypothesis figure 5.1 described below).

Hypothesis: Two hypothetic cell lines express constitutively active receptors (marked *) but with different expression levels (Figure 5.1). Cell line 1 contains lower gene expression level of hSmo* and mGli1 mRNA compared to cell line 2 (Figure 5.1 A). When analysing the mGli1/hSmo* ratio in cell line 1 and 2 (Figure 5.1 B), it seems that they are of same value, but as shown in A the cell lines have different expression levels. Selecting a cell line for further studies, cell line number 2 are preferred, because this cell line contains higher expression levels of hSmo* and mGli1 mRNA compared to cell line 1.

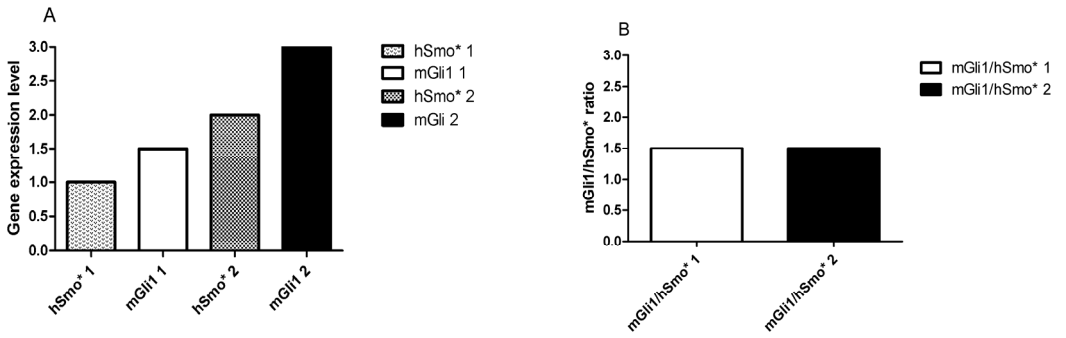


Figure 5.1: Two hypothetic cell lines (1 and 2) containing constitutively active Smo-receptors (hSmo*) with different expression levels of hSmo and mGli1. A, gene expression levels of hSmo* and mGli1 in cell line 1 and 2. B, mGli1/hSmo* expression ratio in cell line 1 and 2.

The data from the real-time RT-PCR was analysed according to this hypothesis. For example, when analysing data from the cell lines containing p3xFLAG-CMV-10 L514F-Smo, the mGli1/hSmo-data showed that L514F 1-6 expressed more hSmo than mGli1 mRNA, but L514F 1, 4 and 5 were approximately equal, indicating that there were greater mGli1 mRNA expression levels in these cell lines. Considering the expression levels of each gene in the monoclonal cell lines, L514F 5 had higher expression level of hSmo and mGli1 mRNA compared to L514F 1 and 4. L514F 5 may therefore be a candidate for further studies, but the expression data from this and the other cell lines showed high variability and the results were difficult to interpret and more studies need to be performed before any conclusion can be drawn. Verification of the various Smo-mutant monoclonal cell lines turned out to be complicated. After analysing the data from the real-time RT-PCR experiments, it is difficult to conclude about the signalling properties of the various mutated versions of Smo. The data from the real-time RT-PCR analyses also showed high variability. A possible explanation is the many technical steps that might influence the final outcome when performing real-time RT-PCR, as discussed above (section 5.1.2.2). To reduce the variability in the real-time RT-PCR experiments equal numbers of cells were plated to achieve cell confluence at the same time. However, not every cell line obtained confluence simultaneously and RNA had to be isolated from cell dishes with varying degree of cell density. Isolation of RNA from cells with unequal density might influence the signalling properties of the Hh-pathway and the amount of mRNA for the Hh-signalling pathway members [91]. This might contribute to the great variability of the obtained data. As mentioned in the Background chapter, Smo is enriched in primary cilia when Ptch-mediated inhibition of Smo is repealed, and cilia formation is formed when cells reach confluence. Smo enrichment in primary cilia is thought to drive activation of target genes like mGli1 and

mPtch [91]. Other contributions to the high variability of the data between the experiments might be unequal RNA quality or cDNA-synthesis. To control for possible genomic DNA contamination, cDNA-synthesis reactions were carried out without reverse transcriptase as a negative control. These samples were always negative in the RT-PCR reactions, indicating that the RNA-samples did not contain genomic DNA. Unstable cell lines might also be an explanation to the great data variability, because when the cells have been in culture for long time the number of transfected cells might decrease in unstable cell lines. Another explanation for the great variability could be that the p3xFLAG-CMV-10 vector may not be a suitable vector for the performed experiments. The pcDNA 3.1 vector seems to maintain the expected signalling properties better than the p3xFLAG-CMV-10 vector, due to the pcDNA3.1 vector gave a trend of constitutive activity of the W535L-Smo as expected from previously reports [66-69]. In addition, design of specific primers targeting the FLAG-tag and hSmo in the p3xFLAG-CMV-10 vector turned out to be difficult. All primers were analysed by the computer programme BLAST to verify their specificity (data not shown), but melting point analysis of the real-time RT-PCR products showed non-specific products, indicating that the primers gave more than one product. Therefore real-time RT-PCR was performed with primers specific to the human Smo inserted into the p3xFLAG-CMV-10 vector. These primers did not prime to endogenously expressed mouse Smo. There was no clear trend of constitutive activity for the W535L-Smo, when the Smo construct was expressed from the p3xFLAG-CMV-10 vector.

Several Western blot analyses were performed trying to detect Smo-protein in transfected NIH/3T3-cell lines. First, Smo-protein expressed from the pcDNA 3.1 vector was studied. This was not successful, due to poor primary antibodies against Smo. With the new p3xFLAG-CMV-10 vector the problem with poor primary antibodies was expected to diminish, because this vector encodes Smo as a fusion protein with three adjacent FLAG-epitopes linked to the N-terminus of Smo. On the Western blot the anti-FLAG M2 antibody gave two unspecific protein bands not corresponding to the predicted size of Smo. The protein bands are unspecific, because the samples transfected with empty vector showed the same bands as for the samples with Smo, indicating that the anti-FLAG M2 antibody binds non-specifically.

6. SUMMARY AND PERSPECTIVES

In summary, the K575M-Smo mutation was successfully generated using a mutagenesis reaction containing two sets of PCR reactions. A trend towards constitutive activity of W535L-Smo was obtained when analysing the signalling properties of the mutated constructs inserted in the pcDNA 3.1 vector, although a significant difference was not obtained. Five mutated versions of Smo (R484W-, L514F-, S533N-, W535L- and K575M-Smo) were analysed, expressed from the p3xFLAG-CMV-10 vector. Analysing the signalling properties of the mutated versions of the Smo-receptor turned out to be complicated as the results showed great variability. The reason for the complications might be:

- RNA isolation from monoclonal cell lines with various densities influence the signalling properties of the Hh-pathway.
- Unequal mRNA and cDNA quality.
- Unstable cell lines.
- Inappropriate expression vector. The pcDNA 3.1 vector seems to maintain the expected signalling properties better than the p3xFLAG-CMV-10 vector.

Based on the variable data from real-time RT-PCR experiments, it is not possible to conclude about the signalling properties of the various mutated versions of Smo, and further studies must be performed. Re-considering the project, perhaps the experiments should have been performed with the expression constructs inserted in the pcDNA 3.1 vector. More experiments should be performed with the monoclonal cell lines at equal cell confluence, aiming to find cell lines expressing comparable levels of transfected Smo. Other analyses should be performed on these cell lines, aiming at determining the signalling properties of the mutated versions of the Smo-receptor.

