MOLECULAR MECHANISM OF INDIAN HEDGEHOG SIGNALLING IN HUMAN SEBOCYTES

Inaugural Dissertation

zur

Erlangung des Doktorgrades

Dr. nat. med.

der Medizinischen Fakultät

und

der Mathematisch-Naturwissenschaftlichen Fakultät

der Universität zu Köln

vorgelegt von

Gilles Frédéric Brice Séquaris

aus Le Chesnay, France

Köln, 2012

Berichterstatter/Berichterstatterin :	Prof. Dr. S. Roth
	Prof. Dr. A. A. Noegel
Tag der letzten mündlichen Prüfung:	11.11.2011

Content

I. Figure index

II. Table index

III. Abbreviations

13-cis-RA:	13-cis-retinoic acid
ACTH:	adrenocorticotropic hormone
ADFP:	Adipophilin
a-MSH:	a-melanocyte-stimulating hormone
Apc:	adenomatous polyposis coli protein
AR:	androgen receptor
Axin 1:	Axis inhibition 1
BCA:	bicinchoninic acid
BCC:	basal cell carcinoma
Bcl9:	B-cell lymphoma 9
Blimp1:	B-lymphocyte-induced maturation protein 1
Bmp:	bone morphogenetic protein
β-MSH:	beta-melanocyte-stimulating hormone
Boc:	brother of Cdo
BrdU:	Bromodeoxyuridine
BSA:	bovine serum albumin
β-TrCP:	beta-transducin-repeat-containing protein
cAMP:	cyclic adenosine monophosphate
cDNA:	copy DNA
Cbp:	CREB binding protein
Cdo:	cell adhesion molecule-related/down-regulated by oncogenes
CEA:	carcinoembryonic antigen
cfu:	colony-forming unit
CKIα:	Casein kinase I alpha
Cycd1:	Cyclin D1
ddH_2O:	double-distilled water
Dhh:	Desert Hedgehog
DHT:	dihydrotestosterone
Disp:	Dispatched
Dkk1-4:	Dickkopf 1-4
Dly:	Dally
dNTP:	deoxyribonucleotide triphosphate
DMSO:	Dimethyl Sulfoxide

DNA:	deoxyribonucleic acid
ΔNLef1:	dominant negative variant of the transcription factor Lef1
Dvl:	Dishevelled
EGF:	epidermal growth factor
ER:	endoplasmatic reticulum
FACS:	fluorescence-activated cell sorting
FAP:	familial adenomatous polyposis coli
FCS:	fetal calf serum
Fzd:	Frizzled
Gas1:	Growth arrest specific 1
Gli1:	glioma-associated oncogene homolog 1, Gli-Kruppel family member GLI1
Gli2:	GLI-Kruppel family member Gli2
Gli2ΔN2:	constitutively active mutant of Gli2 lacking N-terminal repressor domain
Gli3:	GLI-Kruppel family member Gli3
Gli3R:	repressor form of Gli3
GliAct:	activator forms of Gli transcription factor
GliRep:	repressor forms of Gli transcription factor
Glis2:	Gli similar 2
GPCR:	G protein coupled receptor
GPI:	glycosylphosphatidylinositol
Gsk3β:	glycogen synthase kinase 3 beta
Hh:	Hedgehog
HHIP:	Hedgehog interatcting protein
HNF-4a:	hepatic nuclear factor 4 alpha
HSPG:	Heparin sulfate proteoglycan
HSV-TK:	herpes simplex virus thymidine kinase
IGF:	insulin-like growth factor
Ihh:	Indian Hedgehog
Int:	Integration 1
JNK:	c-Jun N-terminal kinase
K15:	Keratin 15
kDa:	kilodalton
kbp:	kilo base pairs
KGF:	keratinocyte growth factor
KRT6a:	Keratin 6

LB-medium:	lysogeny broth medium
LD:	lipid droplet
Lef1:	lymphoid-enhancer-binding factor
LRA:	Luciferase reporter assay
Lgr6:	Leucine-rich repeat-containing G-protein coupled receptor 6
Lrig1:	Leucine-rich repeats and immunoglobulin-like domains protein 1
Lrp:	lipoprotein receptor-related protein
LXR:	liver X receptor
Mc5r:	melanocortin 5 receptor
MeOH:	Methanol
mg:	milligram
ml:	milliliter
mM:	millimolar
MMTV:	mouse mammary tumour virus
MOI:	multiplicity of infection
NBCCS:	nevoid BCC syndrome
ng:	nanogram
OD:	Optical density
pBS:	phosphate-buffered saline
PCR:	polymerase chain reaction
PI3-K:	phosphoinositide
PLC:	Phospholipase C
PKA:	protein kinase A
PKC:	protein kinase C
Porc:	Porcupine
PPAR:	peroxisome proliferator-activated receptor
PPARα:	peroxisome proliferator-activated receptor alpha
PPARβ:	peroxisome proliferator-activated receptor beta
PPARγ:	peroxisome proliferator-activated receptor gamma
PPRE:	PPAR responsive element
PRDM1:	PR domain zinc finger protein 1
Ptc1:	Patched 1
Ptc2:	Patched 2
PUFA:	polyunsaturated fatty acid
PUFA-RE:	PUFA response element

Pygo:	Pygopus
qRT-PCR:	quantitative real-time polymerase chain reaction
RNA:	ribonucleic acid
RND:	resistance nodulation division
RT:	room temperature
SDS:	sodium dodecyl sulfate
SDS-PAGE:	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sFRP 1-5:	secreted frizzled-related protein 1-5
shRNA:	short hairpin RNA
Ski:	Skinny
siRNA:	small interfering RNA
Shh:	Sonic Hedgehog
Smo:	Smoothened
SREBP:	sterol-response element-binding protein
SREBP-1:	sterol response element-binding protein-1
Tgf-β:	transforming growth factor beta
Sufu:	suppressor of fused
TCF:	Transcription factor or T cell factor
Tcf4:	t-cell factor 4
T2:	constitutive active form of ß-catenin
TK:	thymidine kinase
µg:	microgram
µl:	microliter
µM:	micromolar
UV:	ultra violet
V:	volt
Wg:	Wingless
Wif1:	Wnt inhibitory factor 1
Wnt:	amalgam of Wg and Int
Wls:	Wntless
ZFD:	zinc-finger domain

IV. Abstract

The hedgehog signalling pathway plays a crucial role during regulation of hair cycle and homeostasis of mammalian skin. Furthermore, misregulation of this pathway is attributed to a range of skin diseases and cancers. In previous studies Indian hedgehog was identified to be the only Hedgehog ligand expressed in sebaceous glands. Moreover, modulation of hedgehog pathway activity was shown to affect size and number of sebaceous glands in the skin. Thereby, local inactivation of the canonical Wnt pathway seems to be prerequisite for correct sebaceous gland development. However, the underlying molecular mechanism has not been clarified, yet.

Therefore, we hypothesise that Indian hedgehog signalling regulates proliferation and/or differentiation in human sebocytes. Thereby, activation of Hedgehog pathway and inactivation of canonical Wnt signalling might be cross-linked.

To test this, we applied the human sebocyte cell line SZ95 as *in vitro* model by comparing three distinct sebocyte populations depending on their differentiation state: “undifferentiated”, “differentiating” and “terminally differentiated” after induction by arachidonic acid treatment.

Terminally differentiated sebocytes displayed increased expression of IHH without endogenous pathway activation. In contrast, undifferentiated sebocyte displayed increased proliferation rate upon pathway activation by GLI1 and GLI2 expression. Additionally, our findings point to a role of the lipid metabolism in regulating these processes as shown by modulation of gene expression by addition of arachidonic acid.

Importantly, overexpression of GLI1, GLI2 and GLI3 also revealed that each GLI transcription factors preferentially activates a distinct set of established and potentially new Hedgehog target genes in human sebocytes.

Additionally, we identified a new mechanism of mutual regulation between Hedgehog and Wnt pathways on protein level in human sebocytes. More precisely, overexpression of GLI3 and GLI2 transcription factors resulted in accumulation of non-phosphorylated, active β-CATENIN, although Wnt pathway activity was not increased. Conversely, augmented levels of β-CATENIN in combination with GLI1 activator dramatically increased Gli reporter activity.

In summary, we propose the following model where undifferentiated sebocytes are the target cells of Hedgehog signals originating from mature sebocytes and GLI repressors might block endogenous Wnt pathway by interference with β-CATENIN.

V. Zusammenfassung

Der Hedgehog-Signalweg spielt eine entscheidende Rolle bei der Regulation des Haarzyklus und der Homöostase in der Haut von Säugetieren. Darüber hinaus sind Fehlregulationen dieses Signalweges mit einer Reihe von Hautkrankheiten und Hauttumoren assoziiert. In früheren Arbeiten wurde Indian Hedgehog als der einzige Hedgehog Ligand identifiziert, welcher in Talgdrüsen expremiert wird. Zudem wurde mittels Manipulation der Hedgehog-Signalweg-Aktivität gezeigt, dass Größe und Anzahl der Talgdrüsen in der Haut durch diesen reguliert werden. Dabei scheint die lokale Inaktivierung des kanonischen Wnt-Signalwegs eine Voraussetzung für eine korrekte Talgdrüsen-Entwicklung zu sein. Allerdings konnten die zugrunde liegenden molekularen Mechanismen noch nicht geklärt werden.
Daher stellen wir die Hypothese auf, dass der Indian Hedgehog-Signalweg Proliferation und/oder Differenzierung von humanen Sebozyten regelt. Dabei könnten die Aktivierung des Hedgehog-Signalwegs und die Inaktivierung des kanonischen Wnt-Signalwegs miteinander verknüpft sein.

Um dies zu prüfen, verwendeten wir die humane Talgdrüsen-Zelllinie SZ95 als *in vitro*-Modell und verglichen dabei drei distinkte Sebozyten-Populationen in Abhängigkeit ihres Reifegrades: "undifferenziert", "differenzierende" und "ausdifferenziert" nach Induktion mittels Arachidonsäure-Behandlung.

Ausdifferenzierte Sebozyten zeigten eine erhöhte Expression von IHH ohne endogene Signalwegsaktivierung. Im Gegensatz dazu zeigten undifferenzierte Sebozyten erhöhte Proliferationsrate und Hedgehog-Signalaktivität nach Überexpression von GLI1 und GLI2. Zusätzlich konnten wir Hinweise auf eine Rolle des Fettstoffwechsels bei der Regulation dieser Prozesse mittels Genexpressionsanalyse nach Zugabe von Arachidonsäure finden. Interessanterweise, führte GLI1-, GLI2- und GLI3-Überexpression jeweils zur spezifischen Aktivierung von etablierten und potenziell neuen Zielgenen in humanen Sebozyten. Zusätzlich identifizierten wir einen neuen Mechanismus der die gegenseitige Regulation zwischen den Hedgehog- und Wnt-Signalwegen auf Protein-Ebene in humanen Sebozyten ermöglicht. Dabei führte die Überexpression von GLI3 und Gli2 zur Akkumulation von nicht-phosphoryliertem, aktivem β-CATENIN, obwohl gleichzeitig die Wnt-Signalweg-Aktivität gesenkt wurde. Umgekehrt führte die Co-Expression von β-CATENIN mit dem Transkriptionsaktivator GLI1 zu einer drastischen Erhöhung der Gli Reporter Aktivität.

Zusammenfassend, schlagen wir folgendes Modell vor, bei dem undifferenzierte Sebozyten die Zielzellen der Ihh-Signale darstellen, die aus reifen Sebozyten sezerniert werden. Dabei könnten GLI-Repressoren den endogenen Wnt-Signalweg mittels Interferenz mit β-CATENIN blockieren.

1. Introduction

1.1. The Hedgehog signalling pathway in mammals

1.1.1. Biological functions of the Hedgehog ligands

The Hedgehog (Hh) pathway controls a variety of fundamental processes such as pattern formation, differentiation, proliferation and organogenesis in embryonic development, as well as, in adult tissue maintenance. The Hh family of secreted signalling proteins was first characterised in *Drosophila* by Nüsselein-Volhard and Wieschaus in 1980, where a mutation of the single *hh* gene resulted in a “hedgehog” like phenotype of the embryo and revealed its function as morphogen [1]. The *hh* gene has been duplicated during evolution. In mammals, there are three *Hh* genes [2-3]: *Sonic hedgehog* (*Shh*), *Desert hedgehog* (*Dhh*), and *Indian Hedgehog* (*Ihh*). *Shh* is the most broadly expressed mammalian *Hh* signalling molecule and, as consequence, the most studied. In particular, it is involved in neural tube development [3-8], patterning of left-right and dorso-ventral axes of the embryo [9-13], morphogenesis of different organs such as lung [14-17], skin [18-24], limbs [7, 25-27], teeth [23, 28-29], and together with *Ihh* it regulates skeletal development [25, 30-31]. *Shh* knockout mice display severe developmental defects and/or abnormalities in these tissues including foregut [32], pancreas [33], left-right asymmetry [34], lung [14], hair follicle [21, 35] and teeth [36]. *Dhh* expression is largely restricted to gonads, including sertoli cells of testis and granulosa cells of ovaries [37-39]. *Dhh*-deficient mice show no notable phenotypic defects except of complete lack of mature sperms. Thus, infertility found in mutant males confirms a crucial role for Dhh signalling during spermatogenesis [37]. Besides its expression in prehypertrophic chondrocytes in the growth plates of bones [30, 40], *Ihh* is specifically expressed in primitive endoderm [41], gut [42], sebaceous glands [43] and together with *Shh* in mammary glands [44-45]. Deletion of *Ihh* gene reveals a direct role of Ihh, for example, in regulation of chondrocyte proliferation [46], during pancreas [33], sclera and retina development [47], and also in bone formation [48].

Since its discovery, Hh/Gli signal transduction has also attracted considerable interest in the field of cancer research besides its crucial roles in embryogenesis. A series of publications has implicated aberrant pathway activation in the growth and maintenance of common

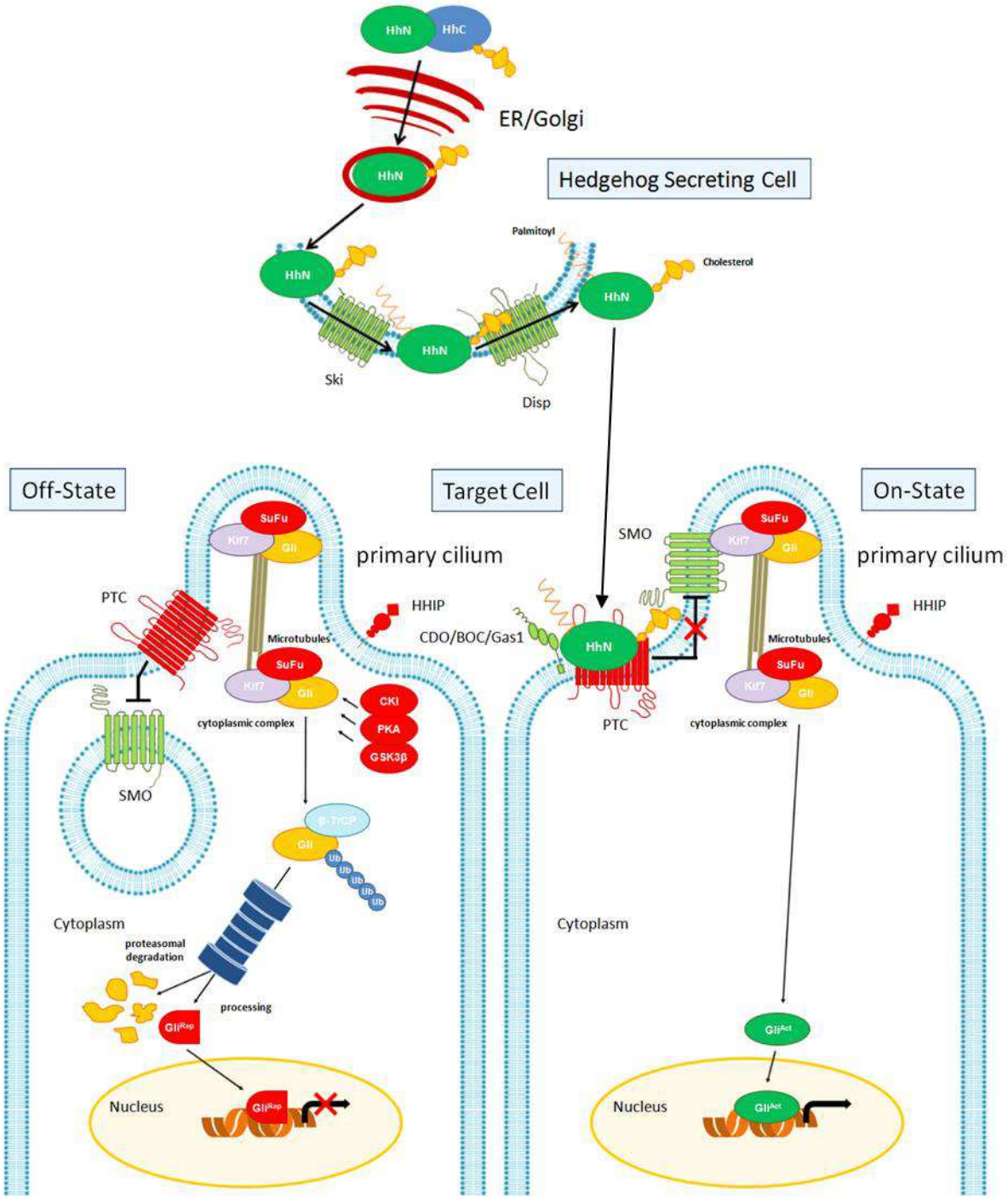


Figure 1: A schematic model depicting the Hh signalling pathway in mammals. [Off-State] In the absence of Hh signal, Ptc inhibits Smo. Gli transcription factors interact with a protein complex that prevents the activator full-length Glis from entering the nucleus. Consequently, cleavage of Gli molecules is induced to generate truncated forms of the transcription factors acting as repressors. [On-State] Hh acts by repressing its receptor Ptc thereby alleviating its inhibitory effect on Smo. Consequently, activator Gli transcription factors translocate to the nucleus inducing expression of target genes.

malignancies such as basal cell carcinoma (BCC), lung, esophageal and biliary cancer, as well as pancreatic and prostate cancer. Specific targeting of Hh/Gli-signalling may therefore offer a highly effective therapeutic strategy for the treatment of a variety of lethal tumours.
Overall, most of the components in Hh signalling are conserved from insects to vertebrates, with a main difference in the number of genes in each species [49]. However, it appears that there are also interspicific differences in the underlying signalling mechanisms [50]. Thus, according to this study, the following pathway description focuses on the actual knowledge regarding the mammal system (Fig. 1):

1.1.2. Production and secretion of the Hedgehog ligand

After translation, the Hh ligands (45 kDa) undergo multiple processing steps that are evolutionary conserved and are required for complete functionality of the Hh protein, as well as, its release from the producing cell [49-55]. These comprise autoproteolytic cleavage of the functional N-terminus from the C-terminal protease domain. Lipid modification by addition of cholesterol and palmitoyl moieties with the help of the transmembrane aclytransferase Skinny hedgehog (Ski) results in the functional Hh ligand (HhN) [51, 56-57].
Then, the lipidated Hh proteins (19 kDa) are secreted by the macromolecule Dispatched (Disp) into the extracellular matrix to exert short and long range effects by building up a concentration gradient [49, 58]. Heparin sulfate proteoglycans (HSPGs) are suggested to be involved in stabilization and transport of Hh [58-59].
Furthermore, in vertebrates the transmembrane Hedgehog interacting protein (Hhip) can bind to Hh and reduce its range of movement [58, 60]. Importantly, *Hhip* itself is a target gene of the Hedgehog pathway [60], thus providing a negative feedback on pathway activation (Fig. 1).

1.1.3. Hedgehog signal reception

Downstream of Hh there are two isoforms of Patched receptors in vertebrates, Patched 1 (Ptc1) and Patched 2 (Ptc2). Both are target genes of the Hh pathway [61-62] and seem to be equivalent in terms of binding the three Hh isoforms [63]. On the one hand, Ptc2 is likely to be dispensable during embryonic development, but on the other hand, Ptc2 deficiency in conjunction with Ptc1 haplo-insufficiency increases the incidence of several types of tumour [64]. As the Ptc amino-acid sequence is distantly related to bacterial resistance-nodulation-

division (RND) pumps, it is noteworthy, that these proteins are homotrimers, and that biochemical and genetic data also confirmed that Ptc functions as a multimer [65-67]. In the absence of the Hh ligand, Ptc receptors inhibit the function of a 7-pass transmembrane protein, called Smoothened (Smo) [68]. Thus, binding of Hh to Ptc results in the alleviation of inhibition on Smo, and consequently transduces the Hh signal to the cytoplasm [68-69]. The nature of this inhibitory effect seems to be catalytic as one molecule of Ptc1 can inactivate approx. 250 molecules of Smo, but the underlying mechanism still remains unsolved [68]. However, it is suggested that reciprocal changes in the subcelluar location of Ptc and Smo occur during pathway activation. Thereby, in the absence of the Hh ligand, Ptc shuttles between the plasma membrane and internal vesicles containing the inactive form of Smo. As consequence, Smo is prevented to move from these vesicles to the cell surface. After binding of Hh, Ptc is internalised from the plasma membrane, while Smo moves in the opposite direction and is released from vesicles to the cell surface by acquiring an active conformation [70-75]. In this context, a fundamental role for the primary cilia arises as subcellular location for coordination of Hedgehog signal transduction specifically in mammals [76-80]. The primary cilium is a single, immotile cell surface projection found on most vertebrate cells [81]. Activated mammalian Smo has been found to accumulate to primary cilia in response to Shh treatment [82], whereas this process is inhibited by Ptc when the Hh ligand is missing [83]. Therefore, endogenous Ptc localises to both, the membrane enveloping the shaft of the cilium, and to a prominent skirt-like domain around the base. One can envision Ptc as gatekeeper controlling precise localisation of Smo.

Hedgehog signal reception by target cells is facilitated by three classes of accessory receptors: the glypican-family of cell surface proteoglycans [84-89], the glycosylphosphatidylinositol (GPI-) anchored membrane-bound Growth-arrest-specific 1 (Gas1) [90] and the transmembrane proteins Cdo (also named Cdon, Cell adhesion molecule-related/down-regulated by oncogenes) as well as Brother of Cdo (Boc) in vertebrates [90-93]. Cdo and Boc belong to the immunoglobin superfamily of single-transmembrane proteins. The lack of these proteins is accompanied with unresponsiveness of cells towards ShhN stimulation, and severe developmental defects have been observed in deficient mice.

1.1.4. The cytoplasmic complex and Gli transcription factors

Upon recruitment to the cell membrane, active Smo interacts with a cytoplasmic protein complex. It consists amongst others of the mammalian ortholog of Cos2 (Costal2), Kif7, the

Suppressor of fused (Sufu) and the protein kinase A (Pka). But the underlying mechanism of how activation of Smo is coupled to these cytoplasmic proteins remains unclear. Importantly, this complex controls activation and proteolytic cleavage of Gli transcription factors, the effectors of the pathway [94-97].

In vertebrates, there are three Gli transcription factors, Gli1 (glioma-associated oncogene homolog 1), Gli2 and Gli3, which mediate Hedgehog signalling [98-99] (Fig. 2). The three isoforms contain five zinc-finger DNA-binding domains, but their N-terminal domains exhibit important differences. Gli1 functions as a strong transcriptional activator. It lacks a repressor domain found in the N-terminus of Gli2 and Gli3. Gli1 expression also depends on Gli2 and/or Gli3-mediated transcription since it constitutes a direct target gene of the Hh pathway. Compared to Gli2, Gli1 exerts the largest part of activator functions by providing a positive feedback-loop. However, Gli2 represents the primary downstream activator and is indispensable to initiate activation of target genes of the Hedgehog pathway. Thereby, both factors display identical or very similar DNA binding specificities [96, 100-102]. By contrast, Gli3 exerts a role as a repressor [97, 103].

In absence of the Hh ligand, Su(Fu) acts as scaffold and brings together the Gli proteins with the negative regulator Pka [104]. Thereby, the Gli transcription factors are retained in the cytoplasmic complex and undergo an initial phosphorylation step mediated by Pka. Subsequent phosphorylation by Gsk3β (glycogen synthase kinase 3 beta) and CKIα (Casein kinase I α) follows. The hyperphosphorylation of the Gli proteins promote recognition and ubiquitination by the ubiquitin E3 ligase β-TrCP (β-transducin-repeat-containing protein) leading to proteolytical processing and generation of a truncated transcriptional repressor form in the case of Gli3 [96-97, 105]. With exception of Gli1, similar processing has been documented for Gli2. But it is still controversially discussed, whether it results in the complete degradation or in the generation of a truncated repressor form of the Gli2 protein [96, 106]. Gli1 contains two degradation sequences, dregon N and dregon C, which allow recognition, as well as, ubiquitination by β-TrCP and hence proteasomal degradation. Contrary to Gli2 and Gli3, the proteasomal degradation is complete and no truncated repressor variant of Gli1 is produced [107]. Moreover, studies on the efficiency of processing revealed that the ratio of Gli2 full-length/truncated form is 6:1, whereas the Gli3 full-length/truncated form ratio is about 1:1. These results demonstrate that Gli2 is inefficiently processed compared to Gli3 [96].

Therefore, the lack of transcriptional Gli activators as well as the production of repressors leads to repression of Hedgehog target genes.

In the presence of Hh ligand, active Smo disables the cytoplasmic complex, and hence, processing and degradation of the Gli transcription factors do not occur. This leads to accumulation of full-length forms of both, Gli2 and Gli3 and up-regulation of the

transcriptional activator Gli1. Consequently, target genes of the Hedgehog pathway are activated [77].

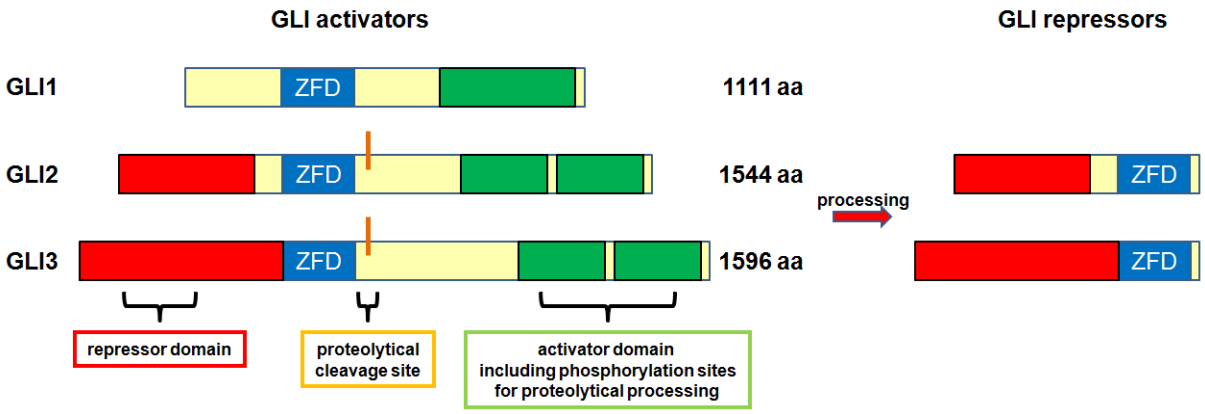


Figure 2: A schematic diagram of protein structure of Gli transcription factors. All members possess the zinc-finger domain (ZFD) and the activator domain in the C-termini. Contrary to Gli1, Gli2 and Gli3 additionally have a repressor domain in the N-terminal part of the protein, and therefore can function as repressors after processing at the proteolytic cleavage site.

1.2. The role of the Hedgehog pathway in skin homeostasis

In mammalian skin, many studies describe an important role for Hh signalling in the regulation of hair cycle and in the homeostasis of sebaceous glands.

In particular, Shh deficient mice lack hair follicles and sebaceous glands [21, 35]. Thus, Shh signalling is required for the control of hair growth and morphogenesis of hair follicles, in part through regulating the Gli transcription factors [108]. Therefore, *Shh* expression and responsiveness are spatially and temporally tightly regulated and only occur in the anagen stage of the hair cycle [109]. *Shh* knockout embryos display hair germs, comprising epidermal placodes and associated dermal condensates, like the wild type controls. However, the progression through subsequent stages of hair follicle development is blocked in skin of mutant mice [110]. Consistently, reduced expression of the target genes *Gli1* and *Ptc1* are found in Shh deficient dermal condensates, which fail to evolve into hair follicle dermal papillae [111].

Previous work in our group demonstrated that transgenic mice expressing a dominant negative variant of the transcription factor Lef1 (ΔNLef1) under the control of Keratin 14 promoter exhibit enhanced sebaceous differentiation accompanied by increased expression

of *Ihh* in the skin [43]. Noteworthy, the β-catenin binding site is lacking in ΔNLef1, rendering the transcription factor unresponsive to Wnt/β-catenin pathway and blocking downstream target genes. *Ihh* expression is particularly found in the more differentiated compartment of sebaceous glands, whereas *Gli1* expression mainly in sebocyte precursors, suggesting a paracrine signalling mechanism *in vivo*. Additionally, *in vitro* studies in the human sebocyte cell line SZ95 revealed that PTC1 and IHH are up-regulated during differentiation and blocking signalling by cyclopamine treatment inhibits their proliferation and stimulates their differentiation [43].

Epidermal stem cells, in particular their daughter cells, termed transit amplifying cells (TA), are able to give rise to multiple lineages in skin, comprising the hair follicles, the associated sebaceous glands and the overlying interfollicular epidermis. Skin-specific inhibition of the Hh pathway by expression of a repressor variant of Gli2 (K5Gli2ΔC4) leads to a selective suppression of sebocyte development [112]. On the other hand, its overactivation by skin-specific expression of Smo (K5M2Smo) results in an enhancement in both size and number of sebaceous glands [112]. In this context, ectopic Hh signalling has also been found to induce sebaceous gland development from footpad epidermis, which is normally devoid of hair follicles and associated structures.

Shh and Ihh knockout mice die at late embryogenesis due to severe developmental defects [44]. Transplantation experiments of mammary epithelium with hair follicles derived from Shh-null and Ihh-null mice into wild-type skin reveal no difference in mammary gland morphogenesis compared to control skin. Interestingly, hair follicles from Shh-deficient skin fail to evolve while Ihh-deficient skin develops apparently normally [44]. These findings suggest that both Hedgehog ligands have redundant functions in the skin.

1.3. The role of the Hedgehog pathway in human diseases and skin cancer development

1.3.1. Human diseases associated with inhibition of the Hedgehog pathway

The lack of activity of the Hedgehog pathway during embryogenesis have been found in a range of developmental defects like holoprosencephaly (due to loss of Shh or generation of an insensitive variant of Ptc1 by mutation) [113-116], cephalopolysyndactyly, Pallister-Hall syndrome, Postaxial Polydactyly (all three induced by a dominant negative variant of Gli3)

[117-118], Vacterl syndrome (possibly due to combined loss of function of Gli2 and Gli3) [119] and Smith-Lemli-Optitz syndrome (based on a defect of cholesterol biosynthesis, and hence, on inappropriately modified Hh ligands) [120-122].

1.3.2. Human diseases associated with abnormal activation of the Hedgehog pathway

One of the most important diseases associated with activation of the pathway is the Gorlin syndrome (also known as the nevoid BCC syndrome, NBCCS, or the basal-cell nevus syndrome) [123-124]. It is an autosomal dominant disorder usually characterised by the predisposition to develop basal cell carcinomas (BCCs) of the skin, medulloblastomas, and ovarian fibromas. Inactivating mutations of the *PTC1* gene have been identified as cause for this disorder [125-128]. In addition, activating mutations in the *SMO* gene have been detected in 10-20% of sporadic BCCs, which result in a similar effect like inactivation of PTC1 [129-130].

Analogously, these types of mutations were also found in sporadic medulloblastomas, primitive neuroectodermal tumours [131-133] and in a range of other tumour types, like trichoepitheliomas, esophageal squamous cell carcinomas, and transitional cell carcinomas of the bladder [134-135].

Deregulation of Hedgehog signalling pathway has also been shown to be implicated in the development of rhabdomyoma and rhabdomyosarcoma in humans. Thereby, haploinsufficiency of the two tumour suppressor genes *PTC1* and *SUFU* may play a key role in tumour progression [136].

The most frequent and lethal tumour of the central nervous system are glioblastoma [137-138]. *GLI1* was originally characterised as an oncogene for this tumour since amplifications of the chromosomal region 12q13-q14 containing the *GLI1* gene have been identified [138-141].

Taken together, abnormal activation and inactivation of the Hedgehog pathway lead to a variety of developmental defects as well as of tumour formations in humans.

1.4. The Wnt signalling pathway in mammals

1.4.1. Biological functions of the Wnt signalling pathway

Another important signalling network is the Wnt pathway, which diversifies into at least three branches: 1) the β-catenin pathway (canonical Wnt pathway), 2) the planar cell polarity pathway involving JNK (c-Jun N-terminal kinase) and 3) the Wnt/Ca^{2+} pathway including PLC (Phospholipase C) and PKC (protein kinase C) recruitment [142-143]. The name "Wnt" is an amalgam of *Wg* (*Wingless*) and *Int* (*Integration 1*, *Int1*) [144]. *Int1* was identified as proto-

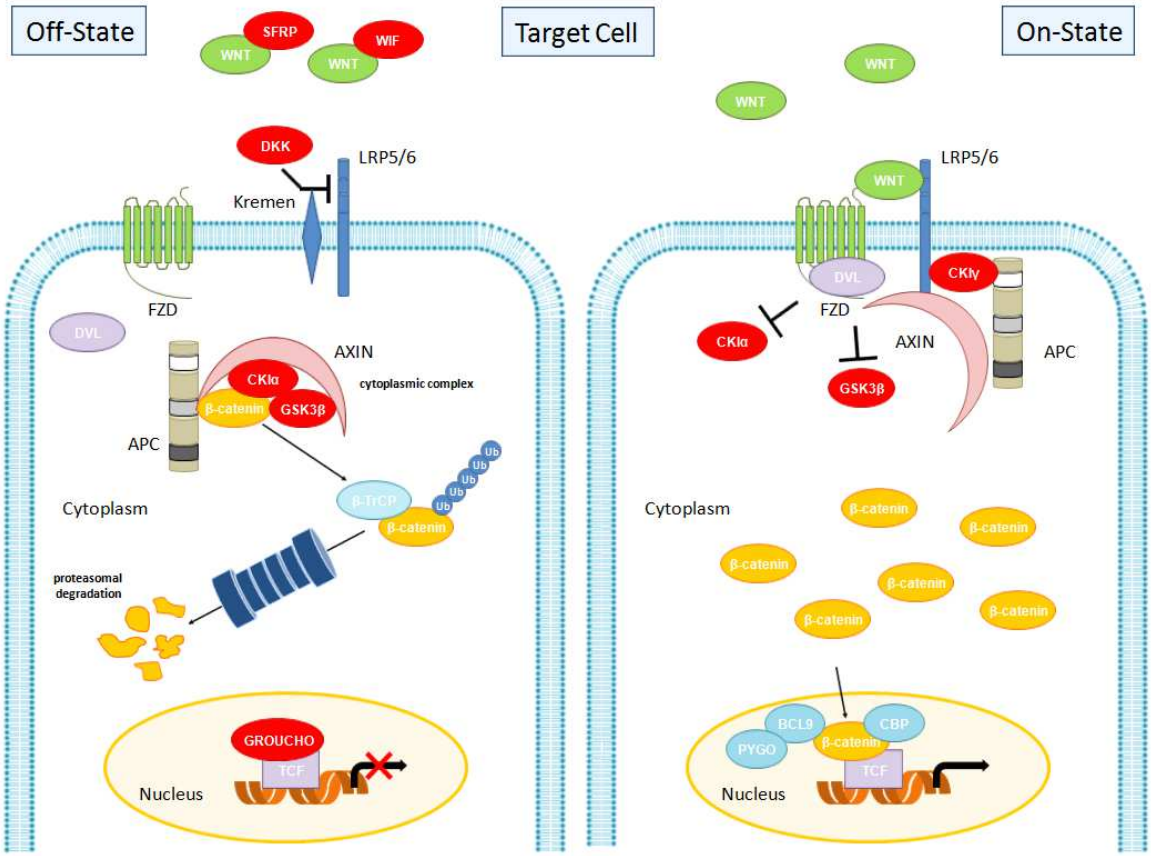


Figure 3: A schematic overview of the canonical Wnt signalling pathway. [Off-state] In the absence of a Wnt signal, β-catenin is captured by Apc and Axin within the destruction complex. There, it undergoes several phosphorylation steps for subsequent degradation by the proteasomes. As a result, nuclear DNA-binding proteins of the Tcf/Lef transcription factor family actively repress target genes by recruiting transcriptional co-repressors (Groucho/Tle). [On-state] Binding of Wnt ligands to the receptor complex containing Fzd and Lrp triggers the inactivation of the cytoplasmic destruction box. This allows β-catenin to accumulate and enter the nucleus, where it interacts with members of the Tcf/Lef family to activate transcription of target genes.

oncogene in association with MMTV (mouse mammary tumour virus) -induced mammary gland tumours by Roel Nusse and Harold Varmus [145]. *Wg* has been identified in *Drosophila* by several mutants displaying segmentation defects in fly embryos [1, 146-147]. This project focuses on the canonical Wnt pathway (Fig. 3), which orchestrates proper tissue development in embryos and tissue maintenance in adults similar to the Hedgehog pathway.
Noteworthy, the key mediator of the canonical Wnt/β-catenin signal transduction cascade is β-catenin. It is a member of the armadillo protein family containing 12 armadillo repeats that are required for protein-protein interactions [148]. Despite its role in the canonical Wnt pathway, β-catenin was first discovered in cell-adhesion complexes, where it is bound to the cytoplasmic domain of cadherins and to cytoskeleton-linked α-catenin. In this context, cadherins are likely to antagonise accumulation of β-catenin in the cytoplasm by recruiting it to the cell membrane. Studies in different model systems demonstrate a negative correlation between the expression levels of cadherins and Wnt signalling activity [149-151]. Moreover, the conformation of β-catenin protein may also be important for directing its binding affinity towards either cadherins or members of Tcf/Lef (Lymphoid enhancer binding factor/T cell factor) transcription factor family. Thereby, a structural variant of β-catenin containing an intramolecular fold-back of the COOH terminus was found to activate Wnt signalling as monomer [152]. In contrast, cadherins were found to preferentially bind β-catenin-α-catenin complexes. Thus, these findings emphasise the dual role of β-catenin and suggest a model of precise regulation of β-catenin to function in cell adhesion and nuclear signalling [153].

1.4.2. Production and secretion of the Wnt ligand

The Wnt ligand family comprises 19 members, which vary in length between 350 and 400 amino acids. The members of this family undergo several posttranslational modifications. Following cleavage of the signal sequence, the Wnt molecules translocate into the endoplasmatic reticulum (ER). For subsequent transport from ER to cell surface, N-linked glycosylation and palmitoylation take place to generate the hydrophobic nature and ensure proper signalling capacity of these ligands [154-163]. These processes are supported by several components like the integral membrane protein Wntless (Wls) (found in Golgi, plasma membrane and endosomes) [164-168], the Retromer (a multiprotein complex that shuttles cargo from endosomes to the trans-Golgi) [169], and Porcupine (Porc) (localised in the ER) [155, 170]. Finally, Wnt ligands are secreted and move away from the signalling cells, thereby establishing a morphogen gradient to regulate short-, as well as long-range targets in a concentration-dependent manner [171-172]. In analogy to the Hedgehog

morphogens, translocation of Wnt molecules is influenced by heparin sulphate proteoglycans, namely Dally (Dly) and Dally-like, which are anchored to the cell membranes via a GPI linker [173-175].

1.4.3. Degradation of β-catenin by cytoplasmic destruction complex in the absence of Wnt ligands

In the absence of a Wnt signal, the main mediator β-catenin is captured by Axin 1 (Axis inhibition 1) and the tumour suppressor protein Apc (adenomatous polyposis coli protein) within the pentameric β-catenin destruction complex [176-177]. In addition, this complex is composed of CkIα and Gsk3β. By bringing together these components, the pentameric β-catenin destruction complex facilitates phosphorylation of β-catenin by the kinases Ck1α and Gsk3β at conserved serine and threonine residues at the amino terminus. In turn, this promotes binding of β-TrCP, which subsequently mediates the ubiquitinylation and efficient proteasomal degradation of β-catenin. Consequently, lack of cytoplasmic free β-catenin ensures that nuclear DNA-binding proteins of the Tcf/Lef transcription factor family (Tcf1, Tcf3, Tcf4 and Lef1) actively repress target genes by recruiting transcriptional co-repressors (Groucho/Tle)[178-180].

1.4.4. Signal transduction by β-catenin in presence of Wnt ligands

Wnt/β-catenin signalling is mediated by a complex of two families of cell surface proteins: the Frizzled (Fzd) family of 7 trans-membrane serpentine receptors and the mammalian lipoprotein receptor-related proteins (Lrp) Lrp5 and Lrp6 [181-187]. Fzd proteins are the primary receptors for Wnt molecules, whereas Lrps are likely to act as co-receptors.
Upon binding the Wnt ligands, phosphorylation of the conserved PPPSPxS motifs is induced in the cytoplasmic tail of Lrps by Gsk3β and CKIα [176-177, 188-189]. Then, Axin 1 is recruited to the plasma membrane to interact with the phosphorylated Lrp, thereby disengaging the pentameric β-catenin destruction complex. Other effects of Wnt stimulation are the polymerisation of Lrps into large structures, namely the Lrp5/6 signalosome, and the recruitment of Dishevelled (Dvl), which further helps to disable the destruction complex by localising its components to the cell membrane. Consequently, unphosphorylated β-catenin is more stable and accumulates in the cytoplasm leading to its translocation into the nucleus [190-192].

In the nucleus, β-catenin forms a transcriptionally active complex with members of the Lef/Tcf family of transcription factors [193-195] by binding to their N-terminus and displacing repressors like Grouchos [178]. Also interaction with co-activators such as Bcl9 (B-cell lymphoma 9) [196-197], Pygopus (Pygo) [198-201] and Cbp (CREB binding protein) [202] or Hyrax [203-204] have been described.

There are four *Tcf/Lef* genes in vertebrates, *Tcf-1*, *Lef-1*, *Tcf-3*, and *Tcf-4*, which have evolved by alternative splicing and promoter usage [205]. They are characterised by high sequence homology in their DNA-binding domains, the high mobility groups (HMG), responsible for specific binding of target genes [205-206]. In addition, the N-terminal β-catenin binding domain is also highly conserved among orthologs which all bind to β-catenin [205].

The broad spectrum of Wnt target genes include proliferation promoting factors like Cycd1 (Cyclin D1) [207] and c-Myc [208], as well as a range of factors, which are part of the canonical Wnt pathway themselves by positively or negatively influencing the signal cascade (e.g. members of the Frizzeld and Dickkopf families, LRP's, Axin 2, β-TrCP and Lef-Tcf transcription factors; see Roel Nusse's webpage for more references: http://www.stanford.edu/~rnusse/wntwindow.html).

1.5. The canonical Wnt pathway in mammalian skin homeostasis and diseases

In mammalian skin, the canonical Wnt/β-catenin signalling pathway is required for skin morphogenesis including its appendages like hair follicles, sebaceous glands, teeth and mammary glands. Thereby, it regulates a variety of processes such as proliferation, differentiation, cell migration, cell polarity and cell adhesion [209].

Skin-specific expression of a constitutively active form of ß-catenin or overexpression of Lef1 results in *de novo* hair follicle formation [22, 210-211]. Accordingly, ectopic expression of these factors promotes differentiation towards hair-shaft but not interfollicular epidermis [212-213].

Loss-of-function mutations in ß-catenin or Lef1 led to a phenotype with a decreased number of hair follicles [214-216]. Specifically, deletion of the transcription factor Lef-1 in mice leads to lethality shortly after birth. Transgenic embryos display a reduced number of hair follicles and mammary glands, abortive tooth growth and missing whiskers [215]. Furthermore, skin-specific expression of a truncated Lef1 variant, ΔNLef1, which lacks the β-catenin binding

site, results in the formation of epidermal cysts and a progressive hair loss in mouse skin [217]. It has been shown that combined interaction of β-catenin and Lef1 is necessary to repress *E-cadherin* expression during hair follicle development [218].

With regard to sebaceous gland development, evidences indicate the necessity of Wnt pathway inhibition for sebaceous fate selection in the skin. For example, ectopic expression of the dominant negative construct ΔNLef1, and thus canonical Wnt pathway inhibition on transcriptional level in transgenic mouse skin results in an augmentation of sebaceous gland differentiation apparently at the expense of hair follicle differentiation [43, 217, 219].

Furthermore, expression of a stable, dominant negative form of β-catenin in basal keratinocytes of transgenic mice blocks hair formation and creates epidermal cysts while inducing hair germ formation in the interfollicular epithelium [220]. This construct lacks the amino terminal degradation domain and the carboxy terminal activation domain but is still able to bind members of the Tcf/Lef transcription factors family.

Suppressing β-catenin expression in basal keratinocytes of conditional knockout mice revealed that β-catenin is also required to govern homeostasis of the self-renewing skin by inducing anagen phase during the hair cycles [24, 216]. During the hair cycle, Wnt activity is detected in the bulge region of hair follicles at the onset of anagen, and, subsequently, in the hair shaft precursor cells along this stage.

The function of the canonical Wnt/β-catenin pathway in cell fate determination is also manifested in different types of skin tumours, where Wnt activity is up- or down-regulated by mutations of pathway components. In particular, increased Wnt signalling favours the development of hair follicle tumours, for example spontaneous pilomatricomas [210, 221] and tricholofolliculomas [222] in mouse and human skin. Thereby, continuous activation of β-catenin signalling is also required to maintain these hair follicle tumours.

By contrast, transgenic mice expressing ΔNLef1 preferentially develop spontaneous sebaceous tumours or squamous epidermal cysts exhibiting sebaceous differentiation [43, 223]. Accordingly, human sebaceous tumours have been found to harbour such inactivating mutations in *LEF1* [224].

In summary, β-catenin levels are responsible for lineage selection of progenitor cells of hair follicles, whereby high levels of β-catenin stimulate the formation of hair follicles, whereas low levels favour differentiation into interfollicular epidermis and sebocytes.

1.6. Crosstalk between the Hedgehog and Wnt signalling pathways

Complex processes like embryonic development and organogenesis, as well as tissue homeostasis in adults require tightly regulated interactions between different signalling pathways (e.g. Wnt, Hh, Tgf-β/Bmp and, Notch). An Imbalance or deregulation of this network is sufficient to promote malformations and cancer.

1.6.1. Crosstalk between Hedgehog and Wnt signalling pathways in invertebrates and vertebrates

First evidence for interconnection between Hedgehog and Wnt signalling pathways was found in *Drosophila*, where reciprocal signals between Wnt and Hh producing cells contribute to the definition of segment boundaries [225].

In vertebrates, dorsoventral patterning of the nervous system is achieved by the combined activity of the Wnt and Hh pathways. On the one hand, Shh/Gli morphogenetic activity specifies the ventral fate of the neural tubes. On the other hand, the canonical Wnt/β-catenin pathway, which is induced by the Wnt1/Wnt3 ligands in this region, counteracts this Gli-dependent program in the dorsal part of the neural tube by directly inducing Gli3 expression. Gli3 limits the graded Shh/Gli ventral activity in the dorsal department by acting as a transcriptional repressor [226].

Another interaction between these pathways has also been identified during neural tube formation. Shh-deficient chicken embryos display reduced Wnt activity under the influence of the Hh-regulated repressor form of Gli3 (Gli3R) [227]. A model has been proposed, where Gli3R antagonizes active forms of β-catenin by physically interacting with the carboxy-terminal domain of β-catenin containing the transactivation domain.

Van den Brink and colleagues reported that Ihh is expressed by mature colonocytes and regulates their differentiation [228]. Thereby, Hh signalling restricts the expression of Wnt target genes to the base of colonic crypts *in vivo*. Additional *in vitro* assays showed that introduction of Ihh into colon cancer cells results in a downregulation of both components of the nuclear TCF4–β-catenin complex thereby abrogating local response to Wnt signals. In turn, expression of Ihh is downregulated in polyps of individuals with familial adenomatous polyposis coli (FAP) owing to activation of Wnt signalling as consequence of a mutation in APC. This observation was supported by transfecting APC mutant DLD-1 colon cancer cells with a dominant-negative TCF4 mutant which restores expression of Ihh in these cells [228].

More recently, Hedgehog pathway was shown to be required for the lethality and intestinal phenotypes of mice with hyperactive Wnt signalling due to loss of Apc function. Thereby, conditional knock-out models were used to inhibit the function of the critical Hh mediator Smo. As a consequence, lethality and intestinal phenotypes of loss of Apc were rescued in these mice [229].

There are also multiple factors apart from typical Wnt and Hh signalling components, which are crucial to simultaneously regulate both, Gli and β-catenin functionality and turnover. For example, β-TrcP marks ubiquitinated Gli proteins, as well as β-catenin for proteasomal degradation after priming by phosphorylation events through the kinases Gsk3β and CkI [105, 230-234]. Another negative regulator of both pathways, Su(Fu), has been shown to directly interact with Gli and β-catenin, thereby impeding their activation capacities [235-237]. On the other hand, Cbp is recruited by Glis and β-catenin and increase their activation potential as transcriptional co-activator [238-240]. Consequently, inappropriate activation of either Hedgehog or Wnt signalling cascade may have severe impact on the local distribution and concentration levels of these common factors. Thus, β-TrcP, Gsk3β, CkI, Su(Fu) and Cpb represent additional interconnection points for mutual regulation of the Hedgehog and Wnt pathways.

1.6.2. Wnt and Hedgehog pathway interaction in the skin and sebaceous gland

Several studies in transgenic mouse models and humans demonstrated that Wnt and Hh pathways also act in concert during development and regeneration of mammalian skin.

Previous work of our laboratory indicated that Hh signalling acts downstream of the canonical Wnt cascade. Particularly, Wnt/β-catenin signalling was blocked in the skin by expression of dominant negative Lef1 (ΔNLef1) under the control of the Keratin 14 promoter in transgenic mice, which leads to loss of Shh and Ptc1 expression in the affected regions due to complete change of the differentiation program from hair type to interfollicular epidermis [217].

Further analysis of this mouse model also revealed enhanced formation of sebaceous skin tumours [43]. Interestingly, inhibition of β-catenin leads not only to sebaceous lineage selection but also to expression of *Ihh* and *Ptc1* in these types of cells.

Augmented *IHH* and *PTC1* expression has also been detected in the human sebocyte cell line SZ95 undergoing differentiation, as well as after transfection of the ΔNLef1 construct into undifferentiated cells.

Smad7 was shown to induce degradation of β-catenin in an inducible mouse model overexpressing Smad7 in the skin [241]. Interestingly, when Smad7 was induced during

embryogenesis, hair follicle development was inhibited, but the formation of sebaceous glands was accelerated. Postnatal expression of Smad7 resulted in a complete block of hair follicle entry into a new growth phase and in hair follicle degeneration. Contrary to this, sebaceous glands of these mice were found to be significantly enlarged. Moreover, Indian Hedgehog expression was also increased in sebocytes of transgenic mice [241]. Thus, increased Indian Hedgehog expression seems to correlate with inhibition of Wnt/β-catenin pathway.

These findings suggest that *IHH* expression requires an inactivated Wnt/β-catenin pathway, proposing a mutually exclusive model of interaction, which could also be applicable for other tissues [43, 242-243].

In addition, other studies demonstrated the requirement of β-catenin for placode formation upstream of Shh (and also Bmp). Thereby, Shh is induced by Wnt signals and subsequently serves as effector for subsequent cell proliferation or migration [210, 213, 216, 244]. This may explain why deletion of β-catenin following the hair follicle formation leads to complete hair loss after the first hair cycle [216]. Accordingly, β-catenin fails to trigger hair follicle formation when blocking Hh pathway by cyclopamine treatment [22].

Conversely, the Hh pathway can also act upstream of the Wnt pathway, as exemplified in studies of transgenic mice and human BCCs. In these cases, Gli1 induces expression of Wnt ligands, and the ligand-driven, canonical Wnt/β-catenin signalling seems to be required for Hh-pathway-driven tumourigenesis [245-246].

1.7. The sebaceous gland

1.7.1. Sebaceous gland development and regeneration

Sebaceous glands are holocrine glands and are located in the skin of mammals. They secrete an oily/waxy liquid, called the sebum, by rupture of individual sebocytes to lubricate the skin and hair. In humans, they reside all over the body except on the palms of the hands and soles of the feet, and are found in greatest abundance on the face and scalp with a density of 400-900 glands/cm^2 contrary to the sparse distribution on the back [247].

The majority of sebaceous glands develop in close association with the differentiation process of hair follicles during the 13^{th} to 16^{th} weeks of gestation from the bulge on evolving hair follicles [248-249]. They are localised to the upper portion of the hair follicles in adults,

building up the pilosebaceous unit. But some glands are also found apart from hair follicles like on lips, oral cavity, genitalia, eye lid and mamillas [248-249].

Ducts are established between hair follicles and associated sebaceous glands to purge the sebum into the hair shaft channel and at least to the skin surface.

At least two pathways have been presented to play crucial roles in the fate decision of sebocytes, and thus in sebaceous gland development, namely the Wnt and its target gene c-Myc as well as Hh signalling pathways. Depending on their activity levels the number and sizes of sebaceous glands are affected. In this context, decreased Wnt signalling through inactivating mutations in Lef1 or β-catenin promotes sebocyte lineage selection at the expense of hair follicle differentiation [217, 219]. Mice overexpressing c-Myc show an increase in sebaceous differentiation and size of sebaceous glands in combination with a reduction of hair follicles [250-252]. Finally, increased Hh pathway activity and decreased Wnt signalling also lead to an increase in total amount of sebaceous glands, which could be shown by mutations in *Ptc1* and *Lef1*, respectively [43, 112, 253].

As a holocrine gland, the sebaceous gland sacrifices own cells for secretion, and hence needs to be replenished by new cells. The question arises: Where are sebocyte progenitor cells localised? And how is the regeneration of the sebaceous gland regulated? In this context, bulge cells were the first adult stem cells of the hair follicle which have been identified and later shown to be capable of forming hair follicle, interfollicular epidermis and sebaceous glands [254-255]. Keratin 15 (K15), a type I keratin, has been described as a marker for epithelial stem cells in the hair follicle bulge of human scalp [256-257] and mouse skin [258]. Interestingly, previous publications [254, 259] and actual work in our laboratory (Phd thesis of Monika Petersson and Petersson et al., submitted) also indicate that K15-positive cells derived from the bulge region can give rise not only to hair follicles but also to the associated sebaceous glands in mice. Besides their important role in the sebaceous gland renewal, K15-positive cells have also been assigned to the development of human hair follicle-related neoplasms with sebaceous differentiation including sebaceomas, sebaceous neoplasms in Muir-Torre syndrome and sebaceous carcinomas [260-261]. Thereby, *K15* expression was also documented in the mantles of normal human vellus hair follicles, the source of matured sebaceous glands and ducts.

In addition, recent findings propose two different compartments of sebaceous gland stem cells directly above the bulge region of hair follicles (Lgr6+ cells) [262] as well as in the hair follicle junctional zone adjacent to the sebaceous glands and infundibulum (Lrig1+ cells) [263].

Another marker to monitor sebocyte differentiation is the transcriptional repressor B-lymphocyte-induced maturation protein 1 (Blimp1) or PR domain zinc finger protein 1

(PRDM1) in rodents and humans, respectively, which was first suggested as sebocyte progenitor marker in mouse skin by Horsley and colleagues [264]. *Blimp1* expression constitutes an important feature of sebocyte progenitors that are distinct from pluripotent stem cells of the hair follicle. A crucial role of these cells in controlling proliferation of sebaceous progenitors was suggested, since ablation of Blimp1 positive population was found to cause increased sebocyte growth rate and sebaceous gland hyperplasia in mouse [264]. However, the role of Blimp1 as a specific progenitor marker for the sebaceous gland is discussed controversially, since Blimp1 has been identified in the outer root sheath of the hair follicle and mature sebocytes, thus dealt more as an early differentiation marker than progenitor marker [265-266].

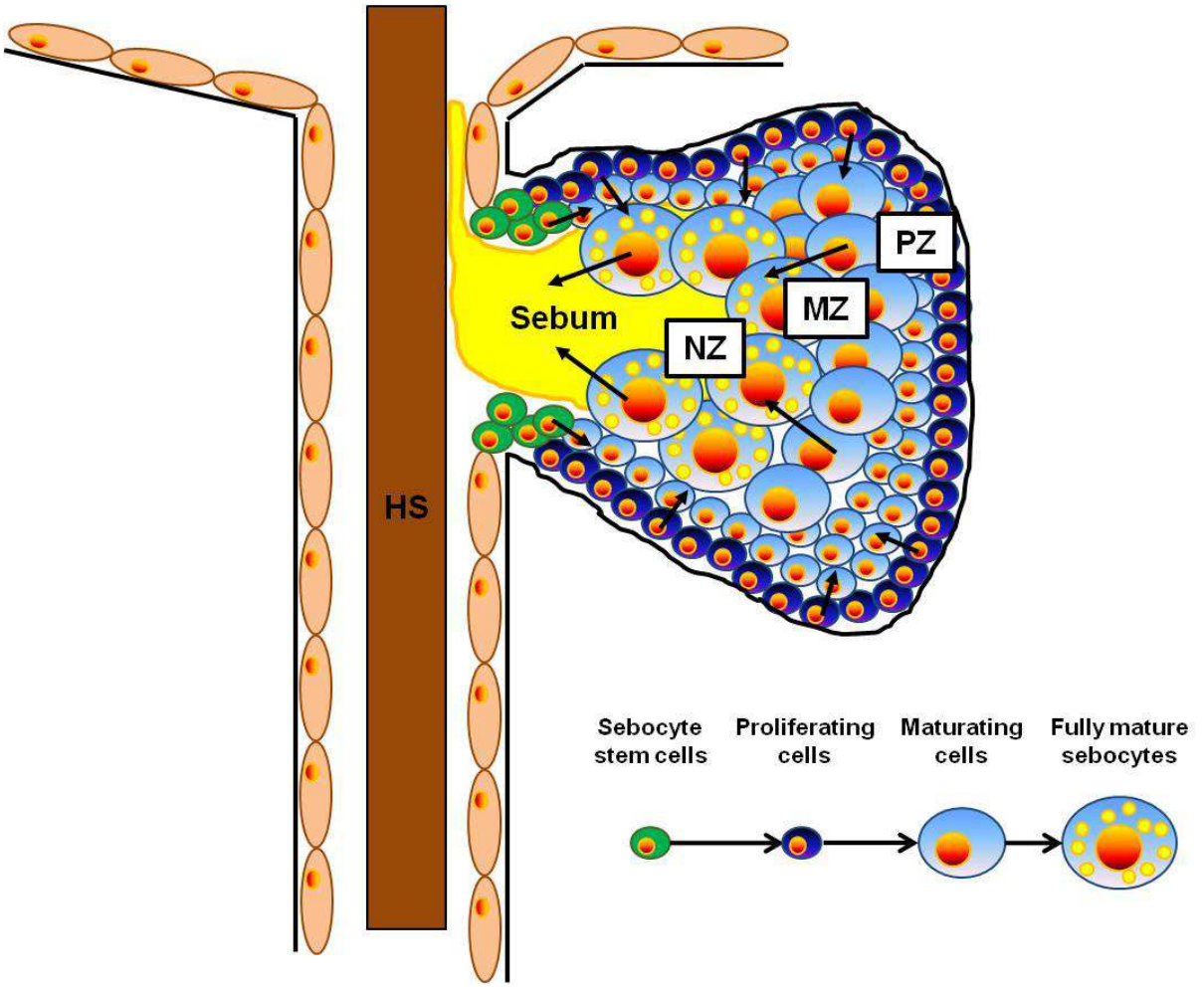


Figure 4: Schematic representation of the pilosebaceous unit. The pilosebaceous unit is made of a hair follicle and sebaceous gland. The outer layer of the sebaceous gland consists of undifferentiated and mitotically active sebocytes. It is assumed that these cells would then migrate to the centre of the gland during the differentiation process, where mature cells burst and release the

sebum into the hair shaft channel by holocrine secretion. *Abbreviations:* HS, hair shaft; PZ, peripheral zone; MZ, maturation zone; NZ, necrosis zone.