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8. APPENDIX

8.1 MATERIALS AND RECIPES

8.1.1 Chemicals and reagents

Chemical/Reagent	Abbreviation	Manufacturer	Cat.no
Absolute (100%) ethanol		Arcus	-
Acrylamide 4K, ultrapure	Acrylamide	AppliChem	-
Ammonium persulfate	APS	Bio-Rad	-
Anti-Smoothened Drosophila Homolog antibody produced in rabbit		Sigma	S9819
Bacto-Agar	Agar	AppliChem	A0949.1000
Bacto-Tryptone	Tryptone	Merck	1.07213.1000
Bacto-Yeast extract	Yeast extract	Merck	1.03753.0500
BCA Protein Assay Reagent	BCA-kit	Uptima	UP40840A
Bovine serum albumin standard 2mg/ml	BSA	Uptima	P36859A
Cryoprotective medium with 15% DMSO		Lonza	12-132A
Dimethyl sulfoxide	DMSO	Sigma	D2650
DNA Ladder, 1Kb Plus		Invitrogen	10787-026
dNTP Set, 100 mM (100 mM each of dATP, dGTP, dCTP and dTTP at neutral pH.		Invitrogen	10297-018
Donkey anti-mouse Ig-IR Dye 800CW		LI-COR Biosciences	926-32212
Donkey anti-rabbit Ig-HRP		GE Healtcare	NA934
DOW corning®high-vacuum silicone grease	Silicone	Sigma-Aldrich	Z273554
Dual-Luciferase® Reporter		Promega	E1980

1000 Assay System*			
Dulbecco`s Modified Eagles Medium, 4.5 g glucose/L	DMEM	Gibco BRL	31966-021
EcoRI-enzyme		Invitrogen	15202-013
Ethidium bromide	EtBr	Sigma	E8751
Fetal bovine serum	FBS	BioWhittaker	DE 14-801F
Formamide		Sigma	F7508
G-418 sulphate (Geniticin)	G418	Gibco BRL	11811-064
Gli-BS-Luc construct		Sasaki H. et al.	-
Glycerol		Gibco BRL	15514-011
HiSpeed® Plasmid Maxi Kit (25)*	Maxiprep kit	Qiagen	12663
Isopropanol		Arcus	1-9516
Lipofectamine 2000 Transfection Reagent	Lf-2000	Invitrogen	18324-012
Lumiglo elite (chemiluminescent substrate kit)*	Lumiglo	KPL	54-71-00
Methanol	MeOH	PROLABO	UN1789
Monoclonal ANTI-FLAG M2 antibody produced in mouse	Anti-flag	Sigma	F1804
N`N`N`N-tetra-metyl-ethylene-diamide	TEMED	AppliChem	A1148,0100
NaCl 0,9% B. Braun		Ecotainer	0082489E
n-butanol		Merck	1.01990.1000
Newborn calf serum	NCS	BioWhittaker (Cambrex)	14-416F
Non-fat dry milk	Dry milk	Normilk	-
Nuclease-free water (not DEPC treated)	nf-H_2O	Ambion	9932
NuSieve 3:1 Agarose	Agarose	Cambrex	50090
Oligo $(dT)_{12-18}$	Oligo dT	Invitrogen	-
One Shot TOP 10 Chemically		Invitrogen	C4040-03

Competent E.coli*			
Optimem	Optimem	Gibco	51985-026
p3XFLAG-CMV-10 expression vector	p3XFLAG	Sigma	E4401
Phosphate buffer salin, 10x powder, ultrapure grade	PBS	Amresco	-
Platinum® SYBR® Green qPCR SuperMix-UDG w/ ROX *	SYBR-green	Invitrogen	11744-500
Platinum® Quantitative PCR SuperMix-UDG w/ROX*	Platinum SM	Invitrogen	11743-500
Ponasterone A	Pon A	Invitrogen	H101-01
Precision Plus Protein Dual Color Standards	Protein standard	Bio-Rad	161-0374
Primers		Invitrogen	-
Probe: GAPDH	GAPDH	Eurogentec	-
QIAquick Gel Extraction kit (250)*		Qiagen	28706
Renilla luciferase pGL4.74-vector	Renilla-luciferase	Promega	
RNase-Free Dnase Set (50)*		Qiagen	79254
RNaseOUT Recombinant Ribonuclease Inhibitor		Invitrogen	10777-019
RNeasy® Mini Kit (250)*		Qiagen	74106
SeaKem LE Agarose	Agarose	Lonza	50004
Smoothened Antibody		Abcam	60016
Sodium hydroxide	NaOH	Merck	1.06469.1000
Sodium dodecyl sulphate, ultrapure, min 99.5%	SDS	Gibco	-
Sodium-ortho-vanadate	Na_3VO_4	Sigma	-
SphI-enzyme	SphI	New England BioLabs	R0182S
SuperScript® VILO cDNA Synthesis kit*		Invitrogen	11754-050

SuperScript® III Reverse Transcriptase*	SSIII	Invitrogen	18080-044
T4 DNA Ligase		Invitrogen	15224-017
TAE buffer**		-	-
Trypsin EDTA/Versene (EDTA)	Trypsin	BioWhittaker	BE17-161E
Tryptan Blue Stain 0.4%	Tryptan blue	Invitrogen	T10282
Tth111I-enzyme	Tth111I	New England BioLabs	R0185S
Tween-20	Tween	AppliChem	A1389,0500
$Vent_R$® DNA Polymerase*	Vent	New England BioLabs	M0254L
Wizard® Plus SV Minipreps DNA Purification System*	Miniprep kit	Promega	A1460
Zeocin		Invitrogen	R250-05
β-mercapthoethanol, min 98%	β-mercapthoethanol	Sigma	M7154
XbaI-enzyme		Invitrogen	R0145S

*Content of kits are specified in 8.1.2 ** Content of reagents specified in 8.1.3

8.1.2 Content of commercial kits

Kit/Reagent	Content/Reagent
Dual-Luciferase® Reporter 1000 Assay System	○ Luciferase Assay Buffer II ○ Luciferase Assay Substrate (Lyophilized Product) ○ Stop & Glo Buffer ○ Stop & Glo substrate, 50X ○ Passive Lysis Buffer, 5X
HiSpeed® Plasmid Maxi Kit (25)*	○ Buffer P1 ○ Buffer P2 ○ Buffer P3 ○ Buffer QBT ○ Buffer QC ○ Buffer QF ○ Buffer TE ○ LyseBlue ○ RNAse A (10 mg/ml or 100 mg/ml solution)
Lumiglo elite (chemiluminescent substrate kit)*	○ LumiGLO Reserve Substrate Solutions A and B ○ Wash solution Concentrate
One Shot TOP 10 Chemically Competent E.coli	○ TOP10 Chemically Competent *E.coli* cells ○ pUC19 Control DNA (10 pg/µl) ○ S.O.C Medium
Platinum® SYBR® Green qPCR SuperMix-UDG w/ ROX	○ Platinum® SYBR® Green qPCR SuperMix-UDG with ROX ○ 50 mM magnesium chloride (MgCl2)
Platinum® Quantitative PCR SuperMix-UDG w/ROX	○ Platinum® Quantitative PCR SuperMix-UDG with ROX ○ 50 mM magnesium chloride
Qiagen Plasmid Maxi Kit buffer	○ Buffer P1, resuspension: 50 mM Tris-Cl, pH 8.0, 10 mM EDTA, 100 µg/ml Rnase A). ○ Buffer P2, lysis: 200 mM NaOH, 1% SDS ○ Buffer P3, neutralisation: 3.0 M potassium acetate pH 5.5 ○ Buffer QBT, equilibration: 750 mM NaCl, 50 mM MOPS pH 7.0, 15% isopropanol ○ Buffer QC, wash: 1.0 M NaCl, 50 mM MOPS pH 7.0, 15 % isopropanol. ○ Buffer QF, elution: 1.25 M NaCl, 50 mM Tris-Cl pH 8.5, 15% isopropanol.
QIAquick Gel Extraction kit (250)	○ Buffer QG (contain chaotropic salts which are irritants) ○ Buffer PE (concentrate)

	○ Buffer EB ○ Loading Dye
RNase-Free Dnase Set (50)	○ DNase I, RNase-Free (lyophilized) ○ Buffer RDD ○ RNase-Free Water
RNeasy® Mini Kit (250)*	○ Buffer RLT (contains a guanidine salt) ○ Buffer RW1 (contains a guanidine salt) ○ Buffer RPE (concentrate) ○ RNase-Free Water
SuperScript® VILO cDNA Synthesis kit	○ 10X SuperScript Enzyme Mix ○ 5X VILO Reaction Mix
SuperScript III Reverse Transcriptase	○ SuperScript III RT, 200 U/µl ○ 5X First-Strand Buffer (250 mM Tris-HCl (pH 8.3 at room temperature), 375 mM KCl, 15 mM $MgCl_2$) ○ 0.1 M DTT
$Vent_R$® DNA Polymerase	○ $MgSO_4$ (100 mM) ○ ThermoPol Reaction Buffer (10X)
Wizard® Plus SV Minipreps DNA Purification System	○ Cell Resuspension Solution (CRA) ○ Cell Lysis Solution (CLA) ○ Neutralization Solution (NSB) ○ Column Wash Solution (CWA) ○ Alkaline Protease Solution ○ Nuclease-Free Water