Accordingly, the sebaceous glands consist of two types of cells (sebocytes) [267-268]: peripheral, immature cells and central, differentiated cells (Fig. 4). The peripheral sebocytes have been characterised as the undifferentiated and mitotically active population of the sebaceous gland and are cubodial or flattened lacking lipid droplets. They have been proposed to be the source of new cellular material in the sebocyte turnover process. During maturation, these cells differentiate into the centre of the gland displaying an increase in cell size, number of endoplasmatic reticulum (ER) and Golgi apparatus as well as an accumulation of lipid droplets [269]. As a result of enhanced lipid production and cell growth, the fully differentiated sebocytes burst and release their content consisting of cholesterol, fatty acids, triglycerides and diglycerides into the hair follicles [268-269]. Sebum composition varies among species. For example, in human sebum there are unique lipids such as squalene and wax esters. These are not found anywhere else in the body [270].

1.7.2. Functions of the sebaceous gland

The sebaceous glands exert different important functions, which comprise the lubrication, maintenance of flexibility and protection against dehydration in hair and skin. Furthermore, a variety of other tasks have been proposed such as transport of fat-soluble antioxidants and pheromones to the skin surface, natural photoprotection, as well as the modulation of pro- and anti-inflammatory processes in the course of the innate antimicrobial defence [269-271]. For example, Vitamin E can act as antioxidant, and may therefore be delivered to the skin surface by sebum secretion to build up a protection against reactive oxygen species, which otherwise would impair the barrier function of the skin [272]. Sebum has also been shown to harm certain micro-organisms as *Streptococcus* [273], thereby minimising the risk of skin infections [274]. Among others, the sebum contains glycerol, which may support the maintenance of skin hydration [275]. With regards to the integrity of hairs, some observations lead to the assumption that sebaceous glands are essential for the correct structural unfolding and maintenance of hair follicle structure. Mutant mice containing a dysfunction of sebocytes, particularly due to a loss of function of lipid metabolic enzymes in the skin, display dehydrated fur and increased hair loss, resulting in an alopecia phenotype with only rudimentary sebaceous glands [276-280]. These findings strengthen the idea of a cross-dependence between hair follicle and sebaceous gland.

1.7.3. Regulation of sebaceous gland function

There are many factors, which regulate these functions (see also review by K. R. Smith and D.M. Thiboutot, JLR, 2008). Among these are androgens, which were shown to induce proliferation and lipid synthesis in the sebaceous glands. Particularly, androgen receptors (AR) are expressed in the basal layer of the gland, where they bind 5a-dihydrosterone (DHT), a product of testosterone [281-282]. DHT is then translocated to the nucleus and initiates the transcription of androgen-responsive genes [283], which are involved in fatty acid, triglyceride, squalene, and cholesterol synthesis, through the lipogenic transcription factor SREBP (Sterol-response element-binding protein) [284-285]. Accordingly in humans, DHT treatment results in enlarged sebaceous glands, whereas individuals lacking the androgen receptor are androgen-insensitive and have no sebum production [286-287].

Conversely, estrogens like estradiol, found in the ovary, adipose tissue and skin, suppress human sebum production, and are applied in the treatment for acne in women, although the underlying mechanism is not fully understood [288]. Estradiol is also a derivate of testosterone synthesised by the action of the enzyme aromatase, and hence may act through a negative feedback loop to antagonise the effect of DHT [289-290]. Thus, a tightly regulated equilibrium between different hormonal signals is crucial for the overall sebum production.

Treatment of acne skin revealed an inhibitory effect of retinoids (e.g. isotretinoin, 13-cis-RA), a class of vitamin A-derivative pharmacological agents, on sebum production. As consequence, treated skin displayed a reduced size of the glands and their retention in an undifferentiated state. Furthermore, treatment of the immortalised human sebocyte cell lines Seb-1 and SZ95 with 13-cis-RA leads to cell cycle arrest and apoptosis [291-293]. Unfortunately, 13-cis-RA is a teratogen and can cause deformities during pregnancy. Thus alternative, non-teratogenic agents have to be found to inhibit sebum production in the treatment for acne.

LXR (Liver X receptor) is another nuclear receptor, which plays a critical role in cholesterol homeostasis and lipid metabolism. It has been found to be expressed in the human sebaceous gland cell line SZ95 and to modulate lipid synthesis and differentiation in these cells [294-295].

PPARs (Peroxisome proliferator-activated receptors) are members of the ligand-activated nuclear hormone receptor superfamily, which includes receptors for thyroid hormones, retinoids, steroid hormones, and vitamin D, and are also involved in the regulation of sebaceous gland function [296]. PPARα regulates sebocyte differentiation and epidermal permeability barrier by increasing lipid metabolism and expression of structural differentiation

markers. PPARβ was found to be the predominant isotype in the skin and to play a major role as modulator of cell differentiation in both keratinocytes and sebocytes. PPARγ was shown to be involved in the regulation of sebocyte function like lipogenesis and sebocyte development [297]. Accordingly, skin with suppressed *PPARγ* expression was found to lack adipose tissue and sebaceous glands [298], underlining the necessity of PPARγ for differentiation of sebocytes.

Insulin and IGF (Insulin-like growth factor) were shown to promote sebaceous gland lipid production [299]. IGF-1 increases the expression of SREBP-1 (sterol response element-binding protein-1), a transcription factor that regulates numerous genes involved in lipid biosynthesis, which in turn stimulates lipogenesis in sebocytes. This is achieved through activation of the PI3-K (phosphoinositide) pathway [300].

Peptide hormones are also involved in sebocyte differentiation through cyclic adenosine monophosphate (cAMP) which acts as signalling mediator inside the cells upon binding of melanocortins to their receptors, for example melanocortin 5 receptor (MC5R) [301]. MC5R is a G protein coupled receptor (GPCR) and has a preferential affinity for α-melanocyte-stimulating hormone (α-MSH), followed by adrenocorticotropic hormone (ACTH) and β-MSH [302]. In human sebaceous glands, *MC5R* expression is found in the differentiated central part of the gland and not in the periphery, where immature sebocytes are localised [303-304]. Furthermore, MC5R is detected in cultured sebocytes after treatment with cholera toxin to promote cAMP formation, and thus differentiation [305]. The physiological role of MC5R remains still a matter of debate. The targeted deletion of this receptor resulted in reduced sebum secretion [306] and has been proposed as marker for terminally differentiated sebocytes [305].

Previous studies have shown that *Stearoyl-CoA desaturase 1* (*SCD1*) null mice show atrophic sebaceous glands as well as narrow eye fissures. The function of this enzyme comprises the conversion of saturated to unsaturated fatty acid. Thereby, accumulation of unsaturated end products as arachidonic acid was found to have a negative effect on Scd1 expression [279, 307-309]. Its expression is regulated by diet, hormones and developmental processes. However, SCD1 has not been described in the context of sebocyte differentiation yet. In our laboratory, it was found to be up-regulated during early sebaceous gland morphogenesis and expressed in differentiated sebocytes in mouse epidermis (unpublished data).

1.7.4. Sebaceous gland associated skin disorders

Dysfunction of sebaceous glands results in skin diseases such as acne, seborrhea, sebaceoma, and sebaceous carcinoma.
Acne occurs most commonly during adolescence, affecting more than 96% of teenagers, and frequently continues into adulthood. The disorder is accompanied by increased sebum production, hyperkeratinisation of the upper follicle and release of proinflammatory factors upon infection by *Propionibacterium acnes* [310]. Thereby, sebum takes a central role serving as nutrient source for *P. acnes*.
Seborrhea is a chronic inflammatory skin disorder that affects the areas of the head and trunk that have a higher density of sebaceous glands. However, the cause of seborrhea remains unknown [311-312]. The yeast Pityrosporum ovale is known for its affinity for sebaceous glands and has been proposed to be the cause of seborrhoea. Growing colonies lead to skin irritation causing redness and flaking.

1.7.5. Sebaceous gland associated tumours

Sebaceoma has been defined as benign neoplasm of basaloid cells with varying numbers of mature sebocytes by Troy and Ackerman [313]. The histology shows irregular shaped cell masses, in which more than 50% of the cells are undifferentiated consisting of basaloid cells together with large aggregates of sebocytes and transitional cells. In addition, cysts and duct-like structures are found and contain holocrine secretion and cellular debris.
Sebaceous carcinoma is an aggressive, but uncommon tumour. It has been first described by Allaire in 1891, and arises from sebaceous glands in the skin [314-315]. Thereby, approximately 75% of these tumours arise in the periocular region, which is rich in a variety of types of sebaceous glands [315-316].

1.7.6. Impact of ageing on sebaceous glands

In addition, ageing also affects the integrity of sebaceous glands. While the number of sebaceous glands remains approximately the same throughout life, their size tends to decrease with age [317]. Thereby, sebum production in the aged is decreased compared to young adults.

1.8. The human sebocyte cell line SZ95 as *in vitro* model

In order to investigate the molecular mechanism of Indian Hedgehog signalling and its role in human sebocytes, the human facial sebaceous gland cell line SZ95 was selected in the present study. This immortalised cell line has been established and characterised by Zouboulis and colleagues in 1999 [318]. Importantly, this sebocyte cell line preserves major characteristics of sebaceous gland cells *in vivo* such as the polygonal cell shape with a large nucleus, as well as the polymorphic cell population of different sizes and intracellular structures indicating different stages of cell maturation [319-321]. The size of cultured sebocytes was shown to significantly increase with progressive differentiation up to 4- to 5.5-fold. In addition, SZ95 cells respond to different signals as androgens [322] and retinoids [292], and express the receptors LRX [294-295], and PPARs [323-324], which are shown to play a role in the regulation of sebaceous gland function *in vivo*.

Importantly, SZ95 cells have the advantage that they can be induced to differentiate upon confluent growth or fatty acid treatment (e.g. arachidonic acid, linoleic acid) culminating in the production of lipids like triglycerides, squalene and wax esters. Subsequently, these products accumulate in cytoplasmic lipid droplets as seen under normal physiological conditions *in vivo*. Accordingly, unconfluent growing sebocytes comprise undifferentiated cells, whereas populations with high confluence additionally consist of differentiating cells. Furthermore, application of arachidonic acid or linoleic acid induces terminal differentiation in SZ95 cells, which results in holocrine secretion of sebum and cell death as typically observed in mature sebocytes [323, 325-329].

1.9. Objectives of this thesis

Previous work of our workgroup suggested that Indian hedgehog (Ihh) is up-regulated in sebaceous tumours and in the human sebaceous cell line SZ95 undergoing differentiation [43]. Therefore, we hypothesise that Indian hedgehog signalling is important for sebocyte proliferation and differentiation, as well as for formation of sebaceous skin tumour. To test this hypothesis the aim of this work is to specifically address the following questions:

- What is the underlying molecular mechanism of Indian Hedgehog signalling in human sebocytes?
- Does Indian Hedgehog signalling regulate proliferation and differentiation in human sebocytes?
- If so, which of the three Gli transcription factors is specifically involved in these processes?
- Does Indian Hedgehog signalling interact with canonical Wnt/β-catenin signalling in human sebocytes?

To address these crucial questions, the human sebocyte cell line SZ95 was utilised. Thereby, further understanding in the underlying molecular mechanism of Hedgehog signalling in this cell type will help to develop specific and potent therapeutics against diseases associated with impaired regulation of sebaceous glands. Moreover, owing to the widespread role of Hedgehog signalling in the whole organism, gained knowledge in these processes could also be applied for other type of tissues and of Hh pathway-dependent disorders in humans.

2. Material and Methods

2.1. Reporter and expression constructs

2.1.1. Reporter constructs

The following luciferase reporter constructs were kindly provided by H. Clevers [330] and H.Sasaki [331], respectively, and used for transient transfection:

TOPFLASH (functional Tcf reporter plasmid):
The TOPFLASH reporter plasmid contains three copies of the wild type TCF binding site (CCTTTGATC) upstream of a thymidine kinase (TK) minimal promoter and the Luciferase open reading frame (Fig. 5A). The plasmid has a size of 5.5 kbp and serves as a sensitive reporter for the Wnt/β-catenin activity.

FOPFLASH (non-functional Tcf reporter plasmid containing mutated TCF binding sites):
The FOPFLASH reporter plasmid contains three copies of a mutated Tcf binding site (CCTTTGgcC) upstream of a TK minimal promoter and the Luciferase open reading frame (Fig. 5B). The plasmid has a size of 5.5 kbp and serves as a negative control for TOPFLASH.

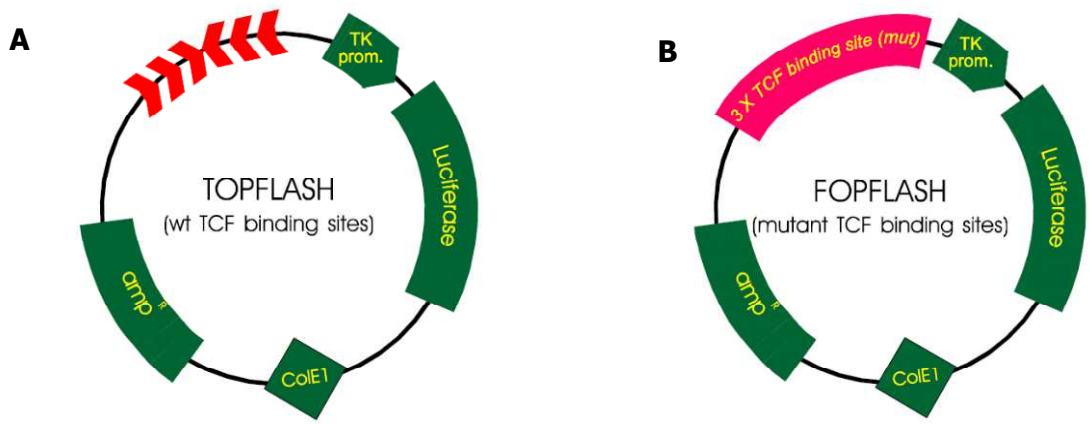


Figure 5: Vector maps of (A) TOPFLASH and (B) FOPFLASH reporter plasmids (Upstate, USA).

8 x 3'Gli-BS Luc (functional Gli reporter plasmid):

The 8 x 3'Gli-BS luciferase reporter construct contains eight copies of the wild type 3'Gli binding site motif (GAACACCCA) upstream of a δ-crystallin basal promoter and the luciferase open reading frame, (Fig. 6). The plasmid serves as a sensitive reporter for the Hh/Gli activity.

8 x m3'Gli-BS Luc (non-functional Gli reporter plasmid containing mutated Gli binding sites):

The 8 x m3'Gli-BS luciferase reporter construct contains eight copies of a mutated variant of the 3'Gli binding site motif (GAAgtgggA) upstream of a δ-crystallin basal promoter and the luciferase open reading frame (Fig. 6). The plasmid serves as a negative control to 8 x 3'Gli-BS luc.

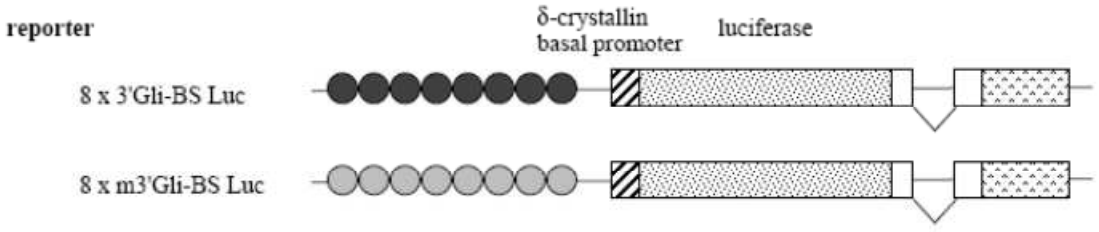


Figure 6: Scheme of Gli reporter constructs for luciferase assay with 8x wild type 3'Gli binding sites (dark cycles) and 8x mutated 3'Gli binding sites (light circles) [331].

pRL-TK (*Renilla* luciferase reporter plasmid):

The pRL-TK plasmid contains the herpes simplex virus thymidine kinase (HSV-TK) promoter to provide low to moderate levels of *Renilla* luciferase expression (Fig. 7). The size of the plasmid is 4 kbp (Promega, USA).This vector was co-transfected as an internal control reporter for transfection efficiency with any of the previously mentioned experimental reporter vectors depending on the experimental settings.

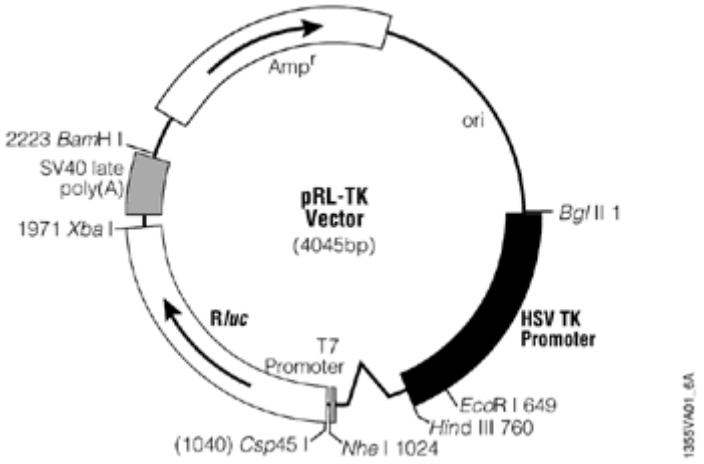


Figure 7: Vector map of pRL-TK (Promega, USA).

2.1.2. Expression constructs

The human full-length Gli expressing vectors were kindly provided by Asa Bergström and Rune Toftgård (Karolinska Institute, department for Biosciences, Sweden).

Human GLI1 HA-tagged expression construct:
Full-length HA-hGli1 cDNA was inserted into the HindIII/SalI restriction site of a pCMV5 vector with ampicillin resistance.

Human GLI2 Myc-tagged expression construct:
Full-length hGli2 cDNA was inserted into pCS2+MT vector with ampicillin resistance and a strong enhancer element (simian CMV IE94).

Human GLI3-GFP fusion protein expressing plasmid:
Full-length hGli3 was cloned in a pEGFP-C2 vector with kanamycin resistance using the SacI/SalI restriction sites.

N-terminally truncated β-catenin expressing plasmid (T2):
The T2 mutant form of β-catenin contains a deletion of the N-terminal 147 amino acids and was cloned in a pCMV5 backbone with ampicillin resistance. This N-terminal sequence includes the phosphorylation sites, which are essential for targeting the β-catenin protein for

degradation. Thus, T2 is a non-degradable and constitutively active variant of this protein [332-333]. The cDNA of this construct originates from *Xenopus,* and, therefore, can be distinguished from the endogenous human β-catenin expression by PCR.

Control vectors pCMV5, pCS2+ and pEGFP-1:
The empty vectors contain an ampicillin (pCMV5, pCS2+) or a kanamycin (pEGFP-1, BD Biosciences Clontech) resistance cassette and were applied as mock controls in transfection experiments.

2.2. Molecular methods

2.2.1. Bacteria strain

TZ101α (Trendzyme, Konstanz)
TZ101α is a chemically competent *E. coli* strain suitable for most applications. Due to a recA mutation, this strain exhibits a reduction of recombination events. An additional endA mutation ensures an improved DNA plasmid quality.
Transformation efficiency > 1 x 108 cfu/µg (cfu: colony-forming unit)
Genotype: F'/ endA1 hsdR17 glnV44 thi-1 recA1 gyrA relA1 Δ(lacIZYA-argF)U169 deoR (Φ80dlacΔ(lacZ)M15)

2.2.2. Chemical transformation of competent bacteria

Most of the chemical methods currently used for bacterial transformation are based on the observations of Mandel and Higa, who showed that bacteria treated with ice-cold solutions of $CaCl_2$ and then briefly heated to 37℃ or 42℃ could be t ransformed with bacteriophage T DNA. The same method was subsequently used to transform bacteria with plasmid DNA and *E. coli* chromosomal DNA as follows:
According to the information given by the provider (Trendzyme, Konstanz), an aliquot of competent TZ101α bacteria was thawed and mixed with 1 µg plasmid DNA. The mixture was incubated on ice for 15 minutes and afterwards, heat shocked for 30 seconds at 42℃.

Subsequently, cells were cooled on ice for 2 minutes before addition of 75 µl SOC-Medium (Invitrogen, Heidelberg). After an incubation period for 1 hour at 37℃ on a shaker, the transformed bacteria were plated on an agar plate containing 50µg/ml ampicilin or 50 µg/ml kanamycin for selection and were grown overnight at 37℃ (agar agar and antibiotics were purchased from Sigma-Aldrich, Hamburg).

2.2.3. Plasmid DNA isolation

After transformation and selection of the bacteria, 5 ml LB medium (Sigma-Aldrich, Hamburg) were inoculated with the appropriate strain of bacteria and incubated overnight at 37℃ on a shaking agitator in the presence of ampicillin (50µg/ml) or kanamycin (50µg/ml). An aliquot (1-2 ml) of the resulting culture was taken to check the correct amplification of the plasmid by restriction digestion following the Mini-Prep kit (Qiagen, Hilden) instructions from the manufacturer. Subsequently, for large scale isolation of up to 500 µg plasmid DNA, 250-500 ml LB medium were inoculated with 3-4 ml of the overnight culture and the culture was grown until it reached an OD_{600} = 0.4. The culture was transferred to centrifugation tubes and left on ice for 15 minutes to stop the growth. The following plasmid DNA isolation was performed using the Maxi-Prep kit (Qiagen, Hilden) according to the manufacturer´s instructions.
The DNA concentration was measured photometrically at 260 nm (Eppendorf, Hamburg) and calculated on the basis that an optical density of 1 equals a concentration of 50 µg/ml double stranded DNA. Purity was determined by measuring OD_{260nm}/OD_{280nm}.
Successfully transformed bacteria strains were stored at -80℃ after addition of 1 ml glycerol to 1 ml culture in a cryogenic vial.

2.2.4. Restriction digestion

Restriction enzyme digestion was used to cut plasmid DNA molecules at defined sites resulting in DNA fragments of defined length. The fragment pattern served as a quality control of the amplified plasmid DNA by the bacteria transformation.

Restriction digestions were performed as follows:

Plasmid DNA	1 – 5 µg
Restriction Enzyme	at least 1 U / µg DNA
10x reaction buffer	3 µl
ddH_2O	ad 30 µl

For some enzymes, the addition of bovine serum albumin (BSA, 1/100 of total volume) was necessary. The digestion was done at the appropriate enzyme reaction temperature (mostly 37℃) for 1 hour to overnight. All restriction enzymes were purchased from NEB (USA).

2.2.5. Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate DNA strands by size, thus allowing identity and quality check of restriction digestion or PCR products. Gels were prepared by boiling 1-2% agarose in 1xTAE. After cooling down to about 70℃, ethidium bromide was added. Ethidium bromide intercalates in double stranded DNA and emits light when it is excited under UV light, which allows visualising the DNA fragments. Gels were run at 120 V for 45-60 minutes. High range or low range DNA ladders (Fermentas, St. Leon-Rot) were used for the determination of the DNA fragment sizes, depending on the expected length of the DNA product.
Additional to non-template and no reverse transcriptase controls, cDNA from total RNA of human fetal thymus (BioChain Institute Inc., USA) was taken for a positive control.

2.2.6. Isolation of mRNA from animal and human cells

Total RNA was purified using the RNeasy Plus Mini Kit (Qiagen, Hilden) according to the manufacturer's protocol, which includes one genomic DNA elimination step.
The total RNA concentration was measured photometrically at 260 nm (Eppendorf, Hamburg) and calculated on the basis that an optical density of 1 equals a concentration of 40 µg/ml single stranded RNA. Purity was determined by measuring the OD_{260nm}/OD_{280nm}.

2.2.7. cDNA synthesis

cDNA synthesis was performed using the QuantiTect Reverse transcription kit (Qiagen, Hilden) at 42℃ for 30 minutes with 1 µg of total RNA according to the manufacturer's protocol, which includes a second genomic DNA elimination step.