8.1.3 Content of reagents, solutions and buffers

Reagent/ solution	Ingredients
dNTP Mix, 10 mM	Same amount dATP, dGTP, dCTP and dTTP
Extraction buffer	35 mM SDS 1 mM Na_3VO_4 50 mM Tris pH 7.4
4x loading buffer (for SDS-PAGE)	12.5 ml 1.0 M Tris-HCl pH 6.8 40 ml 10 % SDS 40 ml glycerol 99% 0.4 ml bromphenol blue ad 100 ml dH_2O Activate with 10% β-mercaptoethanol
5x Running buffer (for SDS-PAGE)	124.5 mM Tris-base 1.24 M glycine 17.34 mM SDS Stored at RT
10x Transfer buffer (for Western blotting)	312.86 mM Tris-base 2.4 M glycine Stored at RT
30% Acrylamidemix	29 g acrylamide 1 g N,N`-methylenebisacrylamide Ad 100 ml with dH_2O Stored in dark bottle at RT
1.5 M Tris-HCl pH 8.8	181.71 g Tris-base 900 ml dH_2O Adjust pH to 8.8 with conc. HCl at RT Ad 1000 ml dH_2O Stored at RT
1.0 M Tris-HCl pH 6.8	60.57 g Tris-base 400 ml dH_2O Adjust pH to 6.8 with conc. HCl at RT Ad 500 ml with dH_2O
1x Transfer buffer activated	70 ml 10x Transfer buffer 490 ml dH_2O 140 ml methanol Stored at 4˚C
6% polyacrylamide gel, 10 ml	5.3 ml dH_2O 2 ml 30% Acrylamide/bisacrylamide ratio 2.5 ml 1.5 M Tris-base pH 8.8 100 µl 10% SDS 100 µl 10% APS (fresh) 8 µl TEMED
10% polyacrylamide gel, 10 ml	4 ml dH_2O 3.3 ml 30% Acrylamide/bisacrylamide ratio 2.5 ml 1.5 M Tris-base pH 8.8 100 µl 10% SDS 100 µl 10% APS (fresh) 4 µl TEMED
5% polyacrylamide gel (stacking gel), 3 ml	2 ml dH_2O

	0.5 ml 30% acrylamide 0.375 ml 1.5 ml Tris-base pH 6.8 30 µl 10% SDS 30 µl 10% APS (fresh) 2 µl TEMED
LB medium	10 g tryptone 5 g yeast extract 10 g NaCl Ad 1000 ml dH_2O Autoclave
NP40 Extraction Buffer	2.5 ml 1M Tris pH 7.5 1.5 ml 5 M NaCl 2.5 ml 10% NP40 0.5 ml 0.5 M NaF Ad 50 ml dH_2O
TAE buffer	0.04 M Tris-acetate 1 mM EDTA

8.1.4 Manufacturers: Instruments

Instruments	**Manufacturer**
ABI Prism® 7900HT Sequence Detection System	Applied Biosystems
Biofuge Fresco	Heraeus Instruments
BlockHeater	Stuart Scientific
Centrifuge A14	Jovan
Centrifuge 5417R	Eppendorf
Controlled environment incubator shaker	New brunswick scientific co., inc. U.S.A.
Countess Automated Cell Counter	Invitrogen
EnVision 2104 Multilabel Reader	Perkin Elmer preciesly
EpiChemi II Darkroom	UVP Laboratory Products
Fluoroskan Ascent FL	Thermo Labsystems
GLC-1 General Laboratory Centrifuge	Sorvall
GloMax®-Multi Detection System	Promega
Microplate reader- Multiscan EX	Thermo Electron Corporation
NanoDrop-1000 Spectrophotometer	Saveen Werner
Odyssey Infrared Imaging System	LI-COR Biosciences
Olympus IX81	Olympus optical co, ltd.
PTC-100 Programmable Thermal Controller	MJ Research Inc.
RC 3B PLUS centrifuge	Sorvall

Ultrospec 2100 pro UV/ Visible spectrophotometer	Amersham Pharmacia Biotec
Varifuge 3.0R	Heraeus Instruments

8.2 PROTOCOLS

8.2.1 Mutagenesis reaction

Mutagenesis, generating K575M-Smoothened (K575M-Smo) in pEF.6 vector:

1. General composition for PCR-reactions:
   3 µl ThermoPol Buffer
   3 µl 10 mM dNTPs
   2 µl DMSO
   1 µl VENT DNA Polymerase
   2 µl 10 mM Primer FW (Forward)
   2 µl 10 mM Primer Rev (Reverse)
   X µl template (1 µg)
   Ad 30 µl dH_2O

In two separated tubes, add the reagents outlined in step 1 with the primer pairs (see table 3.1) and template specified under to generate the PCR-product I and II (see figure 3.1):

I: Primer pair: R199W-FW & K575M-Rev (Table 3.1), Template: pEF.6 Smo wt (Expected PCR-product size1151bp).

II: Primer pair: K575M-FW & P755F-Rev (Table 3.1), Template pEF.6 Smo wt (Expected PCR-product size 558 bp).

2. Amplify the PCR-products using the following program in the thermo cycler (PTC-100TM Programmable Thermal Controller, MJ Research Inc).

94˚C for 5 minutes
94˚C for 30 seconds (denature DNA)
55˚C for 30 seconds* (anneal primers) } 30 cycles
72˚C for 1 minute** (extend primers)
72˚C for 5 minutes
4˚C for ∞

* Annealing: Change temperature according to primers character.

** Extension: Change extension time when needed, approximately 1 min pr. 1000 bp extension.

3. After ended PCR-reaction, analyse and purify the PCR-products from an agarose gel (Protocol 8.2.2 and 8.2.3).
4. Use the flanking primers from step 1 and the purified PCR-products from step 3 to generate the final PCR-product. Prepare the PCR as outlined in step 1.
   III: Primer pair: R199W-FW & P755F-Rev (Table 3.1), Template: Purified PCR-product I & product II (Expected PCR-product size 1689bp). Use the same program as in step 2.
5. Analyse and isolate the PCR-product according to protocol 8.2.2 and 8.2.3.
6. Digest the purified PCR-product from step 5 and pEF.6 Smo wt with the restriction enzymes Tth111I (4000 units/ml) and SphI (10.000 units/ml), as follows:
   30 µl template (~ 1 µg)
   1.5 µl Enzyme 1
   1.5 µl Enzyme 2
   0.5 µl BSA (100 µg/ml)
   5 µl Buffer according to the manufacturer`s specifications (see datasheet)
   Ad 50 µl nf-H_2O
7. Incubate the digestion reaction for 60 minutes at 37˚C.
8. Analyse and isolate the digested products according to protocol 8.2.2 and 8.2.3.
9. Use T4 DNA ligase to ligate the digested and purified PCR-product III and pEF.6 Smo wt from step 8. Use re-ligation of the vector as negative control. Add the following components in two separate tubes:
   1. 2 µl T4 DNA ligase buffer
      1 µl T4 DNA ligase
      2 µl template 1 (e.g. digested and purified vector backbone)
      4 µl template 2 (e.g. digested and purified PCR-product)
      Ad 10 µl nf-H_2O
   2. 2 µl T4 DNA ligase buffer
      1 µl T4 DNA ligase
      2 µl template 1 (digested and purified vector backcone)
      Ad 10 µl nf-H_2O

10. Incubate the ligation reactions for 60 minutes at room temperature.
11. Transform competent bacteria with the ligation mix from step 10 according to protocol 8.2.4 in this section. Alternatively: the ligation mixture could be stored at -20˚C for later transformation of competent bacteria.

8.2.2 Plasmid purification: Agarose gel electrophoresis

1. Mount and level the casting tray. Place comb into place.
2. Prepare 1 % agarose gel:
   1 g agarose
   100 ml 1 X TAE buffer
   10 µl ethidium bromide (10 µg/µl)

Melt the agarose in microwave oven. Gently swirl the beaker and make sure all the agarose has melted. Cool the solution to approximately 50˚C before adding ethidium bromide. All handling with ethidium bromide should be done in ventilated hood due to its mutagenicity. Wear gloves. Mix thoroughly.

3. Pour the solution into the casting tray. Carefully remove any air bubbles. Let the gel polymerize for approximately 30 minutes in room temperature.
4. Transfer the gel to the electrophoresis tank after polymerization, and carefully remove the combs. Cover the gel completely with 1xTAE buffer.
5. Load 30 µl of each sample (or adequate amount) onto the gel. Apply 12 µl of 100 bp DNA ladder in the first well, containing:
   5 µl 100 bp DNA ladder (1 µg/µl)
   5 µl dH_20
   2 µl DNA-loading dye

6. Attach the electrophoresis apparatus to a power supply. Let the sample run through the gel at constant voltage of 100 V for approximately 50 minutes or as long as necessary for complete separation of the fragments.
7. Visualize the bands under UV-light using the Epi Chemi II Darkroom (UVP-Ultra Violet Products, Laboratory Products).

8.2.3 Gel Extraction

QIAquick Gel Extraction Kit Protocol using a microcentrifuge
All centrifugation steps are carried out at 13.000 rpm (17.900 x g) in a conventional tabletop microcentrifuge at room temperature.

1. Weigh the empty colourless tube and set the block heater at 50˚C.
2. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.
3. Put the gel slice in the colourless tube and weigh the tube again.
4. Calculate the weight of the gelslice.
5. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 µl).
6. Incubate at 50˚ C for 10 min (or until the gel slice has completely dissolved). To help dissolving the gel, mix by vortexing the tube every 2-3 minutes during the incubation.
7. Add 1 gel volume of isopropanol to the sample and mix.
8. Place a spin column in a provided 2 ml collection tube.
9. To bind DNA, apply the sample to the column (max 800 µl), and centrifuge for 1 minute.
10. Discard the flow-through and place the column back in the same collection tube.
11. Add 500 µl of Buffer QG to the column and centrifuge for 1 minute.
12. Add 750 µl of Buffer PE to wash the column and centrifuge for 1 minute.
13. Discard the flow-through and centrifuge the column for an additional 1 minute.
14. Place the column into a clean 1.5 ml microcentrifuge tube.
15. To elute DNA, add 30 µl of Buffer EB to the center of the membrane. Let the column stand for 1 minute, and then centrifuge for 1 minute.

8.2.4 Transformation

Competent cells are highly sensitive to changes in temperature and mechanical lysis caused by pipetting. Transformation was started immediately following thawing of the cells on ice. The bacteria suspension was mixed by swirling or tapping the tube gently, not by pipetting.

Transforming chemically competent cells: One Shot TOP10 Competent Cells (Invitrogen)

1. Centrifuge the vials containing the ligation mixture briefly and place on ice.

2. Thaw (on ice) one 50 µl vial of One Shot cells for two sets of ligation mix and carefully pipett 25 µl over to a new clean 1.5 ml tube.
3. Add 5 µl ligation mix to 25 µl competent cells and mix by gently tapping. Store the remaining ligation mixture at - 20˚C.
4. Incubate the cells on ice for 30 minutes.
5. Heat-shock the bacteria for exactly 30 seconds at 42˚C in a water bath without shaking.
6. Transfer the vial immediately to ice and incubate for 2 minutes without shaking.
7. Add 250 µl of pre-warmed (room temperature) S.O.C medium to each vial. Sterilise the top of the S.O.C medium bottle with a gas-flame (S.O.C is a rich medium; sterile technique must be practiced to avoid contamination).
8. Cap the tube tightly, and place the vials in a rack on its side and secure with parafilm to avoid loss of the vials. Shake the vials at 37˚C for exactly 1 hour at 225 rpm in a shaking incubator.
9. Spread 20 µl to 200 µl from each transformation vial on separate, labelled LB agarose-ampicillin (80 µg ampicillin/ml) plates. The remaining transformation mix may be stored at + 4˚C and plated out the next day, if desired.
10. Invert the plates and incubate overnight at 37˚C.
11. Pick single colonies, culture the bacteria 12-16 hours and isolate plasmid DNA with the Wizard Plus SV Minipreps DNA Purification System Kit (Protocol 8.2.5).
12. Prepare DNA for sequencing (protocol 8.2.9).

8.2.5 Small-scale plasmid preparation

Wizard® Plus SV Minipreps DNA Purification System (Promega).

1. Pick one well-isolated colony from a LB-agarose plate described in 8.2.4, and inoculate 3 ml LB-medium with ampicillin (80 µg/ml).
2. Incubate the culture for 12-16 hours at 37˚ C in a shaking incubator (300 rpm).
3. Transfer 1.5 ml of bacterial culture to a 1.5 ml microcentrifuge tube. Store the remaining bacterial culture at 2-8˚ C. Centrifuge the bacterial culture for 5

minutes at 10 000 x g in a tabletop centrifuge. Pour off the supernatant and blot the inverted tube on a paper towel to remove excess media.

4. Add 250 µl of Cell Resuspension Solution and completely resuspend the cell pellet by vortexing or pipetting.
5. Add 250 µl of Cell Lysis Solution, and mix the solution by inverting the tube 4 times. Incubate the solution approximately 1-5 minutes until the cell suspension clears.
6. Add 10 µl of Alkaline Protease Solution and mix by inverting the tube 4 times. Incubate for 5 minutes at room temperature.
7. Add 350 µl of Neutralization Solution and immediately mix by inverting the tube 4 times.
8. Centrifuge the bacterial lysate at maximum speed (~ 14 000 x g) in a microcentrifuge for 10 minutes at room temperature.
9. Insert one spin column into one 2 ml collection tube for each sample.
10. Transfer the cleared lysate (approximately 850 µl) to spin column. Avoid disturbing or transferring any of the white precipitate with the supernatant.
11. Centrifuge the supernatant at maximum speed for 1 minute at room temperature. Discard the flowthrough. Re-insert the spin column into the collection tube.
12. Add 750 µl of column wash solution (previously diluted with 95% ethanol).
13. Centrifuge at maximum speed for 1 minute at room temperature. Discard the flowthrough. Re-insert the spin column.
14. Repeat the wash procedure using 250 µl of column wash solution.
15. Centrifuge at maximum speed for 2 minutes at room temperature.
16. Transfer the spin column to a new, sterile 1.5 ml microcentrifuge tube.
17. Elute the plasmid DNA by adding 100 µl of nuclease-free water to the spin column. Centrifuge at maximum speed for 1 minute at room temperature.
18. Measure the DNA concentration with Nano-drop-1000 (ND-1000 V3.5.2) or with Ultrospec 2100 UV spectrophotometer (Protocol 8.2.6).
19. Store the purified plasmid DNA at -20˚ C.

8.2.6 Quantification of DNA by UV analysis

Ultrospec 2100 pro UV and NanoDrop-1000

Ultrospec 2100 pro:

1. Tune the spectrophotometer to measure ODs at 230 nm, 260 nm and 280 nm.
2. Clean the quartz cuvettes with dH2O.
3. Dilute the plasmid sample 1:10 (DNA) with dH_2O..
4. Calibrate the apparatus using a blank containing only the solvent and then analyze the plasmid at the specified wavelengths. The readout may be as shown in figure 8.1.

Sample	230 nm	260 nm	280 nm	260/230	260/280	Conc.
Ref	0.000	0.000	0.000			
1	0.164	0.346	0.186	2.11	1.86	0.173
2	0.190	0.361	0.200	1.90	1.80	0.181
3	0.166	0.359	0.197	2.16	1.82	0.180
4	0.172	0.355	0.196	2.06	1.81	0.177

Figure 8.1: Example of spectrophotometer readout. The figure shows an example of readout from the Ultrospec 2100 Pro instrument providing absorbance data at 230, 260 and 280 nm. Column 3 shows the absorption at 260 nm (dilution 1:10), and from this we can derive ([DNA] (µg/µl) = OD_{260} x dilution /20) the concentration in column 7. Columns 5 and 6 indicate the purity of the sample which in this case is acceptable (OD_{260}/OD_{280} ratio ~ 1.8 – 1.9, OD_{260}/OD_{230} ratio ~ 1.8 – 2.2).

Nanodrop:

1. Wash the electrodes with water.
2. Calibrate the apparatus by adding 2 µl dH2O.
3. Add 2 µl RNase-free water (blank) to the electrode and measure the concentration.
4. Wipe the electrode dry.
5. Add 2 µl sample, and measure the RNA-concentration (ng/µl).
6. Repeat step 4 and 5 for multiple samples.