2.2.8. Primer design

For quantitative real-time PCR using the SYBR Green technique, all primers were designed to generate a PCR product with similar sizes of 150-300 kb by sharing similar nucleotide sequence length (18-20 kb) and annealing temperatures (57-60℃).
To prevent non-specific binding to residual genomic DNA fragments, sequences spanning more than one target exon were selected if possible. Information on the exon structure of the genes was obtained from the Ensembl database (http://www.ensembl.org/). Primers were designed with the help of Primer3 software (http://frodo.wi.mit.edu/primer3/) and, subsequently, were checked for their quality with FastPCR software (http://www.biocenter.helsinki.fi/bi/Programs/fastpcr.htm) to exclude primer dimer formation. Furthermore, specificity of primer pairs was verified by the nucleotide blast function of the NCBI homepage (http://www.ncbi.nlm.nih.gov/). All primers were purchased from Eurofins MWG GmbH (Ebersberg) and their sequences are listed in the following table (Tab. 1):

Table 1: Primer sequences for qRT-PCR

Target	Primer	Sequence	Product size
ACTG1 (actin gamma 1)	forward reverse	5'-CAGACACCAGGGCGTCAT-3' 5'-CTCGGTCAGCAGCACTGG-3'	251
ADFP (Adipophilin)	forward reverse	5'-CAGAAGCTAGAGCCGCAAAT-3' 5'-CTTCTCCACACTGCCAGTCA-3'	201
α-Tubulin (alpha tubulin)	forward reverse	5'-TCCTTCAACACCTTCTTCAGTG-3' 5'-GCATCTTCCTTGCCTGTGAT-3'	155
AR (Androgen receptor)	forward reverse	5'-GCCTTGCTCTCTAGCCTCAA-3' 5'-CCTCATTCGGACACACTGG-3'	270
AXIN1 (axis inhibition protein 1)	forward reverse	5'-ATGAGGACGATGGCAGAGAC-3' 5'-CCGGCATTGACATAATAGGG-3'	166
AXIN2 (axis inhibition protein	forward reverse	5'-AATGCAAAAGCCACTCCAAG-3' 5'-GCTCACTCTCCAGCATCCAC-3'	222

2)			
β-catenin (beta catenin) (human)	forward reverse	5'-TGGAATGCAAGCTTTAGGAC-3' 5'-TCTGAACCCAGAAGCTGAAC-3'	153
β-catenin (beta catenin) (*Xenopus*)	forward reverse	5'-GGGTGTCCGTATGGAAGAGA-3' 5'-AGCAGTTGCACCTTCAGCTT-3'	229
BLIMP1/PRDM1 (PR domain zinc finger protein 1)	forward reverse	5'-GTGTCAGAACGGGATGAACA-3' 5'-CTTTCCTTTGGAGGGGTTG-3'	268
CBY1 (chibby homolog 1)	forward reverse	5'-GCAGAGACAGGGGTTAGTGG-3' 5'-AAGTGGGATTCAGCAGTGGA-3'	152
CHOP/DDIT3 (DNA damage-inducible transcript 3)	forward reverse	5'-GAGCTGGAAGCCTGGTATGA-3' 5'-TGTGACCTCTGCTGGTTCTG-3'	174
DHH (Desert hedgehog)	forward reverse	5'-CGTGTCGGTCAAAGCTGATA-3' 5'-TCTCCACAGCCACAAATGAA-3'	224
DKK1 (Dickkopf-related protein 1 precursor)	forward reverse	5'-TCCAACGCTATCAAGAACCTG-3' 5'-CAGGCGAGACAGATTTGCAC-3'	227
DKK2 (Dickkopf-related protein 2 precursor)	forward reverse	5'-GCCAAACTCAACTCCATCAA-3' 5'-GCAATACCTCCCAACTTCACA-3'	174
DKK3 (Dickkopf-related protein 3 precursor)	forward reverse	5'-CGAGGTTGAGGAACTGATGG-3' 5'-CCTTCGTGTCTGTGTTGGTC-3'	155
DKK4 (Dickkopf-related protein 4 precursor)	forward reverse	5'-AGGAAAACCAACCCAAAAGG-3' 5'-TCCCTCCAAAAGGACTGG-3'	158
GAPDH (Glyceraldehyde-3-phosphate dehydrogenase)	forward reverse	5'-TCACCAGGGCTGCTTTTAAC-3' 5'-GACAAGCTTCCCGTTCTCAG-3'	152
GLI1 (Glioma-associated oncogene homolog 1)	forward reverse	5'-CCCATTCCAATGAGAAGCCG-3' 5'-GCTTGGCTGTGGCTTCAT-3'	260
GLI2 (GLI-Kruppel family member GLI2)	forward reverse	5'-GTAAGCAGGAGGCTGAGGTG-3' 5'-CACTTGTGGGGCTTCTCG-3'	238
GLI3 (Zinc finger protein GLI3)	forward reverse	5'-CCAACTCCTTGGTCACGATT-3' 5'-AGCTCTGTTGTCGGCTTAGG-3'	168
HHIP (Hedgehog interacting protein)	forward reverse	5'-GCTCTGTCGAAACGGCTACT-3' 5'-TCACTCTGCGGATGTTTCTG-3'	200
IHH (Indian hedgehog)	forward reverse	5'-AAGGCCCACGTGCATTGC-3' 5'-TCGCTGAAGGTGGGGCTC-3'	179
K6a	forward	5'-TGAGATCGACCACGTCAAGA-3'	242

(Keratin 6A)	reverse	5'-ACTCCTCACCCTCCAGCAG-3'	
K15 (Keratin 15)	forward reverse	5'-AGCCCAGAATGCGACTACAG-3' 5'-CTGCATCTCCAGGTCAGTCC-3'	246
MAPK1 (Mitogen-activated protein kinase 1)	forward reverse	5'-CTAACAGGCCCATCTTTCCA-3' 5'-CTCGTCACTCGGGTCGTAAT-3'	299
MC5R (Melanocortin 5 receptor)	forward reverse	5'-CTCAACCTGAATGCCACAGAG-3' 5'-GTCTGCTATCACTAGGTGCTTG-3'	291
c-Myc (Myc proto-oncogene protein)	forward reverse	5'-CCTACCCTCTCAACGACAGC-3' 5'-CTCTGACCTTTTGCCAGGAG-3'	248
PTC1 (Patched homolog 1)	forward reverse	5'-GAGCAGATTTCCAAGGGGAAGG-3' 5'-ACTCGTCCTCCAACTTCCACC-3'	221
PTC2 (Patched homolog 2)	forward reverse	5'-TCACAGGCCAACTTCTACCC-3' 5'-GCCCAGATACTGTTCCCAGA-3'	258
PPARγ (Peroxisome proliferator-activated receptor gamma)	forward reverse	5'-AGCCTTTTGGTGACTTTATGGA-3' 5'-AGGACTCAGGGTGGTTCAGC-3'	213
SCD1 (Acyl-CoA desaturase)	forward reverse	5'-GATGTCTATGAATGGGCTCGTG-3' 5'-CCAGGTTTGTAGTACCTCCTCTG-3'	218
SFRP1 (Secreted frizzled-related protein 1 precursor)	forward reverse	5'-CATGCAGTTCTTCGGCTTCT-3' 5'-GCTTCTTCTTCTTGGGGACA-3'	266
SFRP2 (Secreted frizzled-related protein 2 precursor)	forward reverse	5'-GCTTGAGTGCGACCGTTT-3' 5'-CTTTCGGACACACCGTTCAG-3'	273
SFRP3 (Secreted frizzled-related protein 3 precursor)	forward reverse	5'-AAACTGTAGAGGGGCAAGCA-3' 5'-CCCGTGGAATGTTTACCAGA-3'	194
SFRP4 (Secreted frizzled-related protein 4 precursor)	forward reverse	5'-TCTATGACCGTGGCGTGTG-3' 5'-TTGTAATGAGCGGGACTTGA-3'	330
SFRP5 (Secreted frizzled-related protein 5 precursor)	forward reverse	5'-GGACAACGACCTCTGCATC-3' 5'-TGGGCTCCAATCAGCTTC-3'	201
SHH (Sonic hedgehog)	forward reverse	5'-AGGGCACCATTCTCATCAAC-3' 5'-GGAGCGGTTAGGGCTACTCT-3'	202
SMAD7 (Mothers against decapentaplegic homolog 7)	forward reverse	5'-GCAACCCCCATCACCTTA-3' 5'-CCCCACTCTCGTCTTCTCCT-3'	245

SMO (Smoothened homolog)	forward reverse	5'-CCGGGACTATGTGCTATGTCA-3' 5'-ACTCTGCCCAGTCAACCTG-3'	217
TBP (TATA box binding protein)	forward reverse	5'-TGCACAGGAGCCAAGAGTGAA-3' 5'-CACATCACAGCTCCCCACCA-3'	132
WIF1 (Wnt inhibitory factor 1 precursor)	forward reverse	5'-CCGAAATGGAGGCTTTTGTA-3' 5'-TGGTTGAGCAGTTTGCTTTG-3'	188

2.2.9. Polymerase chain reaction (PCR)

Endpoint PCR samples were set up by adding 100-500 ng cDNA and 0.25 µM of gene specific primers to 12.5 µl REDTaq™ (Sigma-Aldrich, Hamburg) in a 25 µl reaction volume. REDTaq™ is a PCR readymade, 2-fold concentrated reaction mix containing dNTPs, Taq DNA polymerase and $MgCl_2$. The template DNA was denaturised at 95°C for 2 minutes followed by 30-40 amplification cycles each consisting of 1 minute incubation at 95°C, 30 seconds incubation at 55°C for annealing the primers and 30 seconds at 72°C for the elongation step.

2.2.10. Quantitative real-time PCR (qRT-PCR)

Gene expression and down-regulation by siRNA were determined by quantitative real-time RT-PCR on an ABI StepOnePlus Real-Time PCR System (Applied Biosystems, Darmstadt) with the RT^2 SYBR Green qPCR Master Mix (SuperArray, BIOMOL GmbH, Hamburg) according to the manufacturer's instructions. All measurements were standardized with the corresponding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA internal control. For validation, GAPDH was used as an endogenous control for the comparison of expression levels of other potential reference/housekeeping genes in an initial experiment (Fig. 8). As shown below, the expression of these genes did not vary significantly in undifferentiated and differentiated SZ95 cells, so GAPDH was employed as the normalisation gene of choice.

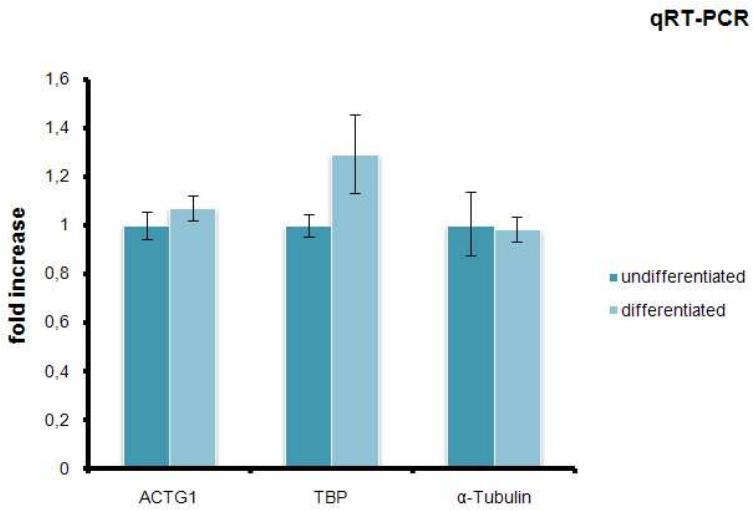


Figure 8: Validation of GAPDH as reference gene. Expression levels of alternative reference/housekeeping genes in undifferentiated and differentiating SZ95 cells were determined by quantitative real-time PCR after normalisation over GAPDH in the appropriate samples. Results are presented as mean values ± SD of triplicates from an experiment that was repeated at least three times.

Depending on the experimental setting, cDNA of either undifferentiated, mock transfected or non-target siRNA treated cells was used as a reference sample.
In addition to the non-template and no reverse transcriptase controls, cDNA from total RNA of SZ95 cells, only treated with EtOH, was taken for the solvent control of arachidonic acid treated cells.
The primers used for expression analysis are displayed in table 2.

2.3. Cell culture

2.3.1. Cell lines

Immortalized human sebaceous gland cell line SZ95:
In this study, SZ95 cells derived from facial human sebaceous glands were chosen as an *in vitro* model to investigate the role of Wnt and Hh signalling pathway during sebocyte differentiation [318]. This cell line has the benefit of reflecting various features of the human sebaceous gland, such as androgen responsiveness [268]. The differentiation of SZ95 cells is induced by cell density. Cultures of unconfluent growing populations consist of

undifferentiated, proliferating cells, whereas high density cultures consist of proliferating and differentiating cells. As seen under normal physiological conditions *in vivo*, the process of SZ95 cell differentiation culminates in the production of lipids like triglycerides, squalene and wax esters that accumulate in cytoplasmic lipid droplets. Application of arachidonic acid (AA) or linoleic acid (LA) was found to induce terminal differentiation leading to holocrine secretion and death of the cells [318, 327, 334].

Human colon adenocarcinoma cell line SW480:

The SW480 cell line was established from a primary adenocarcinoma of the colon [335]. These cells are epithelial like in morphology and used as an *in vitro* model for colorectal cancer to study tumour markers, carcinoembryonic antigen (CEA) production, biochemistry of tumorigenicity, pharmacodynamics, hormone sensibility and as a positive control for the nuclear protein β-catenin. In this thesis, SW480 cells were used as transfection and expression control, because this cell line can be easily transfected with high efficiency in contrast to SZ95 cells.

Hedgehog pathway reporter cell line Shh Light II:

Shh Light II cells were derived from the NIH/3T3 cell line and are employed as stable Gli reporter transfectants [336]. These cells include the GLI-responsive Firefly luciferase reporter from H. Sasaki [331], and a pVgRXR vector (Invitrogen, Karlsruhe) encoding a Zeocin resistance marker for selection. These cells respond to the activation of the hedgehog signalling cascade in presence of hedgehog ligands resulting in an increase of luciferase activity which can be measured by a luminometer.

2.3.2. Cultivation of SZ95 cells

SZ95 cells were cultured in Sebomed medium (Biochrom, Berlin) containing 10% FCS (Biochrom, Berlin) supplemented with 3 ng/ml keratinocyte growth factor (rHuKGF, Pan Biotech GmbH, Aidenbach), and 20 ng/ml epidermal growth factor (EGF, Biochrom, Berlin) at 37°C in a humidified incubator with 5% CO_2. Undifferentiated/immature SZ95 cells were typically seeded at a density of 2-4 x 10^5 cells per 25-cm^2 flask and passaged before reaching confluency. To induce differentiation, SZ95 cells were cultured at high density for 1-3 days or treated with 100 µM arachidonic acid or 100 µM linoleic acid both purchased from Sigma-Aldrich (Hamburg) [318, 327, 334]. For Smoothened or GLI inhibitor assays, 5-50 µM

cyclopamine (Biomol, Hamburg) or 10-100 µM GANT61 (Calbiochem, Darmstadt) were added to the growth medium, respectively. For stimulation of the Hedgehog pathway, 1-5 µg recombinant SHH ligand protein per 500 µl growth medium were applied on cells grown in 24-well cell culture plates. Subsequently, proliferation was assessed by BrdU incorporation assays.

2.3.3. Cultivation of SW480 cells

SW480 cells were cultured in DMEM (GIBCO, Karlsruhe) containing 10% FCS (PAA, Cölbe) supplemented with 100 U/ml penicillin (Biochrom, Berlin) and 100 µg/ml streptomycin (Biochrom, Berlin). The cells were grown at 37°C in a humidified incubator with 5% CO_2.

2.3.4. Cultivation of Shh light II cells

Shh light II cells were cultured in DMEM (GIBCO, Karlsruhe) containing 10% FCS (PAA, Cölbe) supplemented with 100 U/ml penicillin (Biochrom, Berlin), 100 µg/ml streptomycin (Biochrom, Berlin), 0.4 mg/ml G-418 (Sigma-Aldrich, Hamburg) and 0.15 mg/ml Zeocin (Invitrogen, Karlsruhe). Cells were grown at 37°C in a humidified incubator with 5% CO_2.

2.3.5. Thawing and freezing cells

For long-term storage, cells were trypsinised, spinned down after which the pellet was resuspended in freezing medium containing 10% DMSO (dimethyl sulfoxide) in FCS (see FCS used in growth media) and transferred to cryogenic vials. These were transferred into a freezer box, allowing controlled cooling down with a rate of 1°C per minute which improves the survival of the cells. After 24 hours, the cells were transferred to liquid nitrogen (-196°C).
For thawing, frozen cells were immediately transferred from liquid nitrogen into a 37°C water bath as quickly as possible in order to prevent the formation of ice crystals during rehydration. Then, the cells were promptly washed to remove DMSO included in the freezing medium. Therefore, 5 ml of cell culture medium was added, after which the cells were

pelleted by centrifugation for 5 minutes at 800 rpm. Cells were resuspended in an appropriate volume of culture medium and transferred to a culture dish or flask.

2.3.6. Transient transfection of cells by lipofection

For luciferase reporter and/or expression assays, SZ95 and SW480 cells were transiently transfected with the Lipofectamine 2000 reagent (Invitrogen, Karlsruhe). Therefore, cells were plated in 24 well plates ($1x10^5$ cells per well), and cultivated for 24-48 hours before transfection. Transfection was performed as specified by the manufacturer's protocol. OptimemI (GIBCO, Karlsruhe) was used as serum-free, and thus, transfection medium. For the transfection mixture, 500 ng of the appropriate plasmid DNA (GLI1-HA/pCMV5, GLI2-Myc/pCS2+, GLI3/pEGFP-C2, T2/pCMV5, empty plasmids as mock control), in combination with 500 ng Firefly (8x3'Gli-BS/m8x3'Gli-BS; TOP/FOP) and 500 ng of Renilla (pRLTK) reporter plasmids for luciferase assays, were added to 2 µl Lipofectamine 2000 diluted in 100 µl OptimemI. After 5 hours, the transfection medium was replaced by normal growth medium and cells were cultured under normal conditions for further 48 hours before collection for analysis. For larger scale transfections, cell number and amounts of transfection agents were adapted to the size of the cell culture plate.
For siRNA knockdown assays, cells were transfected with HiPerFect transfection reagent (Qiagen, Hilden) according to the manufacturer's protocol (also see 2.3.7).

2.3.7. siRNA knockdown assay

For knock-down experiments, SZ95 cells were seeded as previously described (2.3.6) and transfected with 37.,5 ng siRNA Oligos (Qiagen, Hilden) in combination with 3 µl HiPerFect transfection reagent (Qiagen, Hilden) according to the manufacturer's protocol. After 24-48 hours, the cells were collected for further analysis. Human-specific siRNA directed against Gli transcription factors as well as AllStars negative control siRNA (including the fluorescent label Alexa Fluor 488) and anti mouse/human MAPK1 positive control siRNA (Tab. 2) were purchased from Qiagen. The knockdown efficiency was verified on transcript level by quantitative real-time PCR.

Table 2: List of siRNA oligonucleotides

Oligo name	Catalogue no.	Manufacturer
Hs_GLI2_7	SI03078215	Qiagen, Hilden
Hs_GLI2_8	SI03091445	Qiagen, Hilden
Hs_GLI3_2	SI00003577	Qiagen, Hilden
Hs_GLI3_3	SI00003584	Qiagen, Hilden
Allstars neg. ctrl siRNA AF 488		Qiagen, Hilden
Mm/Hs MAPK1 ctrl siRNA		Qiagen, Hilden

2.3.8. Generation of stable cell lines with shRNA expressing constructs by lentiviral transfection

MISSION shRNA transduction-ready lentiviral particles directed against the human GLI transcription factors, MISSION control transduction-ready lentiviral particles containing non-target shRNA and TurboGFP expression vector (Tab. 3) were purchased from Sigma-Aldrich (USA) and used for the infection of SZ95 cells according to the manufacturer's protocol for gene silencing in mammalian cells. Thereby, the multiplicity of infection (MOI) was set to 5 (number of transducing particles per cell) according to the concentrations of the viral supernatants provided by the manufacturer. After successful infection, the cells were cultured in selection medium containing 1 µg/ml puromycin (Sigma, Hamburg).

Table 3: List of shRNA lentiviral transduction particles

Name	Clone ID	Manufacturer
SHVRS GLI2 NM_005269	TRCN0000033329	Sigma-Aldrich, USA
SHVRS GLI2 NM_005269	TRCN0000033330	Sigma-Aldrich, USA
SHVRS GLI2 NM_005269	TRCN0000033331	Sigma-Aldrich, USA
SHVRS GLI2 NM_005269	TRCN0000033332	Sigma-Aldrich, USA
SHVRS GLI2 NM_005269	TRCN0000033333	Sigma-Aldrich, USA
SHVRS GLI3 NM_000168	TRCN0000020504	Sigma-Aldrich, USA
SHVRS GLI3 NM_000168	TRCN0000020505	Sigma-Aldrich, USA
SHVRS GLI3 NM_000168	TRCN0000020506	Sigma-Aldrich, USA
SHVRS GLI3 NM_000168	TRCN0000020508	Sigma-Aldrich, USA
SHC002V Non-Target Control		Sigma-Aldrich, USA
SHC003V TurboGFP Control		Sigma-Aldrich, USA

2.3.9. Luciferase reporter assays

Luciferase reporter assays were performed with the Dual-Luciferase Reporter Assay kit from Promega (Mannheim). 24-48 hours after transfection, cells were lysed, and the lysates were treated according to the manufacturer's protocol. 10 µl of protein lysates were transferred to a 96-well luminometer plate. After addition of the firefly substrate LARII (supplied by kit), the luciferase activity was measured by a luminometer (TriStar LB941 from Berthold Technologies GmbH & Co. KG, Bad Wildbad). Stop & Glo solution (supplied by kit) was added to initiate the *Renilla* luciferase reaction in the same sample as transfection control. The measurement results were normalized for the transfection efficiency by dividing the firefly luminescence activity (F_{RLU}) with *Renilla* luminescence activity (R_{RLU}). All data are represented as-fold increase of the negative control activity value (m8x3'Gli-BS or FOPFLASH), which is set as one.

2.3.10. BrdU proliferation assay

For BrdU staining of proliferative cells, SZ95 cells were grown on glass cover slips on 24 well plates and exposed for 1 hour to 160 µg/ml BrdU-containing medium before fixation with 4%PFA/1xPBS. Then, cells were permeabilised with 2N HCL and 0.5% Triton-X 100 in 1xPBS for 10 minutes and washed in 1xPBS alone. To prevent unspecific antibody binding, the fixed cells were incubated in blocking buffer containing 3 g BSA, 10% FCS, 5 g milk powder, 10 µl Tween 20 diluted in 1xPBS with a total volume of 100 ml for 30 minutes. After washing with 1xPBS, cells were incubated with the primary monoclonal antibody anti-BrdU clone B44 (Beckton Dickinson, Heidelberg) diluted 1:25 in 1%BSA/1xPBS for 45 minutes. Afterwards, cells were washed with 0.01% Tween 20/1xPBS and incubated with the secondary antibody anti-mouse Alexa 594 (Invitrogen, Carlsbad) at a dilution of 1:500 for 30 minutes. Finally, the cells were washed with 0.01% Tween 20/1xPBS and H_2O before mounting with polvvinyl alcohol (Mowiol, Calbiochem, Darmstadt). All incubation steps were performed at room temperature. Proliferation rate was determined by the ratio of BrdU-positive/proliferating cells to total number of cells which was determined by counterstaining the cell nuclei with DAPI (4',6-diamidino-2-phenylindole, Molecular Probes, Netherland) at a dilution of 1:1000.

2.4. Protein analysis

2.4.1. Total cell protein extraction

Cells were washed with ice-cold 1xPBS after and lysed in 500 µl or 1ml 1xNP40 lysis buffer (1% NP-40, 150 mM NaCl, 4 mM EDTA, 50 mM Tris pH 7.4, supplemented with protease inhibitor cocktail from Roche, Mannheim) was added to each well of a 6-well culture plate or a 6-cm dish, respectively, and left for 15 minutes at 4℃ on a shaker. The lysates were transferred to reaction tubes and centrifuged at 13000 rpm at 4℃ for 15 minutes. The supernatants containing soluble proteins were transferred to new reaction tubes and either directly used for further analysis or stored at -80℃.

2.4.2. Cell fractionation

Cells were grown on 6-cm dishes, washed with ice-cold hypotonic buffer (20 mM Hepes, pH 7.4, 20 mM NaCl, 5 mM $MgCl_2$, 1 mM ATP, supplemented with protease inhibitor cocktail from Roche, Mannheim) and collected on ice with a cell scraper in a sample tube. Afterwards, the swollen cells were homogenized with a glass douncer (10 strokes) and left on ice for 15 minutes. In the following step, the samples were centrifuged at 600 g for 5 minutes at 4℃ and the supernatant containing the cytosolic protein fraction was collected. The remaining pellet was resolved in hypotonic buffer containing 0.5% NP-40 to break up the nuclear membrane and extract the nuclear protein fraction.
Both fractions were either used directly for further analysis or frozen at -80℃.

2.4.3. Protein precipitation (Wessel-Flügge)

The chloroform/methanol precipitation of proteins from solution, including detergent solutions, has been established by Wessel and Flügge (1984). To 0.5 ml lysate 1 volume of methanol and ¼ volume of chloroform were added on ice. Then, the samples were mixed vigorously and centrifuged at 10000 g at 4℃ for 5 minutes. The upper aqueous phase was removed and 0.5 ml methanol was added to the remaining sample which comprises the

lower phase containing chloroform and the interphase containing the precipitated proteins. Again, the solution was mixed and centrifuged at 10000 g at 4℃ for 5 minutes to pellet the proteins and discard the residual chloroform. After air-drying, the protein pellet was redissolved in 50-100 µl 2% SDS/ddH_2O on ice.

2.4.4. Protein quantification

The protein concentration of the lysates was quantified by the bicinchoninic acid (BCA) method using the Pierce BCA Protein Assay Kit (Pierce Biotechnology, Rockford USA) according to the manufacturer's instructions. This method is based on a two step detection system. In an alkaline environment, proteins form complexes with Cu^{2+} ions thereby reducing them to Cu^{1+} ions (biuret method). Subsequently, the reduced Cu^{1+} ions form a soluble purple complex with bicinchoninic acid that has a maximal absorbance at 562 nm.
For the quantification of the protein concentration, samples were diluted 1:10. Applying the protocol, 10 µl of the diluted sample as well as 10 µl of eight BSA samples with known concentrations for the standard curve were incubated with 200 µl supplied BCA-working solution and incubated for 30 minutes at 37℃ in 96 -well plates (TPP, Switzerland). The absorbance of the reaction product was measured by a TriStar LB941 (Berthold Technologies GmbH & Co. KG, Bad Wildbad) at 578 nm. Protein concentration of each lysate was determined in triplicates.
The standard curve was established by plotting the absorbance of the BSA standards against their concentration. The resulting equation of the standard curve was then used to calculate the unknown protein concentration of the analysed samples.

2.4.5. Protein electrophoresis (SDS-Page) and Immunoblotting

Sodium Dodecyl (lauryl) Sulfate Poly-Acrylamide Gel Electrophoresis (SDS-PAGE) provides information about molecular size and purity of proteins as well as about the number and molecular size of subunits. SDS functions as an anionic detergent that binds and denatures proteins, resulting in a net negative charge for all proteins. Therefore, proteins are separated on the basis of their size.

Under reducing conditions, 5-40 µg of lysates were denatured by boiling for 5 minutes at 70℃ and run on NuPAGE Novex Tris Acetate 3-8% or NuPAGE Novex Bis-Tris 4-12% gels as described in the manufacturer's protocol (Invitrogen, Karlsruhe). Afterwards, the proteins were blotted to polyvinylidene membranes (GE Healthcare, München) which were probed with the appropriate primary antibodies diluted according to table 4. The primary antibodies were detected by using horseradish peroxidase-conjugated anti-rabbit, -goat, or -mouse IgG antibodies (see dilution also on table 5),respectively, and visualized with enhanced chemiluminescence (ECL + Western blotting detection kit, GE Healthcare, München), followed by exposure to Hyperfilm ECL (GE Healthcare, München).
Protein band intensities were determined and compared by ImageJ analysis software (http://rsbweb.nih.gov/ij/). Thereby, detection of GAPDH and α-Tubulin proteins served as loading control of each sample.

2.4.6. Immunocytochemistry and lipid staining

For immuno and lipid staining techniques, SZ95 cells were grown on glass cover slips in 24 well culture plates until 60% confluency for analysing undifferentiated cells or 100% confluency for characterising differentiating populations. In some experiments, SZ95 cultures were treated with 100 µM arachidonic acid to induce terminal differentiation of the cells. After 24-48 hours, cells were washed with 1xPBS and fixed in 4% PFA/1xPBS for 10 minutes.
For immunofluorescence staining, the cells were then permeabilised with 0.2% (v/v) Triton X-100 (Merck, Darmstadt) in 1xPBS for 5 minutes. Nonspecific binding was blocked with 10% (v/v) goat (NGS) or donkey serum (NDS) in 1xPBS for 1 hour. Primary antibodies were diluted in blocking solution and incubated at 4℃ overnight or at room temperature for 90 minutes (see table 4 for dilutions). Secondary antibodies were used in accordance with the manufacturer's instructions, whereby optimal dilutions are listed on table 5. Cell nuclei were counterstained DAPI or propidium iodide (PI; Sigma, Hamburg). Coverslips were then mounted with polvvinyl alcohol (Mowiol, Calbiochem, Darmstadt), and immunolocalisation was visualised by Fluorescence Microscope Eclipse E800 (Nikon Instech Co., Ltd. Kanagawa, Japan) or Olympus Confocal Laser Scanning Microscope (Olympus, Münster).
Oil red O (ORO) (Sigma, Hamburg) lipid droplet staining for detection of mature sebocytes was performed by incubating fixed SZ95 cells in 1% ORO in 60% IPA for 30 minutes, washed with H_2O, and counterstained with Harris haematoxylin (Sigma-Aldrich, Hamburg).

Finally, cover slips were embedded in Kaiser's glycerin-gelatine (Merck, Darmstadt) and used for analysis by light microscopy (Nikon Instech Co., Ltd. Kanagawa, Japan).
Analogously, lipid droplets were stained with the fluorescent dye Nile Red for analysis by fluorescence microscopy. To generate the stock solution, 1 µg Nile Red was dissolved in 1 ml MeOH. A working solution was obtained after a dilution of 1:1000 of the stock solution in 1xPBS. Fixed SZ95 cells on cover slips were washed with 1xPBS and incubated in Nile Red working solution for 30 minutes at RT. After washing with 1xPBS, the cover slips were mounted in polvvinyl alcohol (Mowiol, Calbiochem, Darmstadt) and analysed by fluorescence microscopy (Nikon Instech Co., Ltd. Kanagawa, Japan).