8.2.7 Sub-cloning

Generation of EcoRI restriction sites in Smoothened wild type and R484W-, L514F-, S533N-, W535L-, K575M-Smo encoding cDNA:

1. In a PCR-reaction use pEF.6 Smo wt, R484W-, L514F-, S533N-, W535L-, K575M-Smo as template with the primer pEF.6 29U31 (forward; Table 3.1) and pEF.6

202L18 (reverse; Table 3.1). Prepare the PCR reaction as outlined in section 1.2, mutagenesis protocol step 1.

2. Amplify the PCR-products using the thermo cycler-program as outlined in section 1.2, mutagenesis protocol step 2. Change the extension time to 2.5 minutes.
3. Analyse the products on 1% agarose gel, and use QIAquick Gel Extraction Kit to isolate the product from the gel (according to 8.2.2 and 8.2.3). The product size is 2517 bp.

Insertion of R484W-, L514F-, S533N-, W535L-, K575M-Smo and Smo wt in the p3xFLAG-CMV-10 vector:

4. In separate tubes, prepare restriction enzyme digestion of the PCR-products from step 3 and p3xFLAG-CMV-10 vector with EcoRI (10 units/µl) and XbaI (20000 units/ ml) as outlined in mutagenesis protocol step 6 in section 1.2.
5. Isolate the digested PCR-product and p3xFLAG-CMV-10 vector according to protocol 8.2.2 and 8.2.3.
6. Ligate the digested and purified PCR-products and p3xFLAG-CMV-10 vector from step 5 as outlined in section 1.2, mutagenesis protocol step 9.
7. Transform competent bacteria according to protocol 8.2.4 and use re-ligation of the p3xFLAG-CMV-10 vector as a negative control. Growth should not appear on LB-agar-plate, since re-ligated vector is not a circulated plasmid that can express the ampicillin resistant gene.
8. Isolate plasmid-DNA according to protocol 8.2.5.
9. Prepare and send the plasmids for DNA-sequencing (protocol 8.2.9).
10. Analyse the DNA-sequence and make maxiprep of the correct products (protocol 8.2.8).

8.2.8 Large-scale plasmid preparation

HiSpeed Plasmid Maxi Kit (Qiagen)

1. Transfer 1-2 ml culture (from miniprep protocol 8.2.5 step 3) of bacterial cells to 250 ml LB medium with ampicillin in 2 l flask (The flask must be at least 4 times

the volume of the culture). Incubate the bacteria culture at 37°C in shaking incubator (approximately 300 rpm) 12-16 hours.

2. Transfer the bacteria culture to centrifuge cups, and harvest the bacterial cells by centrifugation at 6000 x g for 15 min at 4°C (RC 3B PLUS centrifuge, Sorvall).
3. Remove the supernatant and make sure that all traces of supernatant are completely removed.
4. Resuspend the bacterial pellet in 10 ml Buffer P1 and transfer the suspension to 50 ml tubes (Ensure that RNase A has been added to Buffer P1).
5. Add 10 ml Buffer P2 and mix thoroughly by virgorously inverting the sealed tube 4-6 times, and incubate at room temperature for 5 min. During the incubation prepare the filter cartridge: Screw the cap onto the outlet nozzle of the Maxi cartridgefilter. Place the cartridgefilter in a 50 ml tube.
6. Add 10 ml chilled Buffer P3 to the lysate, and mix immediately and thoroughly by vigorously inverting 4-6 times.
7. Pour the lysate into the barrel of the cartridge. Incubate at room temperature for 10 minutes.
8. Equilibrate a HiSpeed Maxi Tip by applying 10 ml Buffer QBT and allow the column to empty by gravity flow.
9. Remove the cap from the filter outlet nozzle. Insert the plunger gently into the filter Maxi Cartridge and filter the cell lysate into the previously equilibrated HiSpeed Tip. Allow the cleared lysate to enter the resin by gravity flow.
10. Wash the tip with 60 ml Buffer QC.
11. Elute DNA with 15 ml Buffer QF in a 50 ml tube.
12. Precipitate DNA by adding 10.5 ml RT isopropanol to the eluted DNA. Mix and incubate at room temperature for 5 min.
13. Place the precipitator over a waste bottle and transfer the elute/isopropanol mixture into the 30 ml syringe, and insert the plunger. Filter the elute/isopropanol mixture through the precipitator using constant pressure.
14. Remove the precipitator from the 30 ml syringe and pull out the plunger. Re-attach the precipitator and add 2 ml 70% ethanol to the syringe. Wash the DNA by inserting the plunger and pressing the ethanol through the precipitator using constant pressure.

15. Remove the precipitator from the 30 ml syringe and pull out the plunger. Attach precipitator to the 30 ml syringe again, insert the plunger, and dry the membrane by pressing air trough the precipitator quickly and forcefully. Repeat this step.
16. Dry the outlet nozzle of the precipitator with absorbent paper to prevent ethanol carryover.
17. Remove the plunger from a new 5 ml syringe and attach the precipitator onto the outlet nozzle. Hold the outlet of the precipitator over a 1.5 ml collection tube. Add 1 ml TE Buffer to the syringe. Insert the plunger and elute the DNA into the collection tube using constant pressure.
18. Remove the precipitator from the syringe, pull out the plunger and reattach the precipitator to the syringe.
19. Transfer the eluate from step 18 to the 5 ml syringe and elute for a second time into the same 1.5 ml tube.

8.2.9 DNA sequencing

DNA sequencing was performed at Department of Molecular Biosciences, University of Oslo, Norway, having DNA sequencing as a core facility.

1. Add following components were provided in separate tubes with the different primer sets:
   0.6 µg plasmid DNA
   2 µl 10 µM primer (Table 3.1)
   0.7 µl TRIS 100 mM
   Nuclease-free water ad 7.0 µl

8.2.10 Thawing cells

All cell culture procedures were carried out in a laminar airflow (LAF) bench to prevent contamination, using aseptic techniques.

1. Remove a vial of frozen cells from the liquid nitrogen tissue culture library tank. Use forceps and place the vial in a water bath preheated to 37°C, until the cells are just thawed. Wear protective goggles and gloves, because the vial may explode under this procedure.

2. Transfer the thawed cell suspension to a 15 ml tube containing 5 ml preheated (37°C) complete growth medium.
3. Centrifuge the suspension at 160 x g for 5 minutes.
4. Discard the supernatant, and resuspend the pellet in 5 ml complete growth medium and transfer the suspension to a tissue culture dish (60mm).
5. Culture the cells at 37°C in a humidified atmosphere of 5% CO_2.
6. When the cells are approximately 80% confluent, split the cells 1:4 and re-plate the cells on new tissue culture dishes (100 mm).

8.2.11 Splitting cells

For each 100mm dish:

1. Aspirate off the growth medium and carefully wash the cells once with 10 ml 0.9% NaCl.
2. Add 1 ml trypsin-EDTA (200 mg/L) to cover the monolayer. Incubate the cells at 37°C in a humidified atmosphere of 5% CO_2 in air, until the cells detach (approximately 2-10 minutes depending on cell type and confluency).
3. Remove the tissue culture plate from the incubator. Only if necessary give the plate a firmly tap on the side with the palm of the hand to promote the detachment. Check that the cells actually have detached in a microscope.
4. Add 4 ml preheated (37°C) complete growth medium to the plates to neutralise the trypsin, and homogenise the cells by pipetting.
5. Transfer the cell-suspension to a 15 ml tube.
6. Centrifuge the suspension for 5 minutes at 160 x g.
7. Remove the supernatant and resuspend the pellet in preheated complete growth medium.
8. Plate the cells on new plates at a ratio of 1:3 to 1:12 (NIH/3T3), 1:4 to 1:10 (293 EcR Shh) depending on the original confluency (approximately 2 million cells per 100 mm culture dish), or plate a specific number of cells after counting them. See protocol for cell counting. Add 10 ml preheated complete growth medium.
9. Culture the cells at 37 °C in a humidified atmosphere of 5% CO_2 in air.

8.2.11.1 Cell counting

Countess Automated Cell Counter, Invitrogen

1. Transfer 10 µl of trypan blue stain to 1.5 ml eppendorftubes for each cell-line to be counted.
2. Add 10 µl of the cell suspension, from step 7 in protocol for splitting cells (8.2.11), to the 1.5 ml tubes with trypan blue stain, and mix well by pipetting.
3. Add 10 µl of the sample mixture to the chamber ports on one side of the Countess cell counting chamber slide (The two chambers of the slide are labelled "A" and "B" for easy tracking of the samples).
4. Insert the chamber slide into the slide inlet on the instrument. There will be a soft click, confirming that the slide is correctly inserted.
5. Press the "Count Cells" button for the cells to be counted (cells/ml). Live cells have bright centres and dark edges, and dead cells have a uniform blue colour throughout the cell with no bright centres.
6. Seed cells according to table 8.1.

Table 8.1: Cell counting

Experiment	**Cell type**	**Number of cells/dish (well)**	**Culture dish size**
Isolation of RNA for RT-PCR	NIH/3T3	200 000 -400 000 100 000- 300 000	60mm 6-well plate
Gene reporter assay (Lucifease assay)	NIH/3T3	20 000	96-well plate
Generation of Shh-conditioned medium	293 EcR Shh*	$1x10^6$	100 mm

* Culture $1x10^6$ cells in 100 mm dish for 24h, change the medium to 7 ml complete medium (10% FBS-DMEM) supplemented with 5µM Ponasterone A.

8.2.12 Freezing cells

For each 100 mm plate:

1. At 60-70% cell confluence (log phase), split the cells according to protocol 8.2.11, protocol step 1-7.

2. Resuspend the cells in 0.5 ml complete medium (without selection antibiotics), place the cells on ice and add 0.5 ml ice-cold cryoprotective medium containing 15% DMSO.
3. Carefully transfer the cell suspension to a cryotube kept on ice.
4. Place the cryotube in a closed styrofoam box at –70 ºC, to let the temperature slowly decrease over night. Transfer the frozen cells to the liqid nitrogen tissue culture library tank for long term storage.

8.2.13 Transfecting cells

1. In a 100 mm tissue culture plate, seed $5x10^5$ cells in 10 ml complete growth medium.
2. Culture the cells at 37˚C in a humidified atmosphere of 5% CO_2 in air until 70-80 % confluence, usually accomplished within 18-24 hours.
3. For each transfection sample (for each 100 mm dish of cells) prepare the following solutions in two separate sterile tubes:

   Solution A: 6 µg of DNA + 600 µl OPTIMEM.
   Solution B: 15 µl Lipofectamine 2000 + 600 µl OPTIMEM. Mix Lipofectamine 2000 gently before use by inverting the tube 4-6 times.

4. Incubate for 5 minutes at room temperature.
5. Combine the solution A and B from step 3. Mix gently and incubate for 20 minutes at room temperature.
6. Aspirate the growth medium, and wash the cells with 10 ml 0.9% NaCl.
7. Add 4.8 ml 5% NCS-DMEM to each culture dish with cells.
8. Overlay the transfection solution onto the cells.
9. Incubate at 37˚C in a humidified atmosphere of 5% CO_2 in air overnight.
10. Replace the transfection medium with 10 ml complete growth medium per tissue culture plate.
11. Incubate at 37˚C in a humidified atmosphere of 5% CO_2 in air overnight.
12. Selecting stably transfected cells: Replace the growth medium with 10 ml 5% NCS-DMEM supplemented with G-418 (0.8mg/ml) twice a week, until the untransfected cells have died.

8.2.14 Generation of monoclonal cell lines

1. For each polyclonale cell-line, plate the cells at low density (20 000 cells per 100 mm tissue culture dish), and add 10 ml complete medium. Plate one tissue culture plate with 20 000 untransfected cells for control.
2. Grow the cells in the presence of antibiotics (G-418, 0.8 mg/ml) at 37˚C in a humidified atmosphere of 5% CO_2 in air, until the untransfected cells have died.
3. Under the microscope, mark single cell under at the bottom of the tissue culture plate.
4. Let the single cells grow into colonies at 37˚C in a humidified atmosphere of 5% CO_2 in air.
5. Under the microscope, select several independent colonies from the previously marked single cells. Use a pen to make a circle around separate colonies at the bottom of the tissue culture plate.
6. Aspirate medium, and wash the cells with 10 ml 0.9% NaCl.
7. Dip the lower rim of the cloning cylinders  in silicon, and place it over the marked cell colonies.
8. Add two drops of trypsin-EDTA (200ml/L), and incubate the cells at 37°C in a humidified atmosphere of 5% CO_2 in air, until the cells detach.
9. Transfer the detached cells from one cloning cylinder to one well in a 6-well plate, and add 1.5 ml complete growth medium.
10. Culture the cells at 37°C in a humidified atmosphere of 5% CO_2 in air.

8.2.15 Transfection for reporter gene assay (transient transfection)

1. In a 96-well, seed 20 000 cells per well in 200 µl complete growth medium.
2. Culture the cells at 37˚C in a humidified atmosphere of 5% CO_2 in air until 70-80 % confluence, usually accomplished within 18-24 hours.
3. Transfection-mix per well:
   30 µl OPTIMEM
   0.1 µg pGL4.74 Renilla Luciferase
   0.2 µg Gli BS-luciferase
   0.75 µl Lipofectamine 2000

Dilute Lipofectamine 2000 in OPTIMEM, mix and incubate for 5 minutes at room temperature before adding the DNA to the Lipofectamine 2000/OPTIMEM-solution, and incubate for 20 min.

4. Rinse the cells in 0.9% NaCl and completely remove the solution.
5. Add 70 µl complete growth medium to each well.
6. Add 30 µl transfection-mix to each well.
7. Culture the cells at 37˚C in a humidified atmosphere of 5% CO_2 in air overnight.
8. Stimulate cells according to protocol 8.2.16.

8.2.16 Stimulation and harvesting of cells for gene reporter assay

1. Use the transiently transfected cells from protocol 8.2.15. Aspirate the medium and wash with 100 µl 0.9% NaCl.
2. Add 200 µl 0.5% NCS-DMEM to cells seeded in rows A-D (in a 96-well plate), and treat the cells in rows E-H with a mixture of 100 µl Shh conditioned medium, and 100 µl 0.5% NCS-DMEM . Carefully add the stimulation mixture into the wells and tilt the plate to make sure that the medium are evenly distributed in the wells.
3. Incubate overnight at 37˚C in a humidified atmosphere of 5% CO_2 in air.
4. Perform the gene reporter assay (protocol 8.2.17).

8.2.17 Gene-reporter assay: Luciferase assay

Dual-Luciferase® Reporter 1000 Assay System.

Cell lysis:

1. Thaw 5X PLB and prepare sufficient amount of 1X PLB (20 µl per well) by diluting in dH_2O.
2. Completely remove the medium from the cells. Wipe the 96-well plate on a paper.
3. Add 200 µl 1xPBS to each well to rinse the cells
4. Completely remove PBS.
5. Add 20 µl 1xPLB to each well and place the 96-well plate on a shaker at room temperature for 20 minutes.
6. Dispense 10 µl cell lysate into white opaque luminometer microtiter 384 plate (and 20 µl for 96 well-plate).

Reagent preparation:

7. Prepare Luciferase assay reagent II. Resuspend lyophilized Luciferase Assay Substrate in 10 ml of Luciferase Assay Buffer II and label “LAR II”. When using a 384-well plate, 25 µl “LAR II” is needed for each well, and for 96-well plate, 50 µl is needed per well. About 1 ml “LAR II” in excess is also needed to tube volume for luminometer for both plates.

Dual-luciferase measurement:

8. Turn on the luminometer and prime both injectors with LAR II and Stop & Glo Reagent.
9. Program the luminometer to perform 2-second premeasurement delay, followed by a 10-second measurement period for each reporter assay, and initiate the measurement:
    - Injecting Luciferase Assay Reagent II (LAR II)
    - Measuring firefly luciferase activity for 10 seconds
    - Injecting Stop and Glo Reagent
    - Measuring Renilla luciferase activity for 10 seconds

After use wash the injectors

10. Place the tubes for both injectors in a 50 ml tube containing dH_2O. Select flush/wash injectors from the menu. Repeat this wash step with 70% EtOH, and one more time with dH_2O. Perform a final “wash” with air to empty and dry the injector tubes.