2.4.7. Antibodies

WB = Western Blot
IF = Immunofluorescence

Table 4: Primary Antibodies

Name	Epitope	Host	Dilution	Reference/ Manufacturer
monoclonal α-ABC clone 8E7	Human active ß-catenin amino acids 36-44	Mouse	WB: 1:3000 IF: 1:100	Van Noort et al., 2002, Milipore, Schwalbach
monoclonal anti-β-catenin	Mouse β-catenin amino acids 571-781	Mouse	WB: 1:3000	BD Transduction Laboratories, USA
VB2	β-catenin last 15 C-term. amino acids	Rabbit	WB: 1:1000 IF: 1:100	Braga et al., 1995
monoclonal anti-Ihh clone EP1192Y	Human N-terminus	Rabbit	WB:1:3000	Abcam, Cambridge, UK
polyclonal anti-gli1 (sc-6153)	Human N-terminus	Goat	WB: 1:1000 IF: 1:50	Santa Cruz Biotechnology, USA
polyclonal anti-gli2 (ab7195)	Human Gli2 amino acids 1193-1209	Rabbit	WB: 1:3000 IF: 1:100	Abcam, Cambridge, UK
polyclonal anti-gli3 (sc-20688)	Human Gli3 amino acids 1-280	Goat	WB: 1:3000 IF: 1:50	Santa Cruz Biotechnology, USA

monoclonal anti-HA (clone 3F10)	HA peptide sequence (YPYDVPDYA) derived from influenza virus)	Rat	WB: 1:3000	Roche Applied Science, Mannheim
monoclonal anti-c-Myc (9E10)	c-Myc amino acids 410-419	Mouse	WB: 1:3000	Abcam, Cambridge, UK
polyclonal anti-GFP (ab290)	full-length protein of *Aequorea victoria*	Rabbit	WB: 1:3000	Abcam, Cambridge, UK
polyclonal anti-Gapdh (ab9485)	Human full-length protein	Rabbit	WB: 1:3000	Abcam, Cambridge, UK
monoclonal anti-TBP (1TBP18)	Human Synthetic peptide	Mouse	WB: 1:3000	Abcam, Cambridge, UK
monoclonal anti-α-tubulin	Native chick brain microtubules	mouse	WB: 1:3000	Calbiochem, Darmstadt
polyclonal anti-MC5-R (sc-7644)	Human C-terminus	Goat	IF: 1:200	Santa Cruz Biotechnology, USA
polyclonal anti-SCD (sc-23016)	Human internal region	Mouse	IF: 1:200	Santa Cruz Biotechnology, USA
polyclonal anti-PRDM1/Blimp1 (ab13700)	Human amino acids 778-789	Goat	IF: 1:200	Abcam, Cambridge, UK
monoclonal anti-BrdU	detects BrdU = analog of thymidine	Mouse	IF: 1:25	BD Pharmigen, USA

Table 5: Secondary Antibodies

Name	Epitope	Host	Dilution	Reference/ Manufacturer
Alexa 488 anti-mouse	mouse IgGs	Donkey	IF 1:1000	Invitrogen, USA
Alexa 594 anti-rat	rabbit IgGs	Goat	IF: 1:1000	Invitrogen, USA
HRP-conjugate anti-mouse IgG	mouse IgGs	Sheep	WB: 1:3000	GE Healthcare, Buckinhamshire, UK
HRP-conjugate anti-rabbit IgG	rabbit IgGs	Donkey	WB: 1:3000	GE Healthcare, Buckinhamshire, UK
HRP-conjugate anti-rat IgG	rat IgGs	Goat	WB: 1:3000	GE Healthcare, Buckinhamshire, UK

2.5. Additional Software

ABI StepOnePlus Software V.2.0, Applied Biosystems, Darmstadt, Germany
Clone Manager Professional 8, Scientific & Educational Software, USA
Lucia G & G/F V.4.82, Laboratory Imaging s.r.o., Czech Republic
Photoshop, Adobe, USA
Winglow, Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany

3. Results

3.1. Characterisation of undifferentiated, differentiating and terminally differentiated human sebocytes by using SZ95 cell line as *in vitro* model

We hypothesized that Ihh signalling plays a role in regulation of proliferation and/or differentiation in sebocytes. To test this, a flexible and comparable model system to the *in vivo* situation was required to investigate and compare different sebaceous differentiation states. As Ihh knockout mice early die during embryonic development [40], we tested if the human facial sebaceous gland cell line SZ95 would allow us to perform these analyses *in vitro*. As previously mentioned these cells share typical characteristics with the sebocytes *in vivo* and can be retained to an undifferentiated state when grown in low density. On the other hand, they can be easily induced to differentiate when cultivated to high confluency or treated with specific fatty acids (e.g. arachidonic acid and linoleic acid) [268, 318, 327].
By taking advantage of these features, it was first necessary to identify and characterise all sebocyte populations in correlation with differentiation grade in initial experiments. For that reason, we first histologically analysed the differences in cytosolic lipid droplet accumulation as classical marker of sebaceous differentiation. Furthermore, we extended these analyses by determination of expression levels of well established (e.g. MC5R, AR, PPARγ) and potentially new (e.g. SCD1, PRDM1) molecular markers of sebocyte differentiation. Finally, we wanted to combine the obtained observation to establish a histological and molecular profile of human sebocytes according to their differentiation state.

3.1.1. Lipid droplet production and cell size as indicators of sebocyte differentiation

First, we addressed the question, whether lipid production correlates with maturation progress and/or increased cell size is required for human sebocyte differentiation *in vitro* like it has been suggested from *in vivo* observations [268]. Therefore, the differentiation states of unconfluent, confluent and arachidonic acid treated cell populations were investigated and compared with each other by cell shape analysis and by staining of intracellular lipid droplets under the light microscope.

.

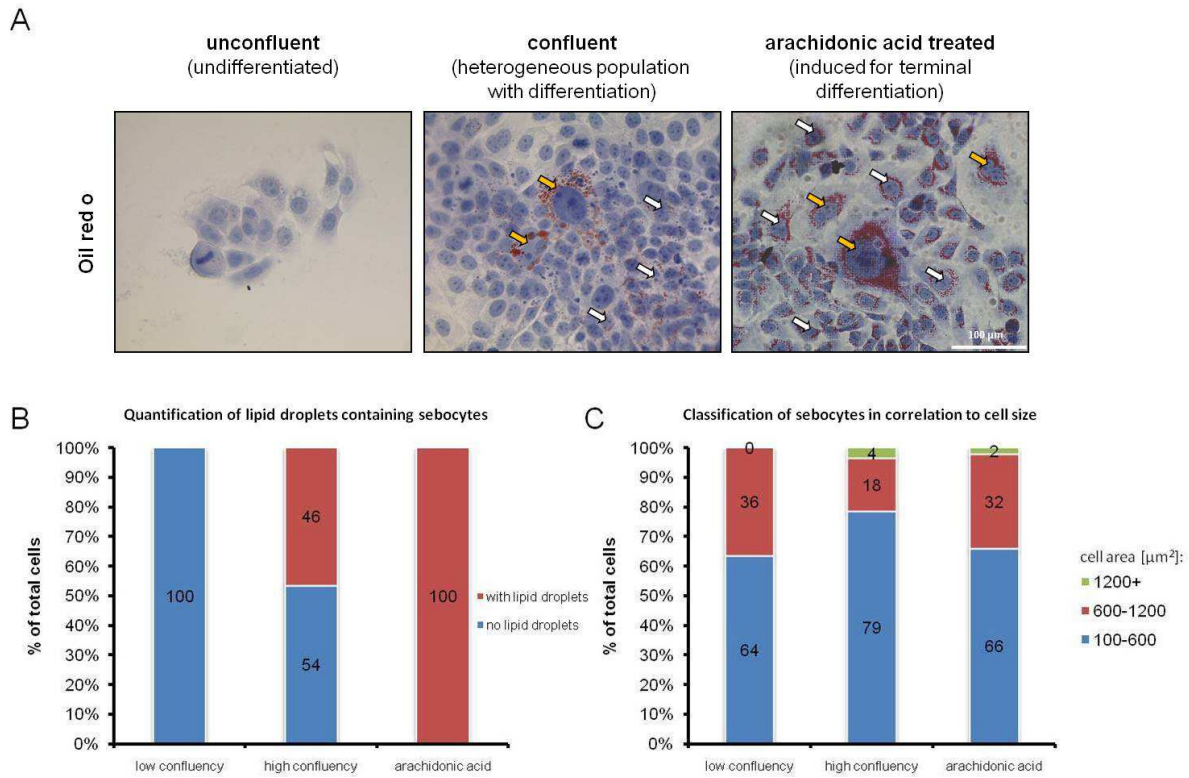


Figure 9: Characterisation of SZ95 cell differentiation on structural level. (A) Oil red o staining of intracellular lipid droplets in SZ95 cells under unconfluent (left) and confluent (middle), as well as, after arachidonic acid (right) treatment. White arrows mark differentiated cells with increased intracellular lipid droplet content. Gold arrows indicate terminally differentiated sebocytes with a large cell volume and an excess load of lipid droplets. (B) Quantification of sebocytes with or without visible lipid droplets by oil red o staining. (C) Classification of sebocytes according to their cell size.

No lipid droplets were detected in unconfluent SZ95 cells attesting their undifferentiated state (Fig. 9 A, left and Fig. 9 B), although 36% of this population displayed a medium-size cell area ranging from 600 to 1200 µm^2 (Fig. 9 C). In contrast to this, lipid droplets were found in 46% of sebocytes growing to high confluency (Fig. 9 A, middle and Fig. 9 B), whereby the majority of these cells (79%) exhibited decreased cell areas ranging from 100-600 µm^2, while only 18% of this population could be assigned to the group of 600-1200 µm^2 (Fig. 9 C). Importantly, an additional group of large-sized sebocytes with a cell area exceeding 1200 µm^2 could be detected in confluent populations with a share of 4% of total cells. These cells particularly displayed strong accumulation of intracellular lipid droplets, thus representing sebocytes with an advanced differentiation status. Strikingly, 100% of all sebocytes treated with arachidonic acid (or linoleic acid, not shown) showed lipid droplet formation (Fig. 9 A, right and Fig. 9 B). Notably, 2% of this population exhibited a large size of about 1200 µm^2,

whereas 32% of these cells were middle-sized (600-1200 µm^2) and the rest even small-sized (100-600 µm^2).

As increased accumulation of lipid droplets did not correlate with increased cell size, lipid droplet production was handled as primary criterion for classification of mature sebocytes. A reason for the missing link between cell size and differentiation could be that sebocytes growing to high confluency do not have enough space to spread due to high population density in their environment. Also did fatty acid treated sebocytes not exhibit larger cell sizes although they were induced to differentiate. Hence, a large cell size is not a prerequisite for lipid production and differentiation.

Based on these findings, we defined three populations which we used as representative sebaceous differentiation stages in the present study as follows:

The first one comprised unconfluent cells representing the undifferentiated reference population. The second one consisted of confluent cultures including cells undergoing differentiation and more likely mimicking the heterogeneous *in vivo* situation of the sebaceous gland. Finally, SZ95 cells treated with arachidonic acid served as a differentiation control.

For convenience, unconfluent populations are named "**undifferentiated" (undiff.)**, while confluent populations **"differentiating" (diff.)** in the following data figures. Accordingly, SZ95 cells treated with arachidonic acid to induce terminal differentiation are termed as **"terminally differentiated" (term. diff.)**.

3.1.2. Correlation between expression of molecular markers and sebocyte differentiation state

In order to establish a molecular readout of the three pre-defined differentiation states in human sebocytes, a range of established and potentially new markers from the literature or own work, respectively, have to be selected. Therefore, quantitative real-time PCR and immunofluorescence staining were performed to analyse and compare the expression pattern of these markers in undifferentiated, differentiating and terminally differentiated cells. Later on, these markers would be used to detect changes in differentiation behaviour after manipulation of human sebocytes.

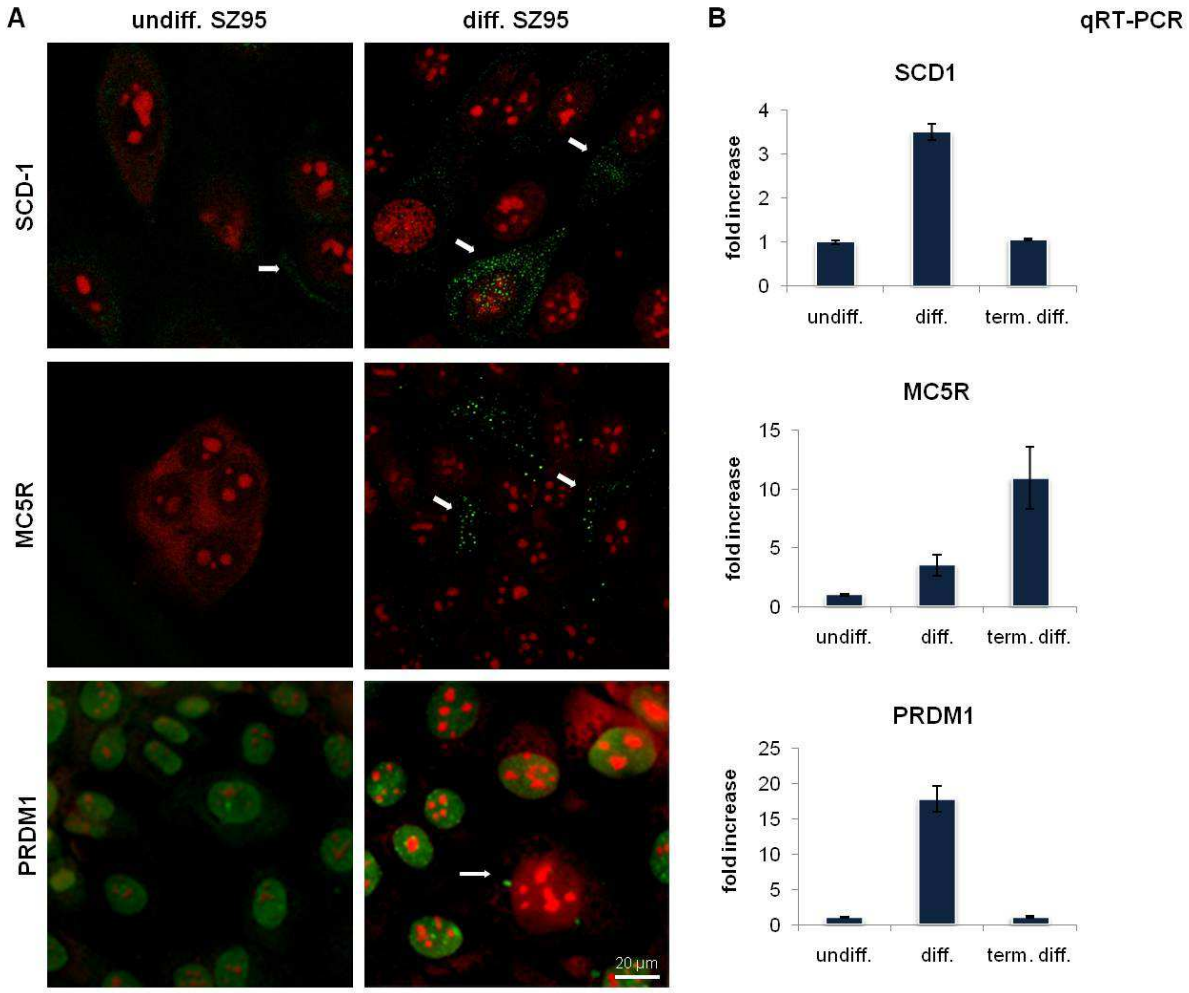


Figure 10: Characterisation of SZ95 cell differentiation on molecular level. (A) Immunostaining for SCD-1, MC5R and PRDM1 in SZ95 cells. White arrows mark SCD-1 and MC5R positive cells, or individual large cells lacking BLIMP1 staining. (B) Determination of expression levels of indicated markers in undifferentiated, differentiating and terminally differentiated cultures by quantitative real-time PCR. Results are presented as mean values ± SD of triplicates from an experiment that was repeated at least three times

MC5R has been previously identified in human and rodent sebaceous glands and proposed to be essential for differentiation (see chapter 1.7.3.). Böhm and colleagues could not detect *MC5R* transcripts in SZ95 cells by *in situ* hybridisation [337]. In accordance with Böhm's findings, we could confirm the lack of MC5R mRNA and protein in undifferentiated cells by quantitative real-time PCR and immunostaining, respectively, where no or very weak expression was found (Fig. 10 A+B, second row). Interestingly, in accordance with observation by Zhang and colleagues, *MC5R* transcript and protein levels were increased in differentiating SZ95 cells by 3.5-fold (Fig. 10 A+B, second row). Moreover, terminally differentiated SZ95 cells displayed an up-regulation of MC5R transcript levels by even 11-

fold suggesting MC5R to be expressed only at late differentiation state in human sebocytes (Fig. 10 B, second graph).

As mentioned before (see chapter 1.7.1.), Blimp1 was identified as marker for sebocyte differentiation. Consistent with these observations, expression of *PRDM1* was significantly increased by 18-fold in differentiating SZ95 populations, but not in cells induced to terminally differentiate when compared to undifferentiated sebocytes (Fig. 10 B, lower graph). Interestingly, immunostaining also confirmed increased protein levels in differentiating sebocytes in comparison with undifferentiated SZ95 cells (Fig. 10 A, lower pictures). But prominent large-sized cells found in differentiating cultures completely lacked PRDM1 protein staining suggesting that these cells were mature sebocytes, and that PRDM1 is only expressed in the early phase of the differentiation process (Fig. 10 A, lower pictures).

Next, we analysed the curse of expression levels of SCD1 (see chapter 1.7.3.) in SZ95 cells. In differentiating SZ95 cells, *SCD1* expression was found up-regulated 3.5-fold compared to undifferentiated cells (Fig. 10 B, upper graph). However, after arachidonic acid treatment, its expression was similar to the level of undifferentiated cells, probably due to end-product repression [309] (Fig. 10 B, upper graph). Additional immunostaining for SCD1 protein revealed an analogical increase in protein levels ranging from undifferentiated to differentiating stage (Fig. 10 A, upper pictures). Thus, *SCD1* expression occurs at early stage of differentiation in highly confluent SZ95 populations, but vanishes in terminally differentiated cells, at least by repression through arachidonic acid.

We also selected K15 as potential marker for sebocyte progenitors (see chapter 1.7.1.). In order to assess its expression profile in human sebocytes, quantitative real-time PCR analyses were performed to determine its expression in undifferentiated, differentiating and terminally differentiated SZ95 cells.

Surprisingly, *K15* transcript levels were increased by 5.4-fold in differentiating SZ95 populations, whereas expression in undifferentiated and terminally differentiated cultures did not differ significantly (Fig. 11).

Adipophilin (ADFP), or adipose differentiation-related protein (formerly ADRP), has been initially described as a protein of lipid droplets (LDs) and marker of lipid accumulation in adipocytes [338]. So far, this protein is required for correct lipid transport, LD formation and turnover in the cell [339-341]. Because adipocytes share several features with sebocytes, this protein was taken in consideration as differentiation marker for the SZ95 cells.

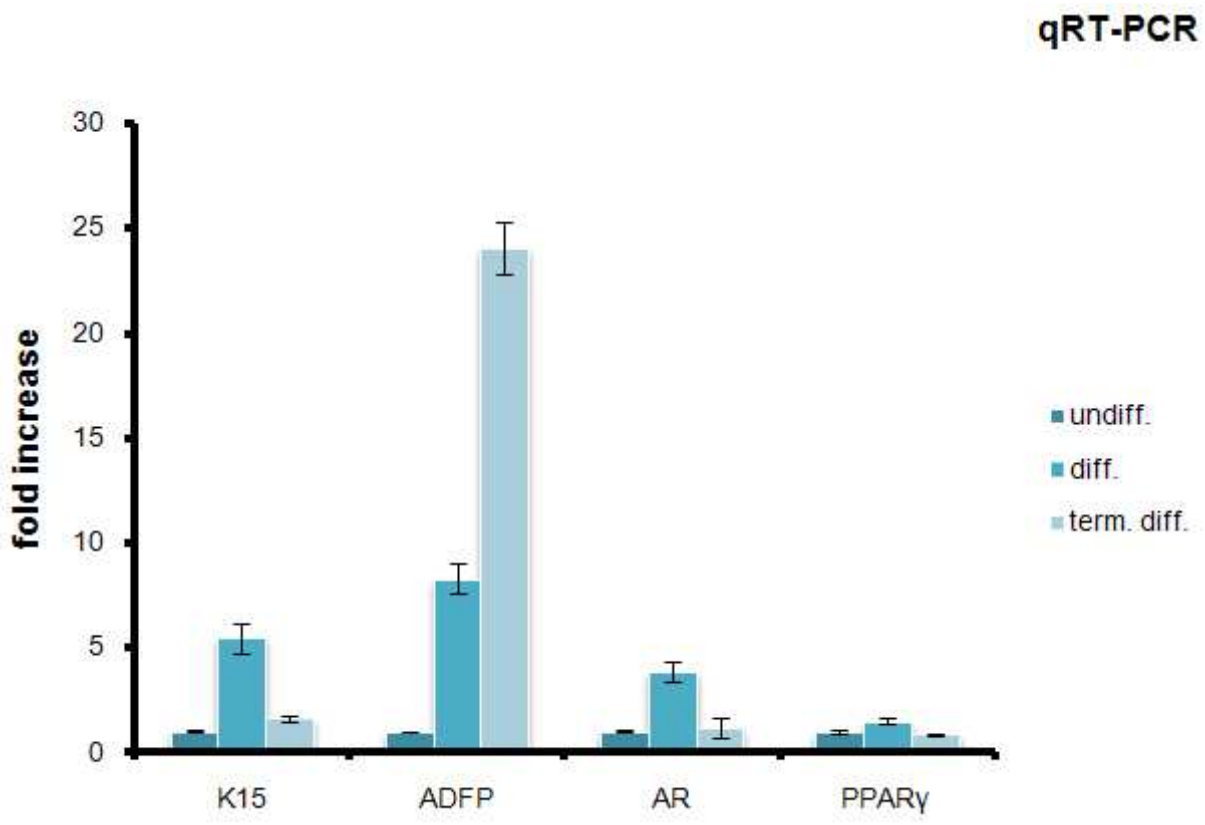


Figure 11: Expression pattern of differentiation markers in SZ95 cells. Expression of the progenitor marker Keratin 15 and differentiation markers Adipophilin, Androgen receptor and PPARγ were determined by quantitative real-time PCR in undifferentiated, differentiating and terminally differentiated cultures. Results are presented as mean values ± SD of triplicates from an experiment that was repeated at least three times.

In fact, expression of *ADFP* correlated positively with number of lipid droplets seen in mature sebocytes. More precisely, *ADFP* expression was up-regulated by 8.3-fold in differentiating SZ95 populations compared to undifferentiated cells (Fig. 11). Furthermore, *ADFP* transcript levels were even increased by 24-fold in terminally differentiated SZ95 cells. Thus, analogous to oil red o staining (Fig. 9), strong *ADFP* expression reflected the enhanced differentiation status of differentiating and terminally differentiated SZ95 cells on molecular level.

Additionally, we analysed AR expression as regulator of sebocyte proliferation and lipid production (see chapter 1.7.3.) at different stages of sebaceous differentiation. In differentiating SZ95 cells, *AR* expression was increased by 3.8-fold compared to undifferentiated cells, and thus confirmed increased differentiation in this population (Fig. 11). Interestingly, no increase of *AR* transcript levels could be detected in terminally differentiated

SZ95 suggesting that AR is not required for terminal differentiation after induction by arachidonic acid which could be a down-stream event of AR activity (Fig. 11).

As previously described (see chapter 1.7.3), PPARγ has been shown to be required for the differentiation process in rodent sebocytes [297-298].

Assuming that this could also be applied for the human system, the expression of *PPARγ* was examined in the three different populations of SZ95 cells. Interestingly, PPARγ expression did not differ significantly in undifferentiated, differentiating and terminally differentiated sebocytes (Fig. 11). It was found to be up-regulated by only 1.5-fold in confluent cultures. Thus, determination of PPARγ expression was not conclusive for the characterisation of the three differentiation populations of SZ95 cells.

To sum up, increased expression of the sebaceous progenitor markers PRDM1 and Keratin 15 in addition to that of differentiation markers was observed in differentiating SZ95 cultures, suggesting a heterogeneous character for this population. In contrast to this, the predominant increase in expression of the terminal differentiation marker MC5R and the lipid droplet factor ADFP was found in terminally differentiated cells and approved their terminally differentiated state. As expected, none of the presented sebaceous differentiation markers was up-regulated in undifferentiated sebocytes.

Taking advantage of these findings, a characteristic marker expression profile for each of the three specified SZ95 populations could be created (Fig. 12). Therefore, all collected SZ95 cell samples of this study could be precisely assigned to one of the three differentiation states depending on their matching of molecular marker expression patterns determined by quantitative real-time PCR in addition to staining of lipid droplets.

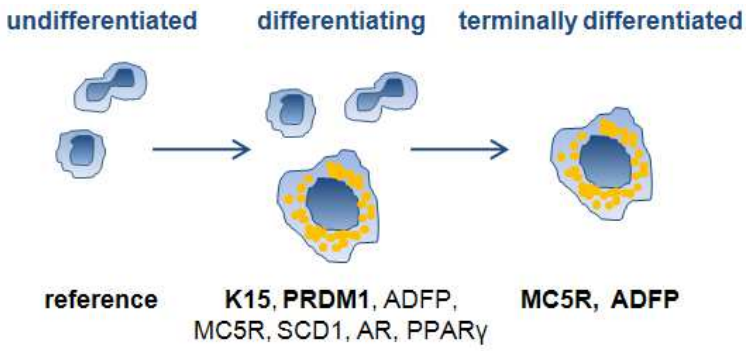


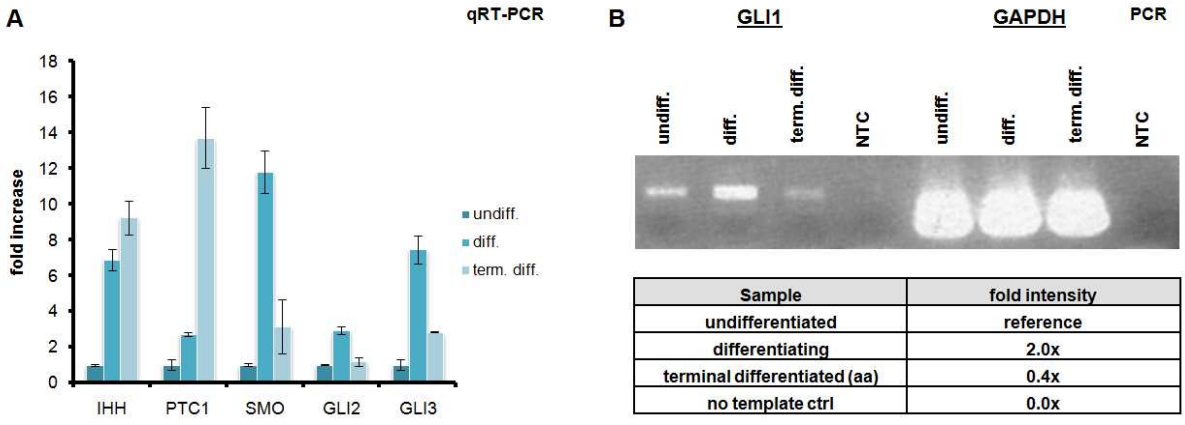
Figure 12: Schematic overview of expression patterns of molecular differentiation markers in SZ95 cells. Undifferentiated sebocytes served as reference and show lowest expression levels of all analyzed markers. Strongly up-regulated and differentiation-state specific markers in comparison to the other populations are highlighted by bold print.

3.2. Investigating the Hedgehog pathway in the human sebocyte cell line SZ95

First of all, it was essential to analyse the expression levels of Hedgehog pathway components and Hedgehog signalling activity at the different differentiation states of human sebocytes. This is prerequisite to identify key players of the Hedgehog pathway in human sebocytes and to get an indication of the underlying signalling mechanism with respect to regulation of proliferation and/or differentiation in these cells.

3.2.1. Hedgehog signalling components are up-regulated in differentiating SZ95 cells

To determine the endogenous expression levels and potential regulation of the hedgehog pathway components in the differentiation process, mRNA from SZ95 cells of each differentiation state was isolated, transcribed into cDNA, and analysed by quantitative real-time PCR. Undifferentiated cells served as reference sample and were used for comparison with the other differentiation stages. Quantitative real-time PCR was the method of choice to achieve the highest rate of target specificity and to directly quantify gene expression.



Sample	fold intensity
undifferentiated	reference
differentiating	2.0x
terminal differentiated (aa)	0.4x
no template ctrl	0.0x

Figure 13: Expression of components of the Hedgehog pathway in SZ95 cells at different differentiation stages. (A) Transcript levels of the key components of the hedgehog pathway, including Indian hedgehog (IHH), Patched 1 (PTC1), Smoothened (SMO), GLI2 and GLI3, were determined by quantitative real-time PCR. Results are presented as mean values ± SD of triplicates from an experiment that was repeated at least three times. (B) Endpoint PCR was applied to monitor expression of GLI1 (total PCR cycle #: 40) in sebocytes by subsequent determination of band intensities with ImageJ software. Note: GLI1 transcript levels were generally too low for detection by

quantitative real-time PCR in the reference sample resulting in bad signal-to-noise ratio. For that reason we had to apply endpoint PCR for GLI1 expression analysis.

Strikingly, all three sebocyte populations were found positive for expression of the ligand *IHH*, its receptor *PTC1*, *SMO*, and the downstream transcription factors *GLI1*, *GLI2* and *GLI3* (Fig. 13).

Interestingly, compared to undifferentiated cells, *IHH* was highly up-regulated by 7- and 9-fold in differentiating and terminally differentiated SZ95 cells, respectively (Fig. 13 A and 14, first row). This finding suggests that *IHH* expression is coupled to differentiation process or, alternatively, represents a direct target by lipid metabolism which has not been documented before. Additionally, increased expression of the Hedgehog ligand would indicate pathway activation in these populations.

On the other hand, expression of the receptor *PTC1* was up-regulated 3-fold in differentiating cells, and 14-fold in terminally differentiated sebocytes (Fig. 13 A). *PTC1* is a target gene of the hedgehog pathway, thus its expression could be partially induced by pathway activation due to increased levels of *IHH* in differentiated populations as stated before. The strong induction in terminally differentiated cells suggests an additional or alternative regulatory mechanism by the lipid metabolism, too.

Transcript levels of co-receptor *SMO* were also increased during differentiation. But in contrast to *PTC1*, *SMO* was more up-regulated, almost by 12-fold, in differentiating cultures and only by 3-fold in terminally differentiated cells compared to undifferentiated sebocytes (Fig. 13 A).

Additionally, mRNA levels of the major downstream transcriptional activators GLI1 and GLI2 were only found increased in differentiating populations by 2- and 3-fold, respectively, suggesting a role in pathway activation in these cells. On the contrary, GLI1 transcript levels even reflected a reduction in expression to 0.4-fold after treatment with arachidonic acid (Fig. 13 B), whereas GLI2 expression did not differ significantly compared with those levels seen in undifferentiated sebocytes (Fig. 13 A). Notably, the transcription factor GLI1 is also a direct target gene of the Hedgehog pathway, and represents a meaningful readout for Hedgehog signalling activity. Thereby, GLI1 reinforces activation of the Hedgehog pathway by a positive feedback loop.

Thus, these findings support the idea that differentiating SZ95 cells are the major state of Hedgehog signalling activity.

Figure 14: Expression analysis of Hedgehog pathway components by endpoint PCR which were not detectable in undifferentiated SZ95 cells. IHH expression levels were identical to those seen by quantitative real-time PCR (compare with Fig. 13), and thus confirmed correct amplification reaction in all samples. GAPDH served as loading control and cDNA from human fetal thymus (HFT) as positive control. SHH, Sonic Hedgehog; DHH, Desert Hedgehog; HHIP, Hedgehog interacting protein; PTC2, Patched 2.

Noticeably, both ligands, Sonic Hedgehog (SHH) and Desert Hedgehog (DHH), as well as the receptors Patched 2 (PTC2) and Hedgehog interacting protein (HHIP) could not be detected in undifferentiated SZ95 cells. Thus, we were not able to compare the expression levels of these genes in the different sebocyte populations. Therefore, cDNA from human fetal thymus cell was employed as positive control template, to validate our primers. Furthermore, expression of these genes was analysed in samples of differentiating and terminally differentiated SZ95 cultures by endpoint PCR analysis (Fig. 14). Analysis of *IHH* expression served as control for reproducibility of the obtained results by qRT-PCR technique (Fig. 13 A).

Strikingly, *DHH* transcripts could not be detected in any of the three differently differentiated SZ95 populations (Fig. 14), thus ruling out a role of this ligand in the regulation of proliferation and differentiation in human sebocytes under physiological conditions.

Conversely, weak expression of *SHH* could be only detected in sebocytes induced to terminally differentiate by arachidonic acid treatment (Fig. 14).

In addition, strong expression of *HHIP* and *PTC2* were found in cells induced to terminally differentiate (Fig. 14). So, not only the production of Hedgehog ligands seems to be increased in these cells, but also the expression of factors known to inhibit the pathway.

The data suggest an important role for the lipid metabolism in regulating the expression of Hedgehog components as demonstrated by the effects of arachidonic acid treatment in terminally differentiated cultures.

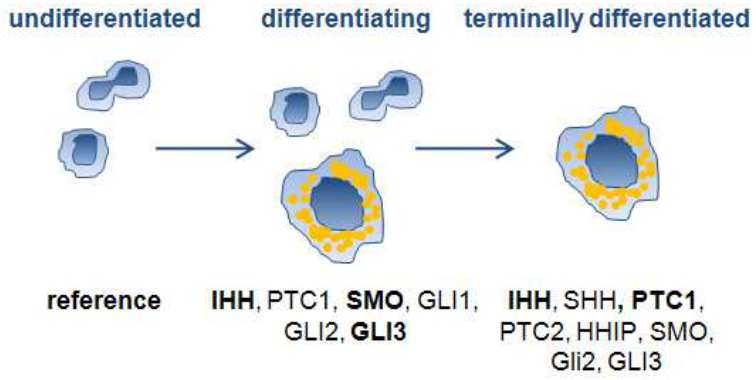


Figure 15: Schematic overview of expression patterns of Hedgehog pathway components in SZ95 cells. Undifferentiated sebocytes served as reference and show lowest expression levels of all analyzed components of the Hedgehog pathway. Strongly up-regulated components are highlighted.

Due to a lack of specific antibodies we were not allowed to study protein production of endogenous Hedgehog ligands and all three Gli transcription factors in the human (and also rodent) system at the time of this work. These proteins share high sequence homology which could not be distinguished by a range of commercial antibodies we tested. Hence, it was not possible for us to analyse these regulation processes on protein level in more detail.

3.2.2. Hedgehog signalling activity is increased in differentiating SZ95 cells

The findings suggested that Hedgehog pathway activation occurs during the differentiation process of sebocytes. To determine the activity of Hedgehog signalling, SZ95 cells were transfected with the Gli-specific luciferase reporter 8 x 3'Gli-BS Luc and the control plasmid 8 x m3'Gli-BS Luc at different differentiation stages. Additionally, pRLTK was introduced into the cells for transfection efficiency control. Importantly, 8 x 3'Gli-BS Luc is stimulated by all three Gli transcription factors, thus monitoring the combined effect of these factors in the different populations.

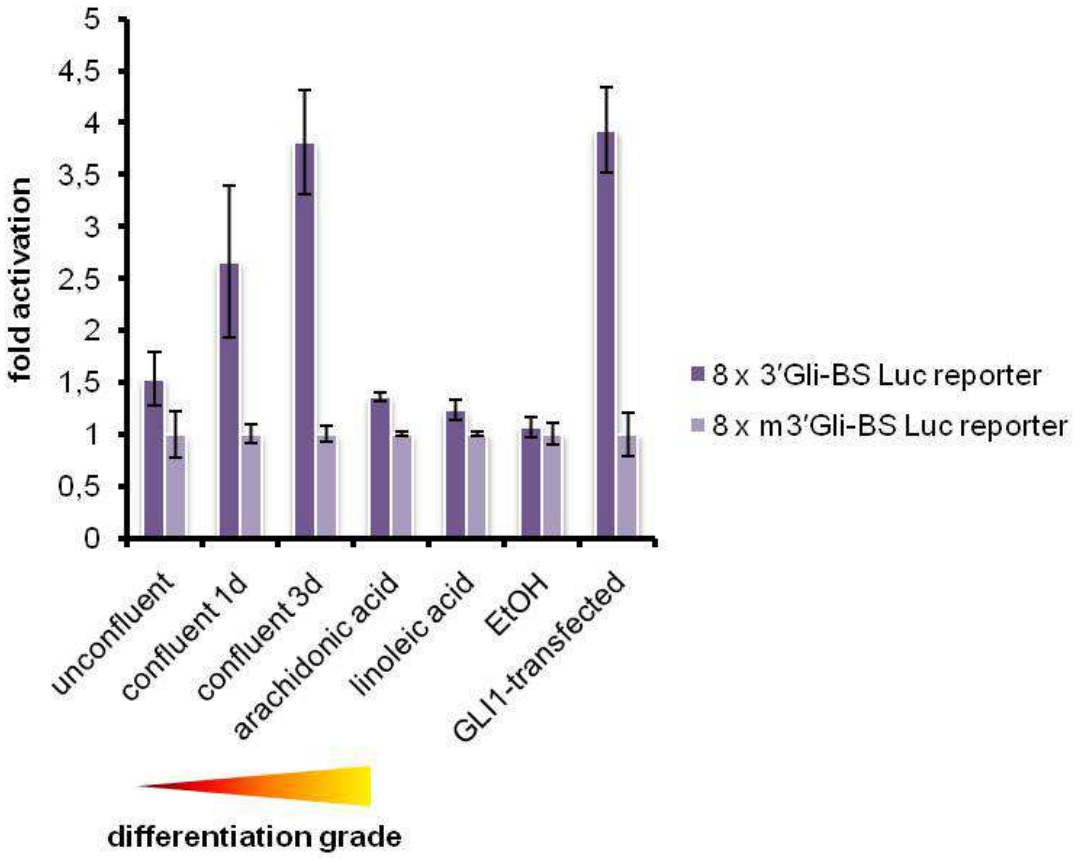


Figure 16: Hedgehog pathway activity in SZ95. Pathway activity was determined by Gli luciferase reporter assay in unconfluent cultures and those with increasing confluency (after 1 day and 3 days), as well as after treatment with fatty acids (arachidonic acid, linoleic acid and solvent control with EtOH). GLI1-transfected cells served as positive control. Results are presented as mean values ± SD of triplicates from an experiment that was repeated at least three times.

The activation levels of the functional Gli luciferase reporter 8 x 3'Gli-BS Luc were compared with those of 8 x m3'Gli-BS Luc. Interestingly, the hedgehog signalling activity increased with the differentiation progress in SZ95 cells (Fig. 16). While undifferentiated cells only showed an 1.5-fold activation, the differentiating cultures showed an activation by 2.7- and 3.8-fold after one day and three days confluent growth, respectively. The activity of the latter condition was even comparable to the 4-fold activation in cells transfected with the human full-length GLI1 expression construct serving as positive control. In contrast to this, terminally differentiated sebocytes exhibited no significant activation of the Hedgehog pathway.

These results confirm a model, where terminally differentiated cells are not susceptible for Hedgehog pathway activation, but, as observed in undifferentiated and differentiating populations, may play a role in transmitting the signal to their environment and in activating

the pathway in undifferentiated and/or lesser differentiated sebocytes by paracrine secretion. Thereby, the increased expression of pathway inhibitors seen in terminally differentiated cells may provide a negative feedback mechanism to prevent pathway activation in this cell type.

3.3. Effects of overexpressed human GLI constructs on proliferation and differentiation in SZ95 cells

To investigate the function of the transcription factors GLI1, GLI2 amd GLI3 in proliferation and differentiation of human sebocytes in more detail, GLI overexpression experiments were performed. Therefore, SZ95 cells were transfected with human HA-tagged GLI1, human Myc-tagged GLI2 or human GLI3-GFP-fusion expression constructs.

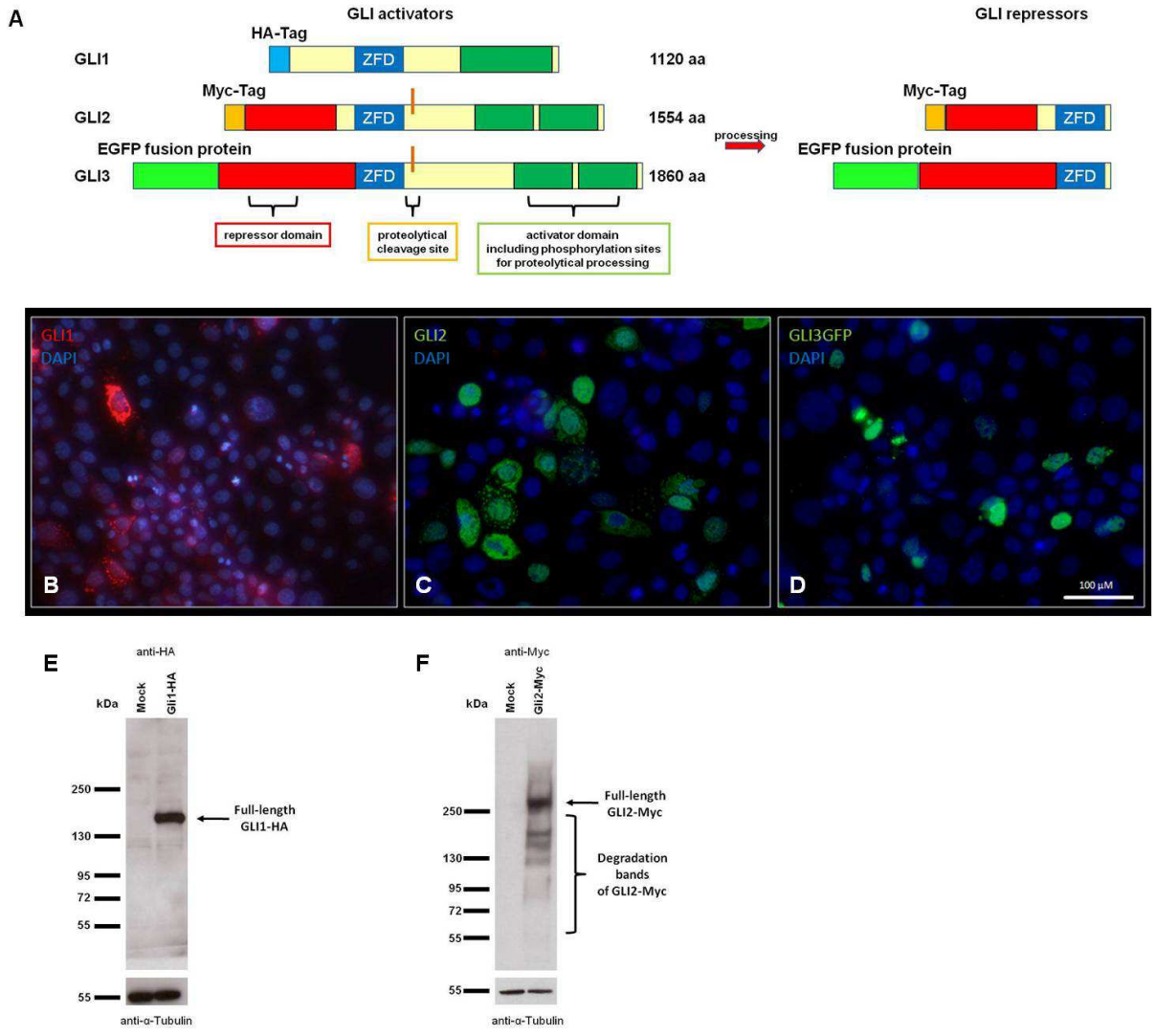


Figure 17: Transfection and detection of Gli expression constructs in SZ95 cells. (A) Schematic diagram of the three human GLI expression constructs. (B) Human Gli1 and (C) human Gli2 expression constructs were detected by immunofluorescence staining for associated tags HA- and Myc-tag, respectively. (D) GLI3 expression was monitored by attached GFP fusion protein. Detection of (E) Gli1-HA and (F) Gli2-MYC by immunoblot. α-Tubulin served as loading control. ZFD: zinc-finger domain.

Positive immunostaining for HA-tag of GLI1 and the MYC-tag of GLI2 expression constructs (Fig. 17 A + B) as well as detection of the GLI3 fusion protein (Fig. 17 A) confirmed their correct expression in SZ95 cells. Expression was also studied by Western blotting. Thereby, GLI2-MYC expression displayed multiple protein bands suggesting that different isoforms are produced and/or the protein is partly degraded in sebocytes besides the expected full-length protein of about 190 kda. In contrast, HA-tagged GLI1 protein displayed the expected single band of full-length protein at approx. 160 kDa. Unfortunately, antibodies directed against GFP for detection of GLI3 expression construct resulted in strong background signals preventing a clear blotting signal.

3.3.1. GLI1 and GLI2 expression induce Hedgehog pathway activation in human sebocytes

Next, the GLI transcription factors were analysed for their ability to stimulate the Hedgehog pathway in human sebocytes. Therefore, undifferentiated SZ95 cells were co-transfected with human GLI1, GLI2 and GLI3 expression constructs and the Gli luciferase reporters as well as pRLTK. First, SW480 was used as control cell line, because these cells were easily transfectable and shown to have an inactive Hedgehog pathway background similar to undifferentiated SZ95 cells [342].
GLI1 expression led to the strongest activation of the reporter construct by 29- and by 8-fold in SW480 (Fig. 18 A) and SZ95 cells (Fig. 18 B), respectively. Compared to this, GLI2 only increased reporter activity by 13- and 3-fold in both cell lines (Fig. 18 A & B), respectively.

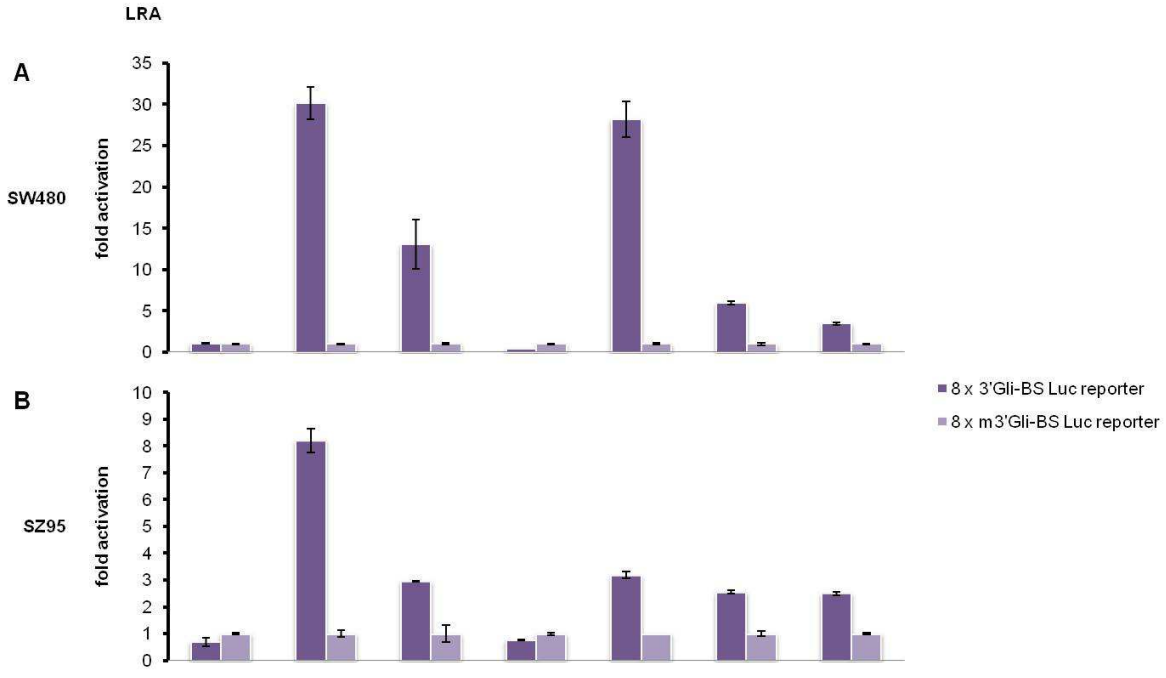


Figure 18: Gli luciferase reporter assay of the three GLI expression constructs in (A) SW480 control and (B) SZ95 cells. GLI1 showed the strongest activator capacity, followed by GLI2. GLI3 displayed strong inhibitory effects, as illustrated by combined transfection with GLI1 or GLI2. Results are presented as mean values ± SD of triplicates from an experiment that was repeated at least three times.

GLI3 overexpression alone displayed no activation at all by reaching only 0.8-fold activation of the Gli reporter construct. Instead, its presence repressed the reporter activity dramatically in SW480 cells to 5.9-fold and 3.5-fold when co-transfected with GLI1 or GLI2 (Fig. 18 A), respectively, thus demonstrating its inhibitory effect on the Hedgehog pathway. Interestingly, reporter activity was significantly decreased to 2.6-fold when GLI3 was co-expressed with GLI1 in human sebocytes compared to GLI1 alone (8-fold). But combined expression of GLI3 and GLI2 (2.5-fold) only resulted in a minor reduction compared to the pathway activation capacity of GLI2 alone (3-fold) (Fig. 18 B).

Furthermore, co-expression of GLI2 with GLI1 (3.2-fold) decreased the signal output of GLI1 alone (8-fold) to almost the same extent in SZ95 cells.

One possible explanation may be that the specific setup of pathway activating molecules, which prevent the degradation and support the maintenance of GLI full-length activators, limits pathway activation capacity in undifferentiated sebocytes. Thereby, remaining full-length GLI molecules are constantly processed to GLI repressors or proteasomally

degraded. Hence, GLI2 molecules may also be partially processed to the repressor form and not only act as activators. As consequence, GLI2 repressor variants could compete with the strong activator GLI1 like GLI3. On the other hand, GLI2 activator variants could interfere with the repressor GLI3 by binding to the same reporter constructs.

3.3.2. GLI1 and GLI2 differentially induce expression of key Hedgehog pathway components in human sebocytes

To further dissect the specific function of the different GLI transcription factors in human sebocytes, the expression levels of key Hedgehog pathway components and target genes were analysed in GLI overexpressing cells. Therefore, mRNA of transfected SZ95 cells at a subconfluent stage was isolated and used for quantitative real-time PCR.

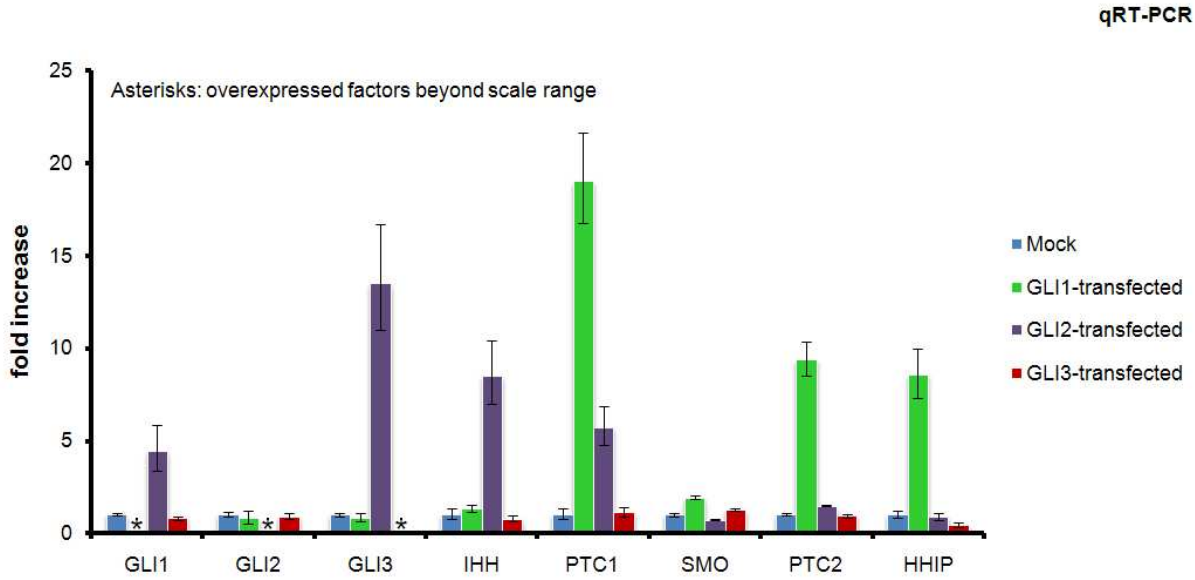


Figure 19: Expression analysis of Hedgehog pathway components after overexpressing the transcription factors GLI1, GLI2 or GLI3 as determined by qRT-PCR. Due to common human origin it was not possible to distinguish between endogenous GLI and GLI construct expression. Therefore, the corresponding values were represented as asterisks. Results are presented as mean values ± SD of triplicates from an experiment that was repeated at least three times.

Additionally, expression of the receptors and target genes *PTC1, PTC2* and *HHIP* were significantly up-regulated by 19-, 9.4- and by 8.5-fold, respectively (Fig. 19, green bars) compared to its expression level in mock transfected cells (Fig. 19, blue bars). Furthermore, GLI1 induced a slight increase of co-receptor *SMO* expression level by 1.9-fold. These

findings imply that GLI1 expression results in both, a major recruitment of the negative pathway regulators PTC1, PTC2 and HHIP as well as in increased expression of the pathway activator SMO. Thereby, the cell could be desensitized or sensitized to Hedgehog signals depending on cellular PTC to SMO ratio.

Similarly, GLI2 overexpression resulted in a significant up-regulation of both target genes *PTC1* and *GLI1* by 5.7- and 4.4-fold, respectively (Fig. 19, purple bars). However, to our surprise GLI2 significantly increased expression of the transcriptional repressor GLI3 by even 13.5-fold. The regulation of *GLI3* expression by GLI2 has not been documented before. Moreover, GLI2 induced expression of the ligand *IHH* by 8.5-fold, the main pathway inductor in human sebocytes. Thus, on the one hand, GLI2 induces the repression of Hedgehog target genes by up-regulating *GLI3* expression. On the other hand, GLI2 represents an important Hedgehog pathway activator by increasing IHH ligand production in human sebocytes.

In contrast, overexpression of GLI3 did not lead to an increase in any component of the Hedgehog pathway which would fit to its role as transcriptional repressor (Fig. 19, red bars). Instead, expression levels were comparable to those detected in the mock-transfected control cells (Fig. 19, blue bars) with exception of *HHIP*. *HHIP* expression was decreased to 0.4-fold of the control level upon GLI3 overexpression. Hence, GLI3 has no or only repressive effect on the transcription of Hedgehog components.

In conclusion, the three GLI transcription factors showed differential effects on expression levels of Hedgehog components (Fig. 20). Interestingly, *GLI3* and *IHH* have not been described as Hedgehog pathway target genes before. The question arises whether they are regulated directly by the GLI transcription factors or indirectly be secondary effects. Promoter studies of these genes will be necessary to answer this question.

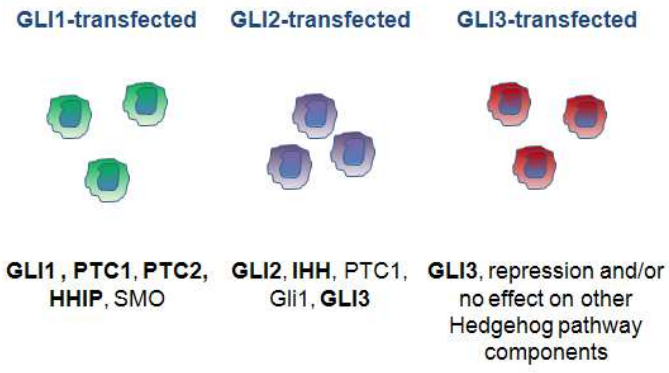


Figure 20: Schematic overview of up-regulated Hedgehog components upon GLI1, GLI2 and GLI3 overexpression in SZ95 cells as determined by quantitative real-time PCR. Strongly up-regulated components are highlighted by bold print.

3.3.3. GLI1 and GLI2 expression promotes sebocyte proliferation

To investigate the function of the individual GLI transcription factors in human sebocytes, we first analyse the impact of GLI1, GLI2 and GLI3 overexpression on cell proliferation. Therefore, undifferentiated SZ95 cells were transfected either with mock, GLI1, GLI2 or GLI3 expression constructs and used for BrdU incorporation assay.

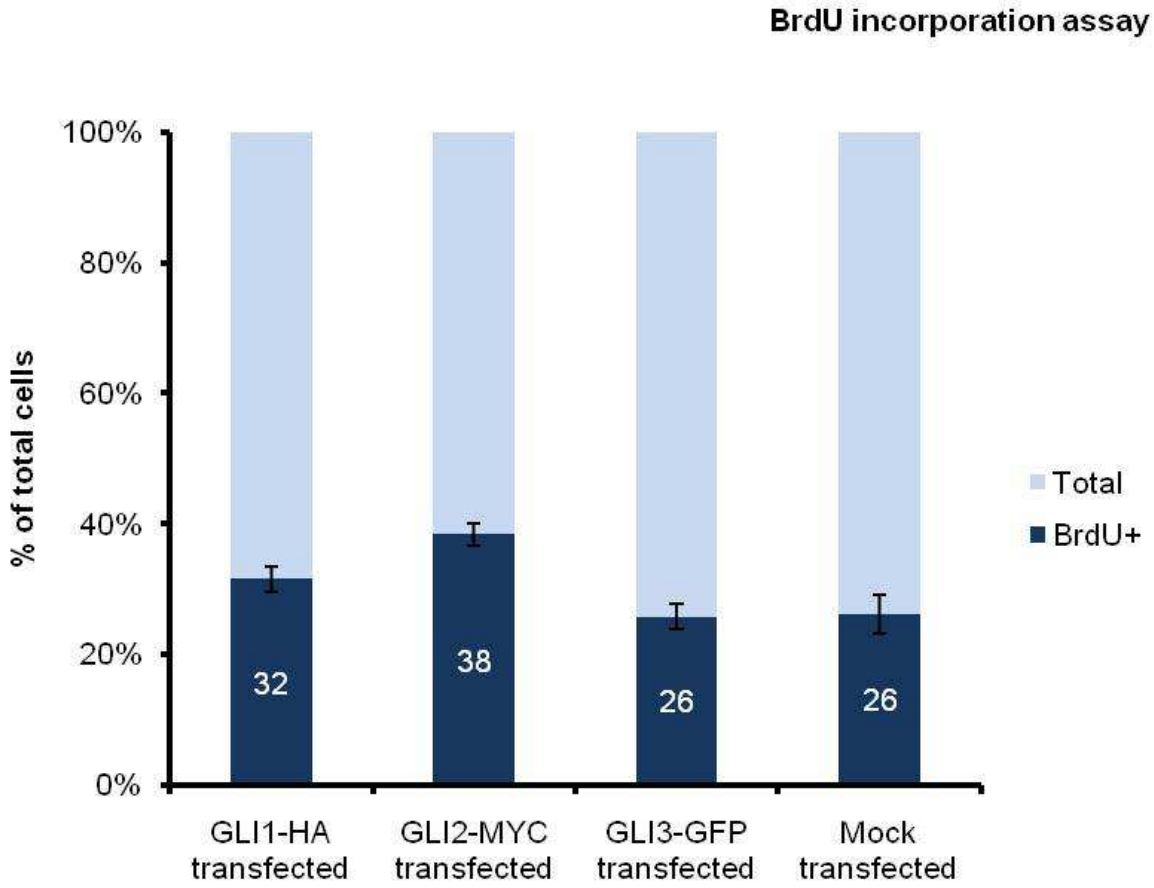


Figure 21: Proliferation assay in SZ95 cells transfected with either GLI1, GLI2 or GLI3 expression constructs. Mock transfected cells served as reference population. BrdU positive cells (BrdU+) are indicated as percentage of the total population. Results are presented as mean values ± SD of triplicates from an experiment that was repeated at least three times.

Strikingly, GLI2 overexpression led to an increase in the proliferation rate by 46.2% (+12% BrdU+ cells) in SZ95 cells in comparison to mock transfected cells as indicated by BrdU incorporation (Fig. 21). Similarly, GLI1-transfected sebocytes displayed an increase of 23.1% (+6% BrdU+ cells) of proliferative cells (Fig. 21). On the contrary, overexpression of GLI3 showed no effect on the proliferation behaviour of SZ95 cells (Fig. 21).