8.2.18 RNA isolation

1. Remove the medium from the wells in a 6-well plate and wash the cells twice with 1 ml 0.9 % NaCl
2. Add 200 µl trypsin-EDTA (200mg/L) to each well.
3. Incubate at 37˚C until the cells detach.
4. Add 1 ml complete growth medium.
5. Transfer the cells to an RNase-free 1.5 ml tube, and centrifuge the cells at 4000 rpm for 5 minutes.
6. Add 10 µl β-mercaptoethanol (β-ME) per 1 ml Buffer RLT.
7. Completely aspirate the supernatant and resuspend the cell pellet from step 5 in 350 µl buffer RLT. Vortex for 1 minute.

8. Homogenize the lysate by passing it through a blunt 20-gauge needle (0.9 mm diameter) at least 5 times. Fit the needle to a RNase-free syringe.
9. Add 350 µl (1 volume) of 70 % ethanol to the homogenized lysate, and mix well by pipetting. Do not centrifuge.
10. Transfer up to 700 µl of the sample to the spin column placed in a 2 ml collection tube. Centrifuge for 15 sec at ≥10 000 rpm. Discard the flow-through.

DNase-digestion (elimination of genomic DNA contaminations):

11. Add 350 µl Buffer RW1 to the spin column. Centrifuge for 15 s at ≥10 000 rpm. Discard the flow-through.
12. Add 10 µl DNase I stock solution to 70 µl Buffer RDD. Mix gently by inverting the tube. Centrifuge briefly to collect residual liquid from the sides of the tube.
13. Add the DNase I incubation mix (80 µl) directly to the spin column membrane. Incubate the samples for 15 min at room temperature.
14. Add 350 µl Buffer RW1 to the spin column. Centrifuge for 15 sec at ≥10 000 rpm. Discard the flow-through.
15. Add 500 µl Buffer RPE to the spin column. Centrifuge for 2 min at ≥10 000 rpm.
16. Place the spin column in a new 1.5 ml collection tube. Add 40 µl RNase-free water directly to the spin column membrane. To elute the RNA, centrifuge the spin column for 1 min at ≥10 000 rpm.
17. Determine the RNA concentration and quality using Ultrospec 2100 pro UV or Nanodrop. (Protocol description 8.2.6).

8.2.19 Determination of RNA purity, quality and concentration

Agarose gel electrophoresis:

1. Prepare 1% agarose gel according to protocol 8.2.2.
2. Prepare the RNA-sample as follows:

   1 µg total RNA

   RNase-free water ad 5 µl

   2 µl formamid

   Incubate the mix at 65˚C for 10 minutes, place on ice for 1 minute. Add 2 µl loading buffer (1:6) to each sample, vortex and briefly centrifuge the sample. Load 9 µl of each sample onto the 1% agarose gel. Apply 12 µl of 100bp DNA ladder in one well.

3. Run electrophoresis at 50-90 V for 35 minutes.
4. Visualize RNA under UV light.

8.2.20 First strand cDNA synthesis

1. Add the following components to nuclease-free microcentrifuge tubes:

Oligo (dT)	1 µl	Mix 1
10 mM dNTPs	1 µl	
Total RNA in RNase-free water	2.5 µg	
RNase-free water ad	11 µl	

2. Incubate the samples at 65 ˚C for 5 minutes (PTC-100 Programmable Thermal Controller, MJ Research) and cool down on ice for 2 minutes.
3. Briefly centrifuge the samples to collect the contents in the bottom of the tubes (Biofuge Fresco, Heraeus Instruments).
4. Make reverse transcriptase (+RT) reaction buffer as follows:

5X First-Strand Buffer	4 µl	Mix 2
0.1 M DTT	1 µl	
RNase Out Recombinant Rnase Inhibitor (40 U/µl)	1 µl	
SuperScript III RT (200 U/µl)	1 µl	

5. Make reaction buffer without reverse transcriptase (-RT) according to (+)RT mix prepared in step 4, but replace SuperScript III RT and RnaseOut by DNase-free water.
6. Mix gently by pipetting.
7. Add 7 µl Mix 2 to each tube from step 4. Mix gently by pipetting.
8. Incubate samples in a Programmable Thermal Controller at:

   25 ˚C 5 minutes

   50 ˚C 60 minutes

   70 ˚C 15 minutes (Inactivate the reaction)

   4 ˚C End

8.2.21 RT-PCR

Making a standard curve for real-time quantitative RT-PCR

Pool 2-5 µl from fifteen to twenty RNA samples, enough to dispense 2.5 µg RNA in six tubes. Carry out first strand cDNA synthesis as described.

1. Pool the cDNA synthesised from the pooled RNA samples.
2. Make the following dilutions in separate tubes (Table 8.2):

	µg/tube	µl cDNA	µl nuclease-free water
Tube # 1	5 µg	120 µl cDNA	165 µl
Tube # 2	2.5 µg	100 µl of tube #1	100 µl
Tube # 3	1.25 µg	100 µl of tube #2	100 µl
Tube # 4	0.625 µg	100 µl of tube #3	100 µl
Tube # 5	0.31 µg	100 µl of tube #4	100 µl
Tube # 6	0.15 µg	100 µl of tube #5	100 µl

Table 8.2: Making a standard curve for real time RT-PCR.

Real-time quantitative Reverse Transcriptase-PCR

1. After reverse transcription reaction make dilution A for analyses of the gene of interest (SYBRgreen) and dilution B for standard curve analyses (GAPDH).
   Dilution A: Dilute each template (+ and – RT) with 75 µl RNase-free water:
   20 µl cDNA sample from protocol 1.7.2.4 step 8 (+ or – RT samples)
   75 µl RNase-free water
   Dilution B:
   5 µl Dilution A (+RT-samples and standard curve samples)
   200 µl RNase-free water
   Vortex the diluted samples. Dilution B is used for highly expressed genes normally used as "normalisation" genes.
   Store the diluted samples at -20˚C.
2. For probe-based RT-PCR (of GAPDH):
   Mix the following volumes (µl) of the given components (Table 8.3):

Components	µl per well	Number of samples (X)x 3 (triplicates) + 2 blanks x3 + 10 (excess)	GAPDH-mix (Column 1 x Column 2)
RNase-free water	3.0 µl/well	Xx3 + 2x3 + 10	X µl
Forward primer (10 µM)	0.45 µl/well	Xx3 + 2x3 + 10	X µl
Reverse primer (10 µM)	0.45 µl/well	Xx3 + 2x3 + 10	X µl
Probe TaqMan (5 µM)	0.6 µl/well	Xx3 + 2x3 + 10	X µl
Platinum®SuperMix (Invitrogen)	7.5 µl/well	Xx3 + 2x3 + 10	X µl
cDNA	3.0 µl/well	Xx3 + 2x3 + 10	X µl

Table 8.3: GAPDH-mix

Use a multi-channel automated pipette and dispense 12 µl per well of the reaction mix in triplicates in a 384-well PCR plate. Add 3 µl cDNA template (dilution B) per well. Keep the samples and plates on ice while working.

3. For SYBR-green based RT-PCR (of hSmo, mPtch, mGli).

   Mix the following volumes (µl) of the given components (Table 8.4):

Components	µl per well	Number of samples * 3 (triplicates) + 2 blanks*3 + 10 (excess)	SYBR-green-mix (Column 1 x Column 2)
RNase-free water	0.6 µl/well	Xx3 + 2x3 + 10	X µl
Forward primer (10 µM)	0.45 µl/well	Xx3 + 2x3 + 10	X µl
Reverse primer (10 µM)	0.45 µl/well	Xx3 + 2x3 + 10	X µl
Platinum®SYBR® Green Super Mix (Invitrogen)	7.5 µl/well	Xx3 + 2x3 + 10	X µl
cDNA	6.0 µl/well	Xx3 + 2x3 + 10	X µl

Table 8.4: SYBR-green-mix

Use a multi-channel automated pipette and dispense 9 µl per well of the above reaction mix in triplicates in a 384-well PCR-plate. Add 6 µl cDNA template (dilution A) per well in triplicates. Keep the samples and plates on ice while working.

4. Cover the PCR plate with optically clear sealing tape (Sarstedt) and centrifuge by using a Varifuge 3.0R (Heraeus Instrument) to collect the sample in the bottom of the well and remove air bubbles.
5. Run real-time quantitative PCR (ABI Prism 7900HT Sequence Detection System, Applied Biosystems) with the following cycling parameters:

50˚C 2 minutes
95˚C 10 minutes
95˚C 15 seconds } 40 cycles
60˚C 60 seconds }

An additional dissociation step was added to the SYBRgreen analyses. The specificity of the RT-PCR products was verified by this melting-point analysis, showing only one top when having specific products.

8.2.22 Protein concentration determination

Prepare samples A-F for a standard curve from BSA protein stock 2 mg/ml, according to table 8.5.

Standard	Final BSA concentration (µg/ml)	BSA	Water
A	1500	375 µl of stock BSA	125 µl
B	1000	325 µl of stock BSA	325 µl
C	750	175 µl of A	175 µl
D	500	325 µl of B	325 µl
E	250	325 µl of D	325 µl
F	125	325 µl of E	325 µl

Table 8.5: Preparation of BSA for standard curve in protein concentration determination assay.

1. Load a flat bottom 96-well plate with 20 µl standard solutions in duplicates, and 5 µl samples in triplicates as shown in figure 8.2.
2. Mix 25 ml of BCAssay Reagent A with 500 µl of reagent B (BCAssay: protein quantitation kit, Uptima) for each 96-well plate.
3. Add 200 µl of the reagent mix to each well.
4. Incubate at 37˚C for 30 min.
5. Allow the 96-well plate to obtain room temperature.
6. Read the absorbance with a plate spectrophotometer at 570 nm.

	1 2	3 4 5	6 7 8	9 10 11	12
A	dH_2O	Sample # 1	Sample # 9	Sample # 17	None
B	Standard F	Sample # 2	Sample # 10	Sample # 18	None
C	Standard E	Sample # 3	Sample # 11	Sample # 19	None
D	Standard D	Sample # 4	Sample # 12	Sample # 20	None
E	Standard C	Sample # 5	Sample # 13	Sample # 2	None
F	Standard B	Sample # 6	Sample # 14	Sample # 22	None
G	Standard A	Sample # 7	Sample # 15	Sample # 23	None
H	Blank	Sample # 8	Sample # 16	Sample # 24	None

Figure 8.2: Layout of a flat bottom 96-well plate for protein measurement.

8.2.23 SDS-PAGE

Sample preparation

1. Based on protein concentration determinations, transfer equal amounts of proteins to 1.5 ml tubes, one for each sample.
2. Dilute the samples with harvesting solution to ensure equal volume of each sample.
3. Add 4x loading buffer (LB), activated with 10 % β-mercaptoethanol, which equals ¼ of the total sample loading volume.
4. Vortex and boil the samples at 95˚C for 5 minutes in a heating block.
5. Centrifuge the samples at 14000 rpm for a few seconds to collect the sample in the bottom of the tube.
6. Load the samples (15 µl) on polyacrylamide gels. The remaining sample volume may be stored at -20˚C for later analyses.

Pouring and running SDS-polyacrylamide gels

1. Wash glass plates, one long with attached spacers and one short for each gel, three times with water and 70% ethanol.
2. Assemble the glass plates and place them in the minigel holder, ensure that the bottom edges of the two plates are well aligned. Blow through the assembled glass plates with high pressure air to remove any remaining paper fibres. Clip the assembled glass plates into the casting stand.
3. Prepare resolving gel solution (see composition in appendix 8.1.3) of desired acrylamide concentration according to the size of the proteins to be separate in a 50 ml tube (add APS and TEMED imidiately before casting to avoid to early polymerization).
4. Mix the solution by gently inverting the 50 ml tube without making any air bubbles, and pour the resolving gel solution between the assembled glass plates (approximately 4 ml). Leave sufficient place for the stacking gel.
5. To ensure a flat surface, carefully overlay the acrylamide solution with 100-150 µl n-butanol.
6. Let the acrylamide solution polymerize for approximately 30 minutes.
7. Wash away the n-butanol with water and remove remaining water with a 3MM filter paper without touching the gel.
8. In a 50 ml tube, prepare 5% polyacrylamide stacking gel solution as outlined in 8.1.3.

9. Pour the stacking gel solution directly on the polymerized resolving gel and carefully insert the teflon comb to avoid trapping of air bubbles. Let the stacking gel polymerize for approximately 20-30 minutes at room temperature.
10. Carefully remove the comb after the stacking gel has polymerised.
11. Place the gels in the electrophoresis chamber.
12. Fill the electrophoresis tank chambers with 1x Running buffer and wash the wells.
13. Load 15 µl/well of protein samples, and 7 µl/well of molecular weight standard solution (Precision Plus Protein Dual Color Standards, Bio-Rad).
14. Attach the electrophoresis apparatus to a power supply, and run the protein samples through the stacking gel (thickness 0.75 mm) at constant current (A) of 20 mA. Increase the current to 45 mA when the blue dye front has reached the resolving gel. Run the gel until the dye front reaches the bottom of the resolving gel.

8.2.24 Western blotting

Wet transfer

1. For each gel, prepare one PVDF-membrane and six pieces of 3MM paper to the size of the resolving gel.
2. To enable correct orientation of the membrane label on the upper right corner with number and F (facial side). Labelling with the letter F ensures the correct orientation of the membrane.
3. Activate the PVDF-membrane by submerging in methanol for approximately 20 seconds.
4. Place the membrane, filter papers and two porous pads for each blot in 700 ml transfer buffer in refrigerator.
5. Prepare a transfer sandwich consisting of one porous pad, 3x 3MM filter papers, polyacrylamide gel, PVDF membrane, 3x 3MM filter papers and one porous pad.
6. Place the transfer sandwich in the transfer apparatus containing pre-chilled transfer buffer with the membrane facing the red electrode (anode). Also add an ice block and a magnetic stir in the apparatus.
7. Electroblot the proteins from the polyacrylamide gel to the membrane for 45 minutes at constant current (400 mA) and constant stirring.

8. After completing the transfer, open the transfer sandwich and directly put the membrane in 5% dry milk solution (or Odyssey blocking buffer).
9. Block the membrane for 1 hour with slow aggitation at room temperature.

Incubation with primary and secondary antibodies

1. Dilute the desired primary antibody in 5 ml 5 % dry milk in PBS (or Odyssey blocking buffer) with 0.05 % Tween 20 (Table 8.6).
2. Place the membrane in a plastic bag sealed on three sides, fill with the primary antibody solution from step 1 and seal the last side of the bag. Avoid trapping air bubbles inside the sealed bag.
3. Incubate membrane over night at 4˚C on a tumbler.
4. Wash the membrane for 3 x 6 minutes, with slow wagging, in PBS added 0.05 % Tween-20, at room temperature.
5. Incubate the membrane in 5% dry milk in PBS (or Odyssey blocking buffer with 0.02 % Tween 20) with appropriate diluted secondary antibody (Table 8.6) corresponding to the primary antibody used in step 1.
6. Incubate at room temperature for 1 hour (Using Odyssey: incubate the nitrocellulose-membrane in a dark box, because the secondary antibodies are sensitive to light).

Substrate incubation

1. Wash the membrane for 3 x 6 minutes in PBS added 0.05 % Tween 20, with slow aggitation at room temperature (Odyssey: Skip step 2 and 4) .
2. Prepare the substrate, LumiGLO Elite Chemiluminescent Kit, according to the manufacturers (KPL) instructions (3 ml LumiGLO Reserve Solution B, 1.5 ml LumiGLO Reserve Solution A).
3. Carefully rinse the membrane in dH_2O before incubating the membrane in the substrate solution for 1 minute.
4. Place the membrane between two clean pieces of transparency film, remove air bubbles and excess substrate.
5. Record an image of the blot with the UVP Bio-Imager or equivalent apparatus immediately after incubation with substrate.
6. Odyssey: Record an image of the blot with the Odyssey Infrared Imaging System after rinsing the membrane in dH_2O (step 2).

Antibody concentrations (All antibodies were diluted in 5% non-fat dry milk phosphate-buffered saline (PBS)/Tween 20 or Odyssey blocking buffer with 0.02 % Tween 20)		
		Antibody dilution
Smoothened protein	Primary antibody (rabbit polyclonal Anti-Smo (Abcam))	1:1000
	Secondary antibody (Donkey anti-rabbit (HRP))	1:2000
Smoothened flag tag protein	Primary antibody (Anti-FLAG M2 antibody (Sigma))	1:1000
	Secondary antibody (Donkey anti-mouse(IRDye))	1:20 000

Table 8.6: Antibody concentrations (cat no., section 8.1.1).