These results suggest that GLI1 and, in particular, GLI2 are both mediators of sebocyte proliferation, whereas GLI3 is not.

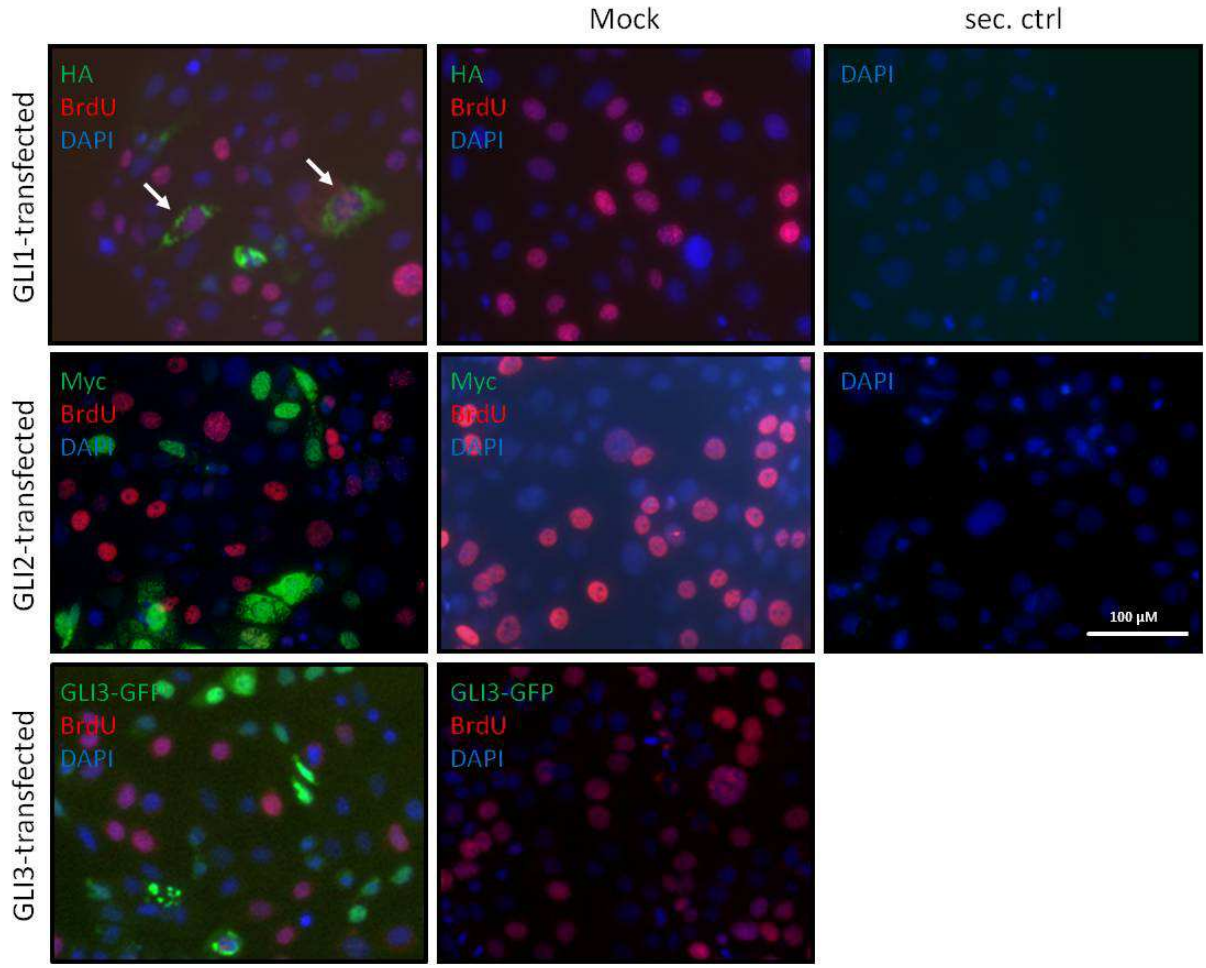


Figure 22: Localisation of BrdU incorporation and GLI expression constructs in SZ95 cells. Cells were stained by using antibodies directed against BrdU in combination with either HA (GLI1) or Myc (GLI2). Accordingly, GLI3 expression was detected by the associated GFP-fusion protein. Mock transfected cells served as negative control. Sebocytes treated with the secondary antibody alone (sec. ctrl) served as control of the specificity of primary antibodies. White arrows mark double stained cells.

Next, we investigated whether GLI expression has a cell-autonomous effect on proliferation or not. Therefore, double staining of transfected SZ95 cells for BrdU and either for GLI1 or GLI2 expression constructs was performed and analysed for co-localisation. Accordingly, staining of BrdU incorporation and expression of GLI3 by the associated GFP fusion protein were analysed in the same manner.

Thereby, GLI1 expression was found in proliferating cells, as depicted by co-localisation with BrdU (Fig. 22, first row, white arrows), suggesting that GLI1 is promoting proliferation in sebocytes.
Strikingly, GLI2 expression did not co-localise with BrdU incorporation (Fig. 22, second row). This raises the question, whether GLI2 increases proliferation rate in sebocytes by a paracrine mechanism, for example by induction of the Indian Hedgehog ligand.
As expected, expression of GLI3 did not co-localise with BrdU positive cells (Fig. 22, lower row). This is in line with the previous finding that GLI3 had no effect on sebocyte proliferation rate (Fig. 21).

3.3.4. GLI2 and GLI3 expression up-regulate the keratinocyte proliferation and sebaceous duct fate marker Keratin 6 and terminal sebocyte differentiation marker MC5R in SZ95 cells

To address the question whether GLI transcription factors also regulate differentiation in human sebocytes, undifferentiated SZ95 cells were transfected either with GLI1, GLI2 or GLI3 expression constructs and analysed for expression of differentiation marker molecules. Therefore, mRNA was isolated and used in quantitative real-time PCR to determine the transcription levels of the selected molecular markers including those initially presented in chapter 3.1.2. which are MC5R, PRDM1, SCD1, K15, ADFP, AR and PPARγ. Furthermore, we chose Keratin 6 a (KRT6a) which has been characterised as bulge marker [343] and is found in hyperproliferative keratinocytes [344]. Interestingly, KRT6a has also been described to be marker of duct fate in sebaceous glands and to be regulated by GLI2 [253].
Strikingly, GLI3 led to a 5.7-fold increase in transcript levels of the terminal differentiation marker MC5R (Fig. 23, red bars). This result suggests that MC5R is up-regulated by the inhibition of a target gene and/or pathway by GLI3 repressors. Moreover, assuming that increased MC5R expression reflects increased differentiation level of sebocytes as previously observed (Fig. 10), GLI3 displays characteristics of a sebaceous differentiation inducing factor.

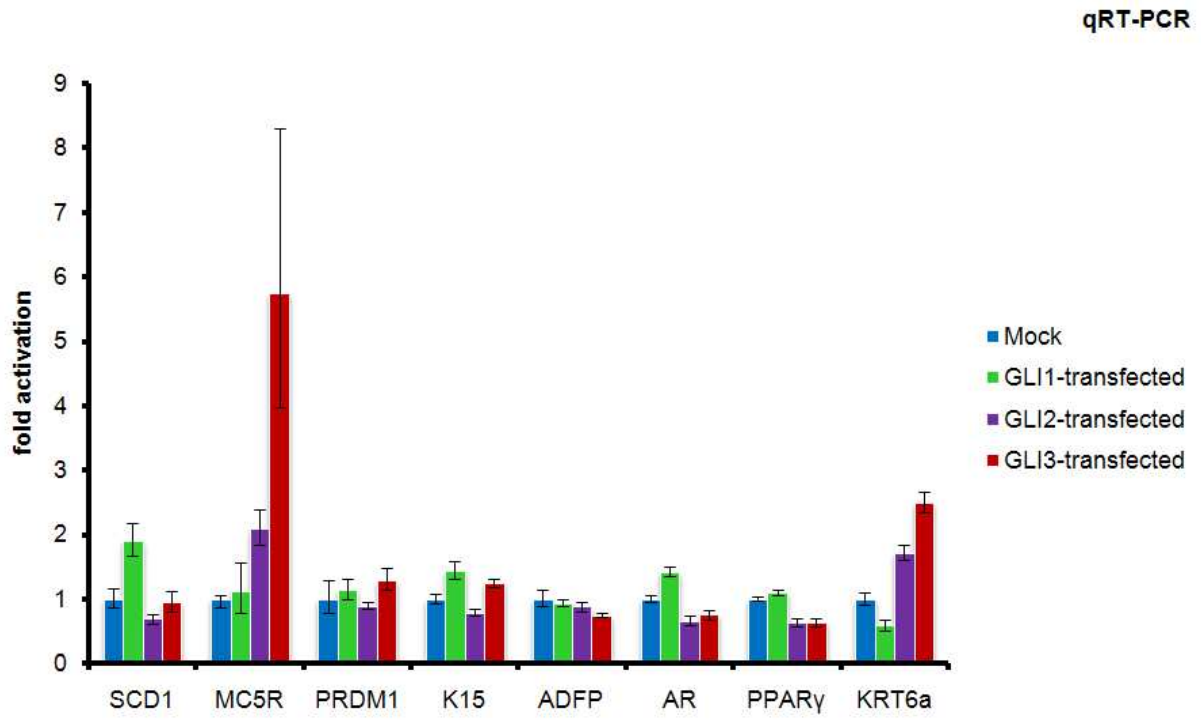


Figure 23: Determination of expression levels of molecular differentiation markers in GLI1-, GLI2-, GLI3- and mock transfected SZ95 cells by qRT-PCR. Results are presented as mean values ± SD of triplicates from an experiment that was repeated at least three times.

In this context, GLI2 was also found to up-regulate MC5R expression by 2.1-fold (Fig. 23, purple bars). Thus, GLI2 represents also a potential regulator of MC5R expression and/or sebocyte differentiation. The question arises whether this regulation process occurs directly by GLI2 or indirectly by induction of GLI3 expression as seen in a previous experiment after overexpression of GLI2 in SZ95 cells (Fig. 19, purple bars).

Similarly, GLI3 and GLI2 increased transcript levels of the sebaceous gland duct marker KRT6a by 2.5- and 1.7-fold, respectively (Fig. 23 red and purple bars).

GLI1 expression resulted only in a modest increase of the early differentiation marker SCD1 by 1.9-fold in SZ95 cells (Fig. 23, green bars). This finding proposes that GLI1 plays a role in the early period of the maturation process of human sebocytes by inducing early lipogenic pathways.

For the rest, transcription of PRDM1, K15, ADFP, AR and PPARγ was not regulated by GLI1, GLI2 or GLI3 expression (Fig. 23).

Thus, a model emerges, where Hedgehog pathway activation stimulates sebocyte proliferation, whereas pathway inhibition promotes sebocyte differentiation (Fig. 24). On the other hand, ADFP expression and lipid droplet formation were not increased upon GLI

overexpression. This indicates that Hedgehog pathway function may be downstream of the lipid metabolism.

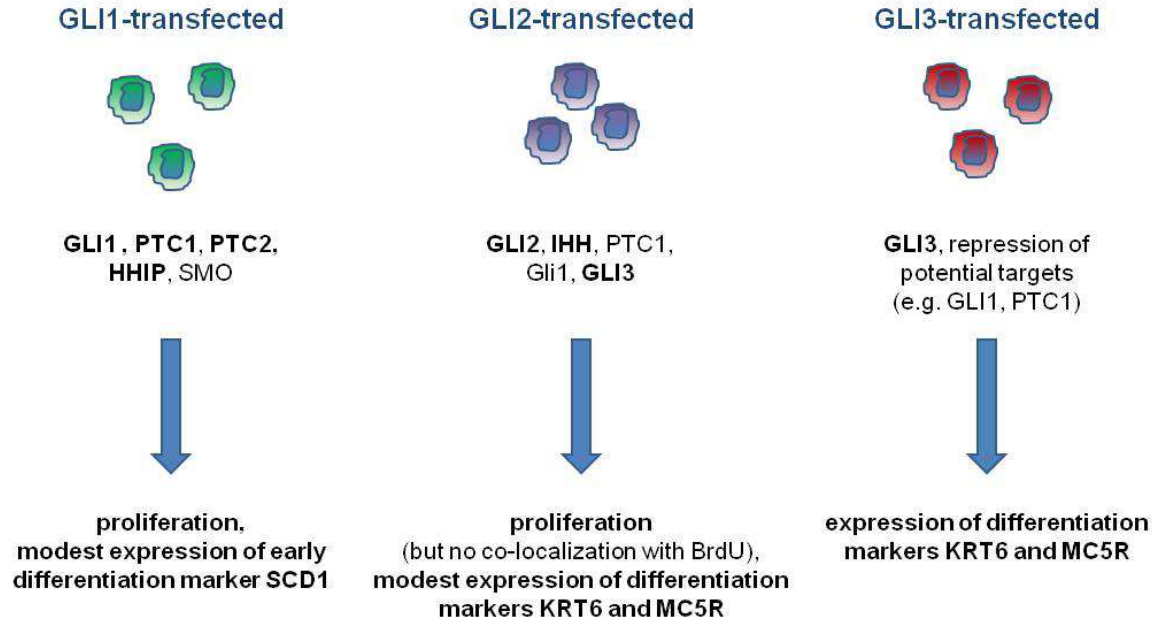


Figure 24: Schematic summary of GLI transcription factor effects in SZ95 cells. Strongly up-regulated components and processes are highlighted by bold print.

3.4. Knockdown of GLI transcription factors in SZ95 cells

As *GLI2* and *GLI3* were shown to be predominantly expressed compared to *GLI1* in the SZ95 cells (Fig. 13), we were interested to investigate the effects of knocking down these two transcription factors in human seboyctes. Therefore, SZ95 cells were either transfected with siRNA oligos or lentivirally infected with shRNA transducing particles directed against human GLI2 and human GLI3 mRNA. Thereby, cells were taken at differentiating stage, because increased expression levels of *GLI2* and *GLI3* were found under this condition (Fig. 13).

3.4.1. Knockdown of GLI2 and GLI3 by siRNA oligos in SZ95 cells

In a first set of experiments, specificity and efficiency of a range of commercially available siRNA oligos with different target sequences directed against human *GLI2* and human *GLI3* were tested in the control cell line SW480. These cells were shown to express these transcription factors endogenously [342]. The aim of these initial studies was to select for the siRNA oligos with the best target specificity and knockdown effect for the subsequent experiments in human sebocytes.

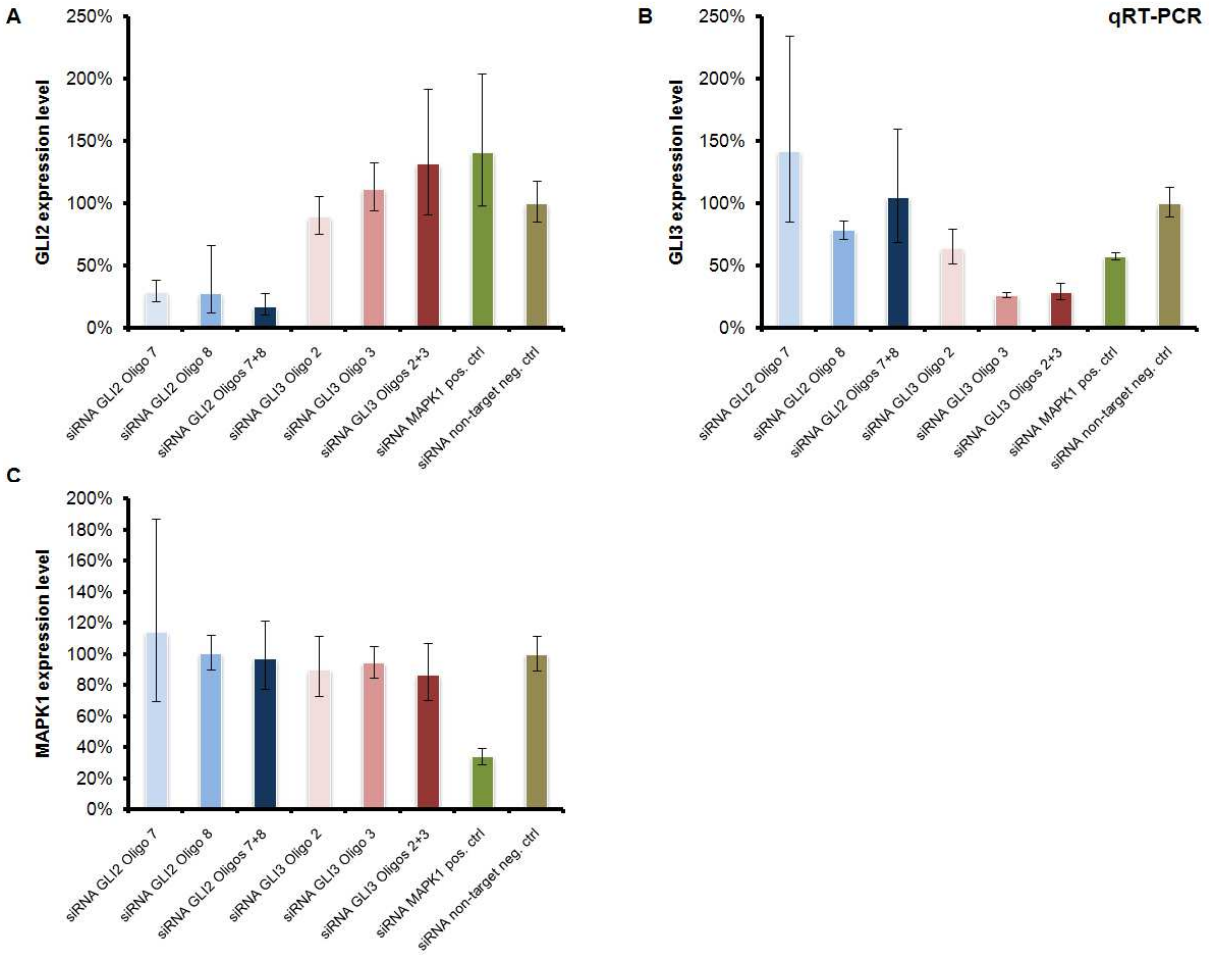


Figure 25: GLI2 and GLI3 knockdown assay by siRNA oligos in SW480 control cell line. qRT-PCR analysis of GLI2 (A) and GLI3 (B) knockdown after transfection of selected siRNA oligos into SW480 cells. (C) MAPK1 siRNA oligos were provided as validated positive control by the manufacturer. SW480 cells treated with non target siRNA oligos served as reference. Results are presented as mean values ± SD of triplicates from an experiment that was repeated at least three times.

To demonstrate that the siRNA knockdown assay works in our hands, we transfected SW480 cells with control siRNA oligo directed against human *MAPK1.* Importantly, this siRNA oligo had been shown to efficiently block expression of MAPK1. As expected treatment with anti-*MAPK1* siRNA oligo significantly decreased *MAPK1* transcript levels, thereby achieving 66% repression. SW480 cells transfected with siRNA directed against human *GLI2* showed a significant decrease in *GLI2* expression of 71% and 72% by employing two distinct target sequences, respectively (Fig. 25, A, oligo 7 and oligo 8). As expected, the combined treatment with both oligos enhanced the knockdown effect, and *GLI2* expression was down-regulated by 83% in comparison to the non-target siRNA control. SiRNA oligos targeting human *GLI3* (Qiagen, Hilden) decreased the GLI3 transcript levels by 36% and by 74%, respectively (oligo 2 and oligo 3) (Fig. 25, B). These results also demonstrated that siRNA oligo 2 was less efficient than siRNA oligo 3. The combined treatment of SW480 cells by these oligos did not lead to an enhanced knockdown effect.

To further check the specificity of the target sequences, expression levels of GLI2 and GLI3 were analyzed in parallel in all assays. Thereby, no unspecific inhibitory effect could be found (Fig. 25, A-C). All in all, the siRNA oligos were specific for their target sequences, and therefore were used to inhibit *GLI2* and *GLI3* expression in human sebocytes.

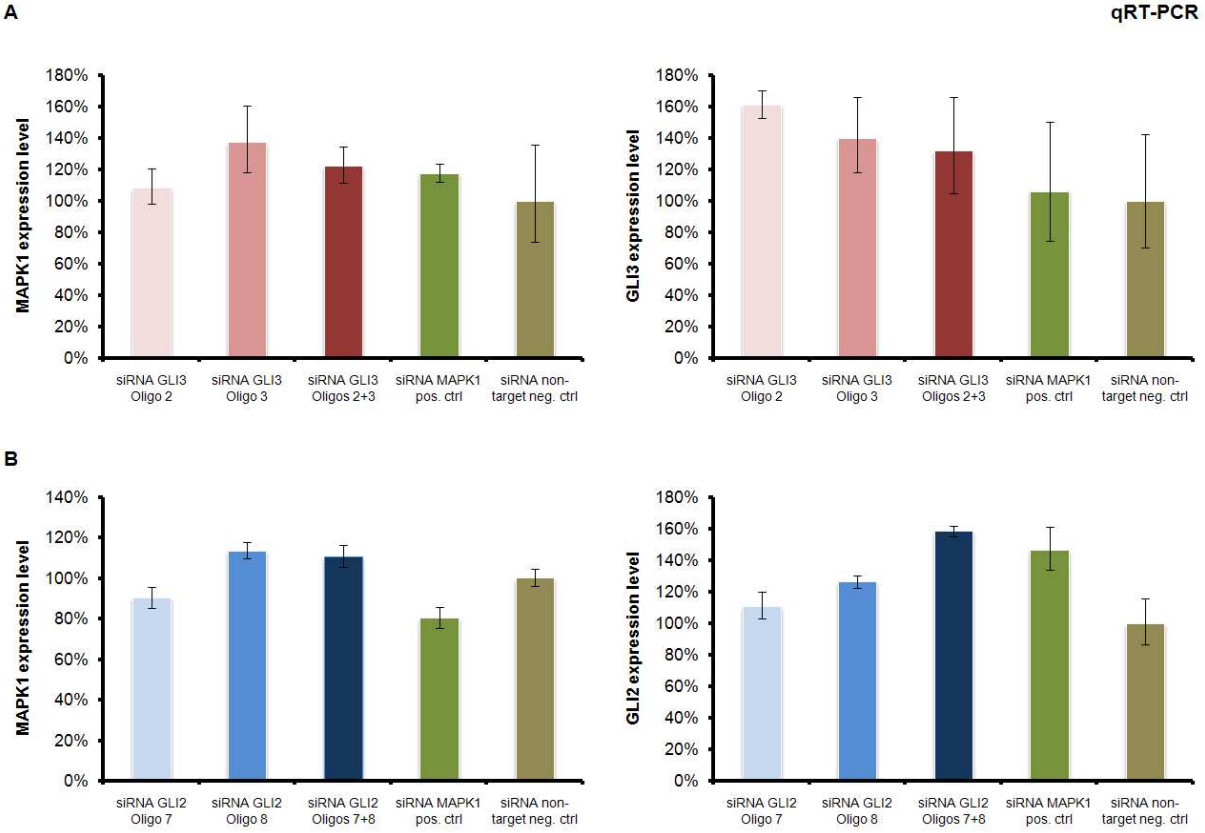


Figure 26: GLI2 and GLI3 knockdown assay by siRNA oligos in SZ95 cells. qRT-PCR analysis of GLI2 and GLI3 knockdown after transfection of selected siRNA oligos by lipofection into SZ95 cells.

MAPK1 siRNA oligos were provided as validated positive control by the manufacturer. Sebocytes treated with non target siRNA oligos served as reference. Results are presented as mean values ± SD of triplicates from an experiment that was repeated at least three times.

Neither siRNA oligos directed against *GLI2* and *GLI3* nor the validated MAPK1 siRNA showed any significant knockdown effect in SZ95 cells (Fig. 26, A and B). Different transfection protocols (lipofection vs. nucleofection) and various transfection conditions were applied without successful knockdown of the target genes. Reasons for these observations may be that sebocytes are generally difficult to transfect because of their high lipid content, and/or this cell line is not as much responsive to siRNA as compared to other cell types.

3.4.2. Knockdown of GLI2 and GLI3 by shRNA transducing lentiviral particles in SZ95 cells

Next we used a different method to knockdown endogenous GLI2 and GLI3 expression by lentiviral particles (Sigma-Aldrich, Hamburg) which transduce shRNA expression against these transcription factors in human sebocytes. Lentiviral shRNA have the advantage to attain efficient transfection rates and to generate stable knockdown cell lines for long-term gene silencing studies.

First, GFPTurbo transducing particles with a range of MOI (multiplicity of infection) from 0.5 to 5.0 were applied to infect SZ95 cells and to test the transduction efficiency. 48 hours later, cells were incubated in selection medium with addition of puromycin for up to 10 days. During this time frame, stably infected clones were selected showing increasing expression of the GFPTurbo control construct. In particular, a MOI of 5.0 turned out to be the best concentration for infecting SZ95 cells with an efficiency of about 80% as observed 10 days after infection (Fig. 27). Therefore, a MOI of 5.0 was used for GLI2 and GLI3 shRNA transducing particles in following experiments.

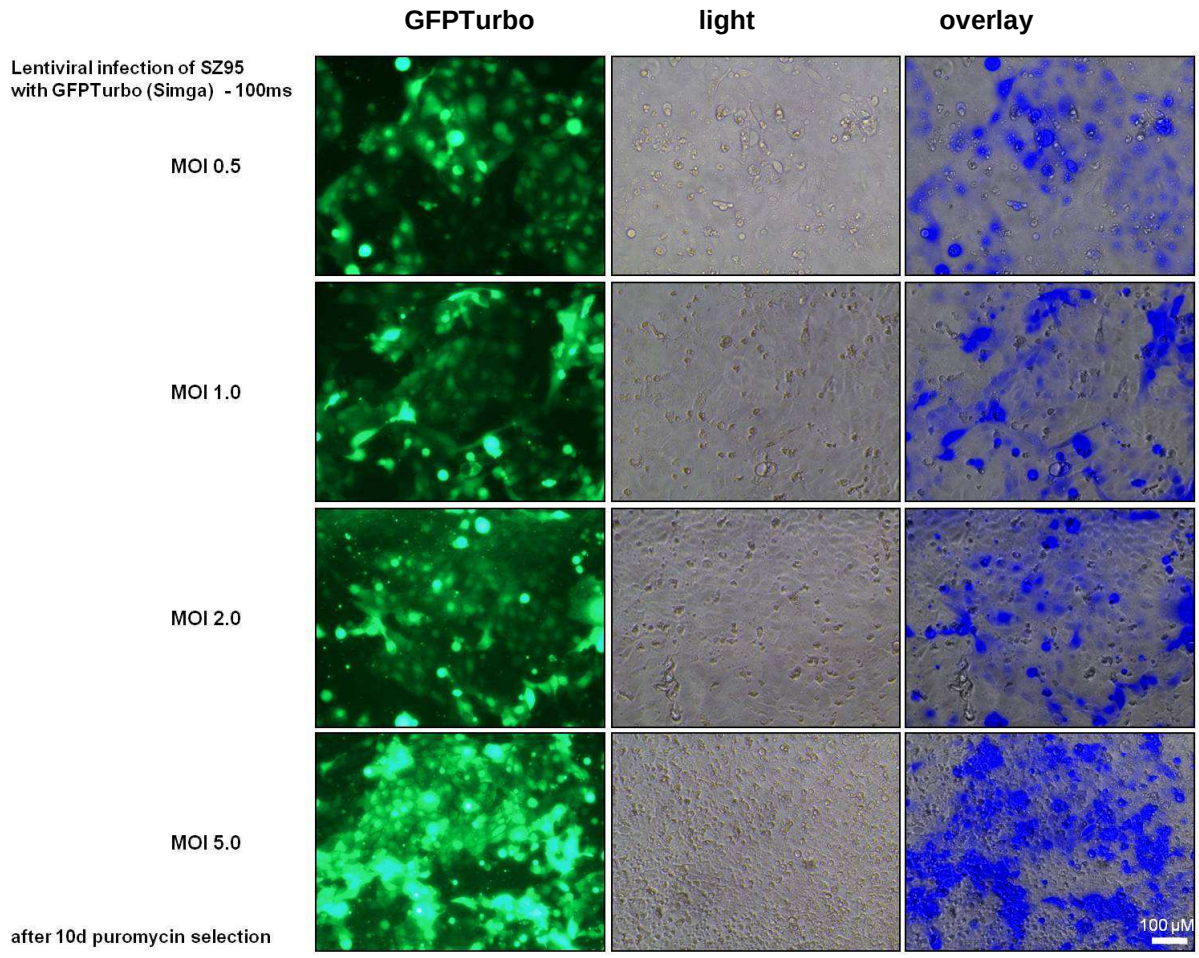


Figure 27: Optimisation of infection rate in SZ95 cells by using GFPTurbo transducing lentiviral particles. MOI of 0.5, 1.0, 2.0 and 5.0 were compared for infection efficiencies. 48 hours after infection cells were treated with puromycin to select infected cells during 10 days.

To knockdown GLI2 expression in human sebocytes we purchased and tested five different sets of shRNA transducing particles each directed against a distinct target sequence of human GLI2 (Fig. 28, target 1-5). SZ95 cells were infected according to the previously optimised protocol with a MOI of 5.0, and selected in puromycin for 48 hours. Afterwards, cells were splitted and cultured as individual subpopulations (Fig. 28, clone A-E). After three weeks of selection, GLI2 transcript levels were determined in remaining vital cultures by quantitative realtime PCR.

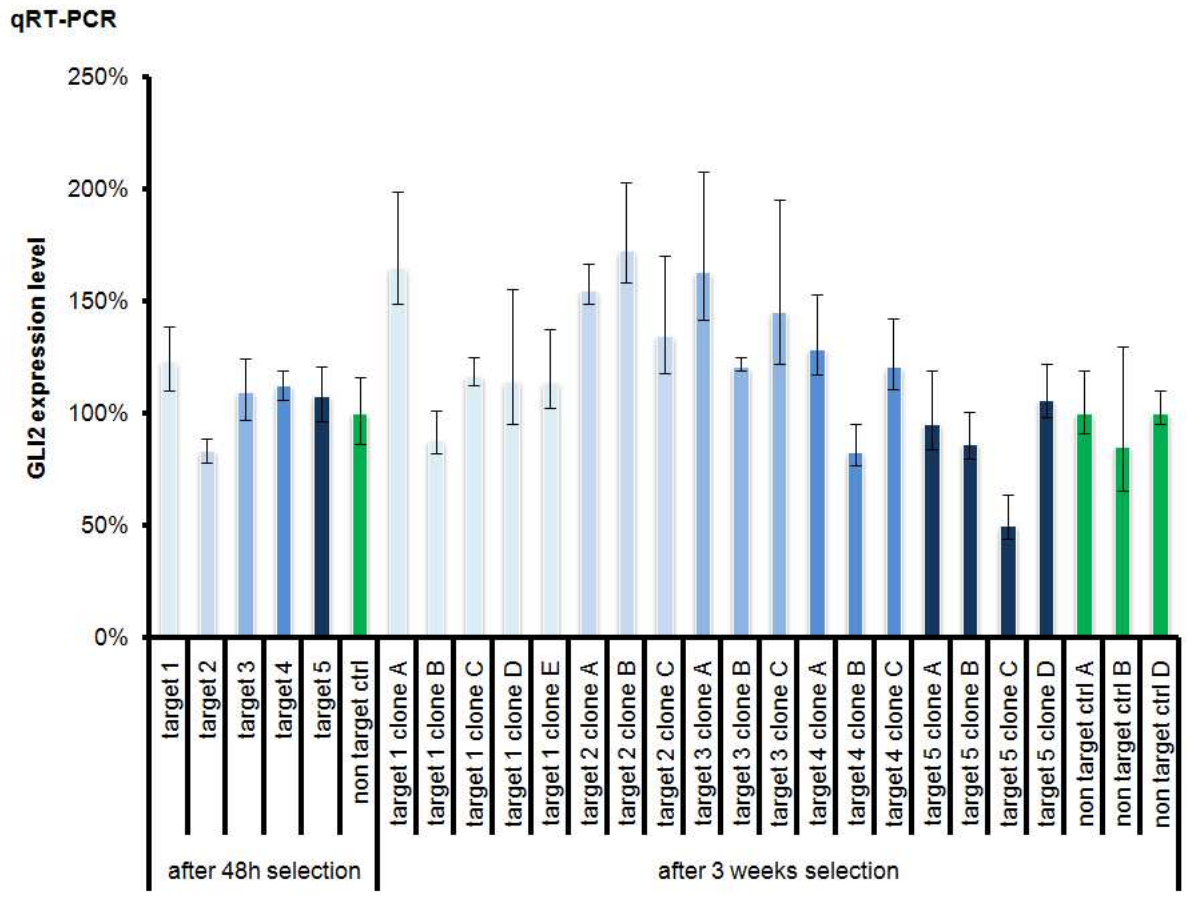


Figure 28: Knockdown of GLI2 by shRNA by lentiviral infection technique in SZ95 cells. qRT-PCR analysis of GLI2 transcripts after infection of SZ95 cells using different GLI2 shRNA transducing lentiviral particles. Different GLI2 shRNA expressing clones were selected by puromycin treatment and checked for GLI2 expression after 48 hours and after 3 weeks. SZ95 clones infected with non target shRNA particles served as reference. Results are presented as mean values ± SD of triplicates from an experiment that was repeated at least three times.

None of the selected GLI2 shRNA clones of any of the five employed target sequences showed a significant knockdown of GLI2 transcripts in SZ95 cells after 48 hours and 3 weeks of puromycin treatment (Fig. 28).

Furthermore, SZ95 cells were infected with a set of four different shRNA transducing lentiviral particles each including a specific target sequence of human GLI3. The same procedure was applied as for infection with particles containing GFPTurbo or anti-GLI2 shRNA.

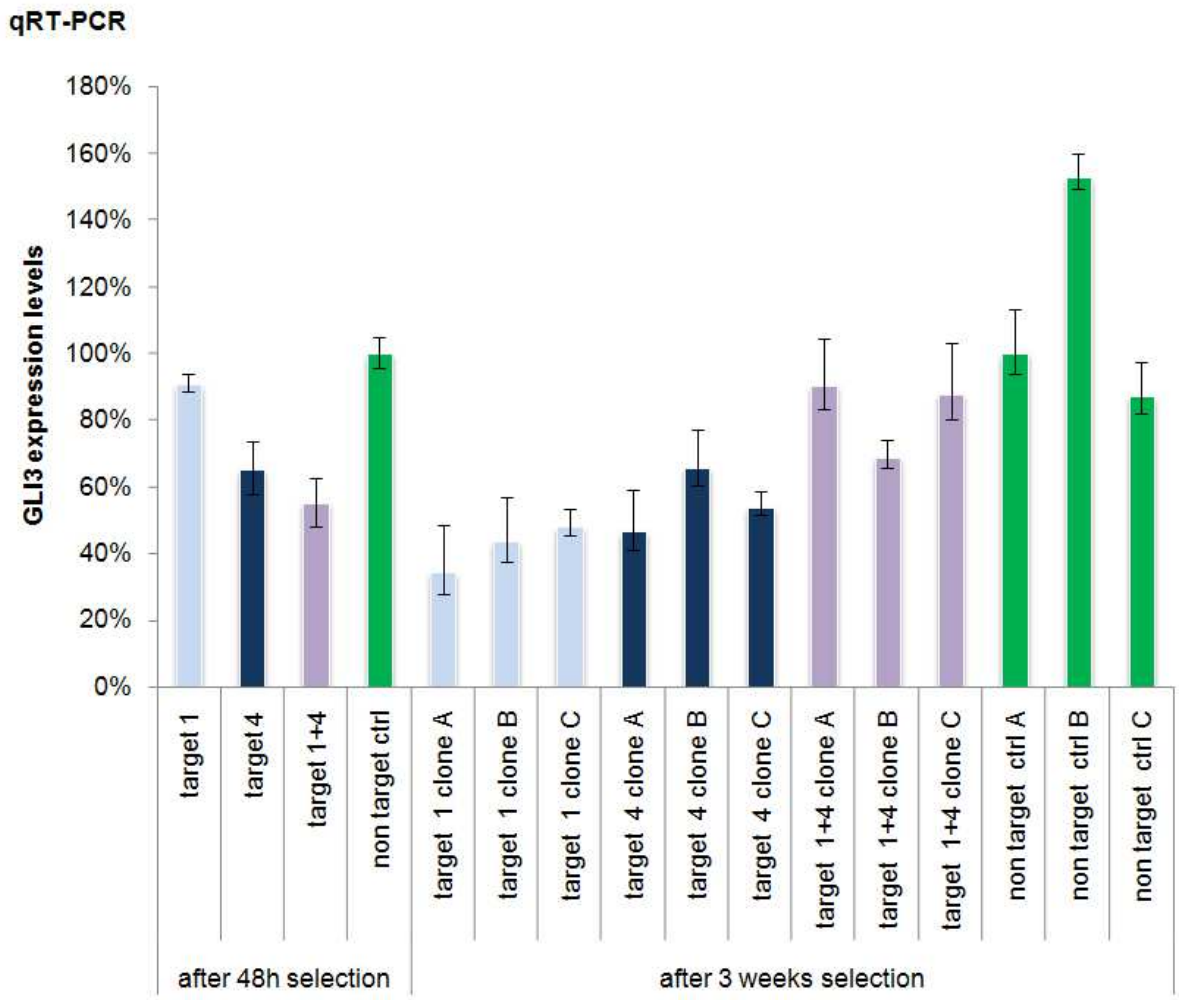
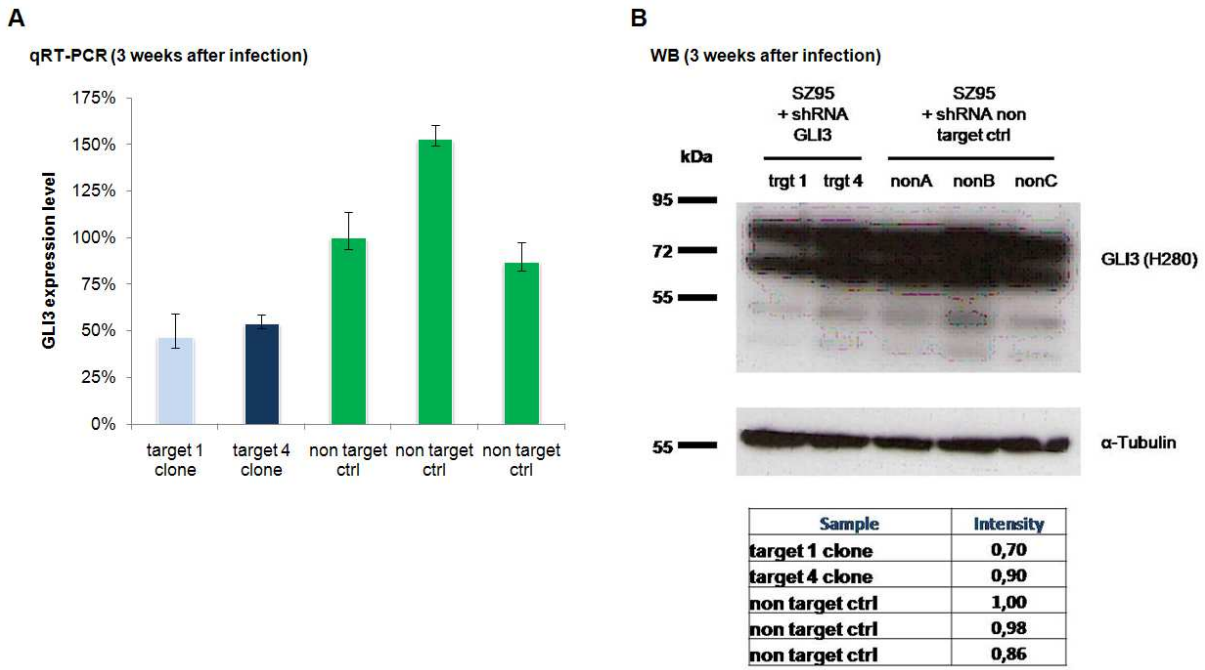


Figure 29: Knockdown of GLI3 by shRNA by lentiviral infection technique in SZ95 cells. qRT-PCR analysis of GLI3 transcripts after infection of SZ95 cells by using different GLI3 shRNA transducing lentiviral particles. Different GLI3 shRNA expressing clones were selected by puromycin treatment and checked for GLI3 expression after 48 hours and after 3 weeks. SZ95 clones infected with non target shRNA particles served as reference. Results are presented as mean values ± SD of triplicates from an experiment that was repeated at least three times.

GLI3 knockdown clones were more promising (Fig. 29). Among these, two target sequences, target 1 and target 4, were shown to down-regulate *GLI3* expression in almost each selected clone by at least 50%. Therefore, only clones of these two target sequences were chosen for further studies.

Combined treatment of target 1 and target 4 resulted in a decrease in GLI3 knockdown efficiency in SZ95 cells compared to single infection (Fig. 29).



Sample	Intensity
target 1 clone	0,70
target 4 clone	0,90
non target ctrl	1,00
non target ctrl	0,98
non target ctrl	0,86

Figure 30: Analyses of selected GLI3 knockdown clones. Comparison of GLI3 mRNA (A) and protein (B) levels after infection of SZ95 cells by using two different GLI3 shRNA transducing lentiviral particles. Clones were selected by puromycin treatment and checked for GLI3 expression after 3 weeks. Results are presented as mean values ± SD of triplicates from an experiment that was repeated at least three times.

To test whether knockdown of GLI3 mRNA (Fig. 29 & Fig. 30 A) was also reflected on protein level, we performed western blot analyses of the corresponding clones (Fig. 30 B). Following 3 weeks after infection, target 1 and target 4 clones displayed an average reduction of GLI3 transcript levels by 66% and by 57% as determined by qRT-PCR, respectively (Fig. 30 A). By applying an antibody specifically detecting GLI3, two bands of repressor variants could be detected with 70 and 89 kDa molecular weight (full-length protein: 190 kDa) (Fig. 30 B). Evaluation of protein bands of GLI3 repressor forms revealed that target 1 clone displayed 30% decrease of GLI3 protein levels, whereas target 4 clone only 10% compared to the non target shRNA reference clones (Fig. 30 B). Thus, GLI3 protein levels were not significantly affected by shRNA knockdown.

One explanation for these observations may be that the *GLI3* transcription levels in the knockdown clones may be still sufficient to produce physiologically normal levels of this protein. On the other hand, GLI3 repressor variants may be efficiently stabilised in these cells.

Taken together, the results obtained from these knockdown assays showed no significant knockdown of GLI protein levels in human sebocytes, and thus did not allow investigating the

consequences of specific inhibition of GLI2 and GLI3 in human sebocytes. Therefore, a different strategy has to be chosen for these analyses.

.

3.5. Application of specific GLI inhibitor GANT61 in SZ95 cells

To investigate the consequences of inhibiting GLI transcription factors, SZ95 cells were treated with specific inhibitors. Recently, Matthias Lauth and colleagues had demonstrated that GANT61 selectively and efficiently inhibits GLI-mediated gene trans-activation downstream of Smoothened by interfering with the DNA binding domain of GLI molecules [345].

3.5.1. GANT61 decreases Hedgehog pathway activation by GLI1 and decreases proliferation rate in SZ95 cells

First, the functionality of the GANT61 inhibitor (Calbiochem, Darmstadt) was tested in the Gli luciferase reporter cell line SHH light II. Therefore, cells were transfected with GLI1 expression construct to induce pathway activation and treated with GANT61 at concentrations raging from 10 to 50 µM.

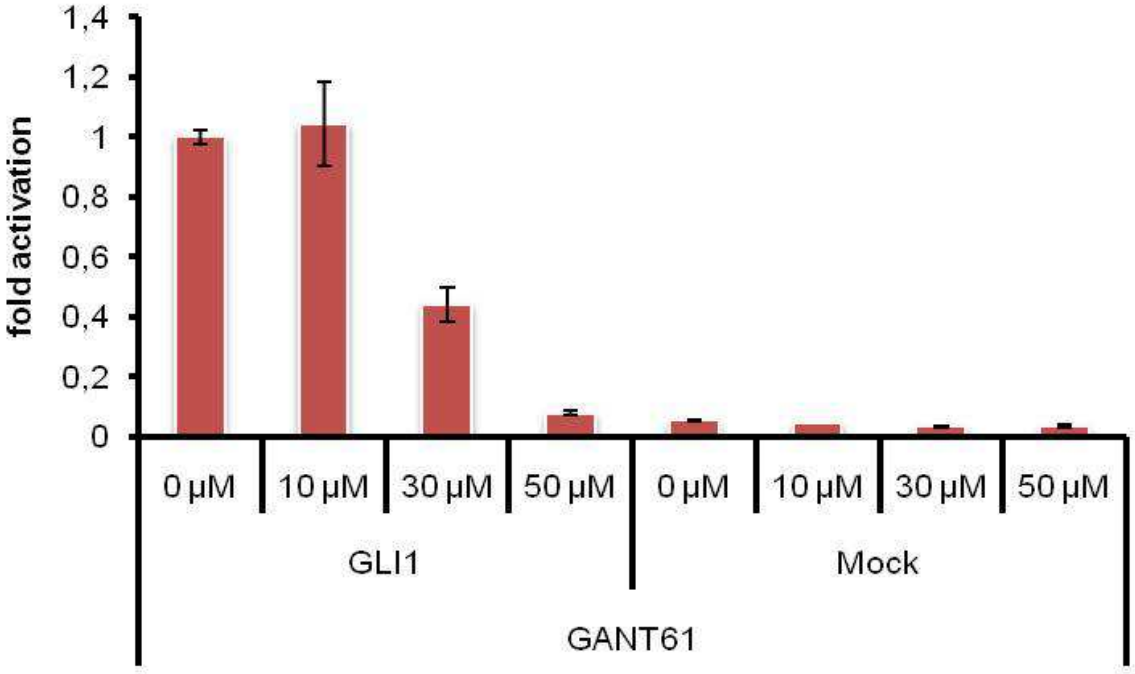


Figure 31: Treatment of SHH light II cells with GANT61 inhibitor. GANT61 efficiently inhibited GLI1-dependent reporter activation at a concentration of 30 µM and higher. Results are presented as mean values ± SD of triplicates from an experiment that was repeated at least three times.

GANT61 treatment displayed a strong inhibitory effect at concentrations of 30 µM and 50 µM. Thereby GLI1-mediated reporter activation was decreased by 56% and by 92% in SHH light II cells, respectively (Fig. 31, left half). A concentration of 10 µM seemed not to be high enough for GANT61 to suppress the activation capacity of GLI1 as no inhibitory effect could be detected (Fig. 31, left half). Mock-transfected cells also showed a slight decrease of background Hedgehog pathway activity upon GANT61 treatment (Fig. 31, right half).

To further study the effect of blocking the GLI transcription factors on proliferation behaviour in human sebocytes, SZ95 cells were treated with GANT61 at a concentration range of 10 to 100 µM. Additionally, SZ95 cells were transfected with GLI1 to directly determine the influence of GANT61 on GLI-induced proliferation.

For comparison, SZ95 cells were treated with different concentration of the Smoothenend inhibitor cyclopamine ranging from 5 to 50 µM. Cyclopamine has been previously shown to decrease the proliferation rate in human sebocytes and increasing their differentiation rate [43].

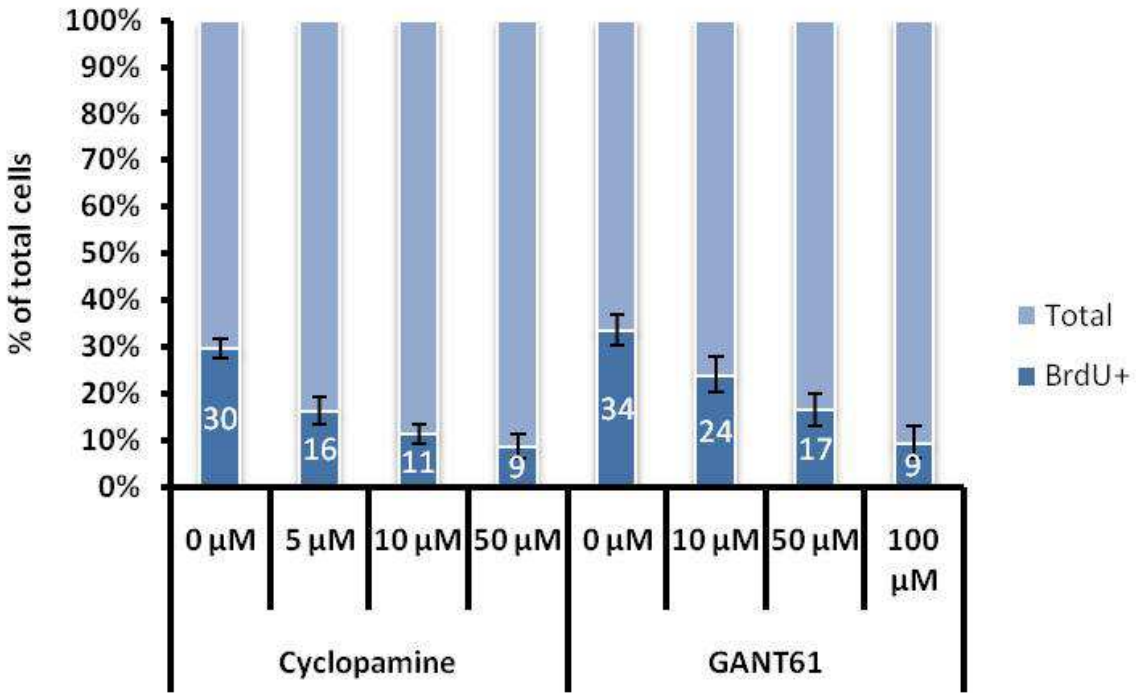


Figure 32: BrdU proliferation assay in GLI1 stimulated SZ95 cells after treatment with Smoothened inhibitor cyclopamine and GLI inhibitor GANT61. Blocking the pathway with both

cyclopamine or GANT61 decreased proliferation in the human sebocytes. Results are presented as mean values ± SD of triplicates from an experiment that was repeated at least three times.

Strikingly, GANT61 treatment significantly decreased the number of BrdU-positive cells from 34% in the solvent control to 9% with negative correlation of applied inhibitor concentrations in GLI1 overexpressing sebocytes (Fig. 32, right part).
The decrease of proliferation rate was similar to that observed in cyclopamine treated SZ95 cells. In this case, the number of BrdU-positive cells decreased significantly from 30% in the solvent control to 9% depending on cyclopamine concentrations (Fig. 32, left part; see also Niemann et al. 2003).
In accordance with the findings of GLI overexpression experiments (Fig. 21), these results demonstrate that GLI transcription factor function is repressed to mediate proliferation stimulation by Hedgehog signalling. In addition, pathway inhibition of Smoothened by cyclopamine treatment modulates the GLI response. This was observed by suppression of sebocyte proliferation following cyclopamine treatment even when GLI1 was overexpressed in these cells (Fig. 32, left part). Thus, depending on the upstream activation state of the Hedgehog pathway, overexpression of GLI1 is not able to rescue the negative effect of upstream inhibition on proliferation. On the other hand, upstream inhibition by cyclopamine may result in constant degradation of GLI1 which is subsequently prohibited to activate target genes promoting proliferation in the SZ95 cells.

3.5.2. Gli inhibitor GANT61 has no effect on lipid production

Next, we wanted to analyse the impact of GLI inhibition, and thus, of GANT61 on the differentiation of human sebocytes. Cyclopamine has been previously shown to inhibit the proliferation and promote the differentiation in these cells [43]. The question arises whether inhibition of GLI function by GANT61 also promotes differentiation in human sebocytes in addition to its inhibitory effect on cell proliferation. Previously, we showed that lipid droplet production can be used as differentiation readout in human sebocytes (see chapter 3.1.1.). Therefore, SZ95 cells were grown on cover slips, treated with 50 µM GANT61 and fixed after 48 hours. Then, cells were subjected to Nile red staining for detection of lipid droplets. Thereby, neutral lipids like cholesteryl esters of lipid droplets emit a green fluorescent signal [346], which can be used to monitor the differentiation state of sebocytes.

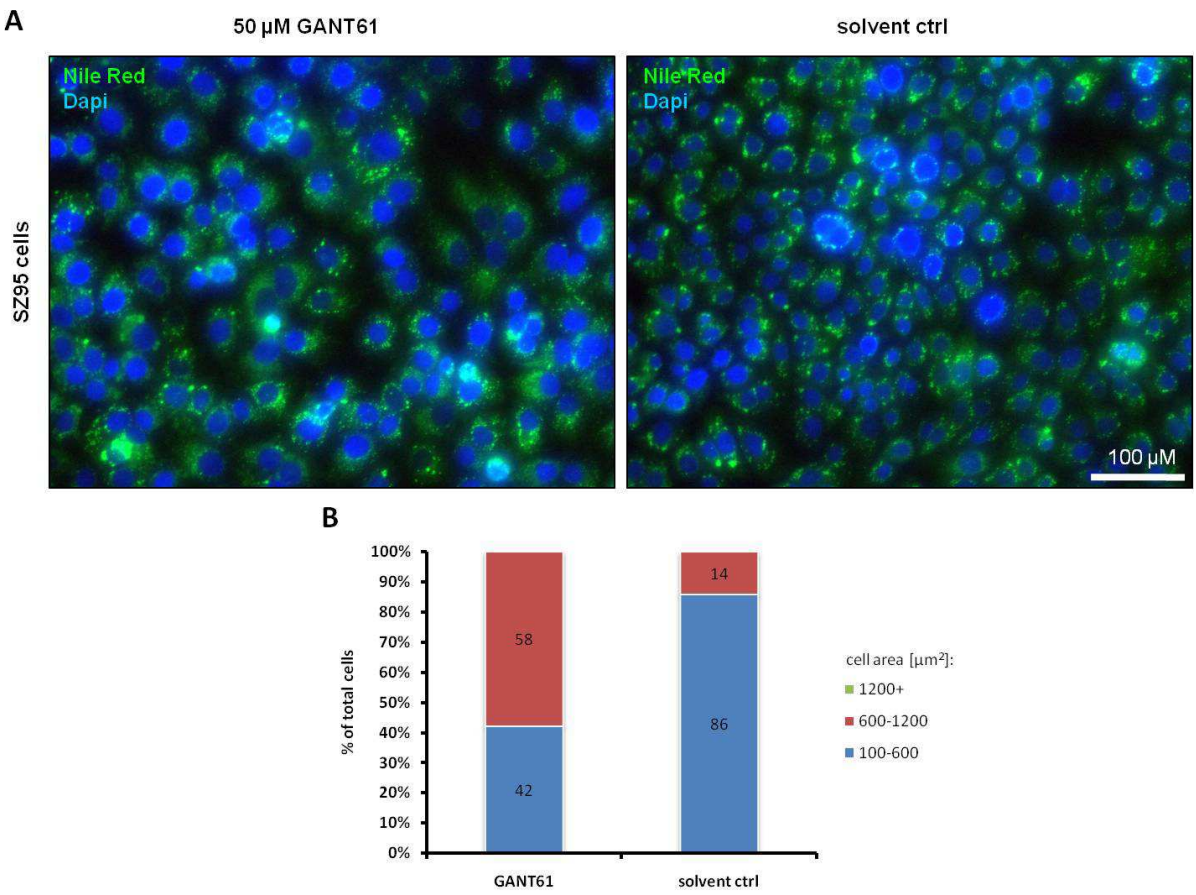


Figure 33: Structural analysis of SZ95 cells treated with 50 µM GANT61 or solvent control (EtOH). (A) Nile red staining of lipid droplets. (B) Classification of sebocytes according to their cell size.

GANT61 treatment of SZ95 cells led to no increase of lipid droplet production compared to the control population (Fig. 33 A). This is in contrast to the positive effect of cyclopamine on lipid droplet formation observed in human sebocytes [43]. But the mean cell size was increased in GANT61-treated sebocytes. Thereby, most of the sebocytes were allocated to the group of 600-1200 µm^2 sized cells instead of 100-600 µm^2 as seen in the control population (Fig. 33 C).

If GLI transcription factors play a major role in regulating lipid droplet formation, and hence, sebocyte differentiation, this process may depend on the function of GLI repressors by inhibiting potential target genes. For example, GLI3 was shown to induce the expression of sebaceous differentiation markers (Fig. 23). Thus, complete blocking of GLI repressor function by GANT61 which interferes with the DNA binding domain is not sufficient to promote lipid production.

3.6. Interaction between Wnt/β-catenin and Hedgehog/GLI pathways in human sebocytes

In addition to the Hedgehog pathway, the canonical Wnt/β-catenin pathway has been shown to play a crucial role during the development of skin and its appendages. Particularly in respect of sebaceous glands, several studies suggest that an inactive Wnt pathway is required for correct sebaceous lineage selection (see chapter 1.5.).

Given the multitude of interactions between the Wnt and Hh signalling pathways during development and homeostasis of several tissues, it is of great interest to address the question whether such kind of interconnection also exists for the regulation of proliferation and differentiation of human sebocytes.

First indications of an interaction between these pathways could be found in previous work of our group, where ΔNLef1, a dominant negative truncated variant of Lef1 incapable to bind to ß-catenin and to transmit its signal, was found to increase *Ihh* expression in SZ95 cells and to promote proliferation in undifferentiated sebocytes [43]. *In vivo*, mice that express ΔNLef1 under control of the K14 promoter develop spontaneous sebaceous-like tumours. In these tumours, transcript levels of Ihh and its receptor Patched 1 were significantly increased.

Therefore, we wanted to investigate the mechanism underlying the down-regulation of Wnt/β-catenin signal transduction and potential interaction with the Hedgehog pathway in human sebocytes.

3.6.1. No significant canonical WNT signalling activity is detected in SZ95 cells

To monitor the endogenous signalling activity of TCF/Lef-dependent WNT/β-CATENIN signalling at different differentiation stages in SZ95 cells, TOPFLASH/FOPFLASH luciferase reporter assays were performed.

No significant changes of WNT/β-CATENIN signalling activity in differentiated SZ95 cells compared to undifferentiated cells were detected. The activation of the TOPFLASH reporter only ranged from 1.3- to 1.9-fold, displaying no or at least low activity levels of the WNT/β-CATENIN pathway in human sebocytes (Fig. 34). Thus, activity of this pathway remains suppressed throughout the differentiation process in these cells. This is in accordance with the model where low Wnt/β-catenin signalling in the skin is necessary for sebaceous differentiation.

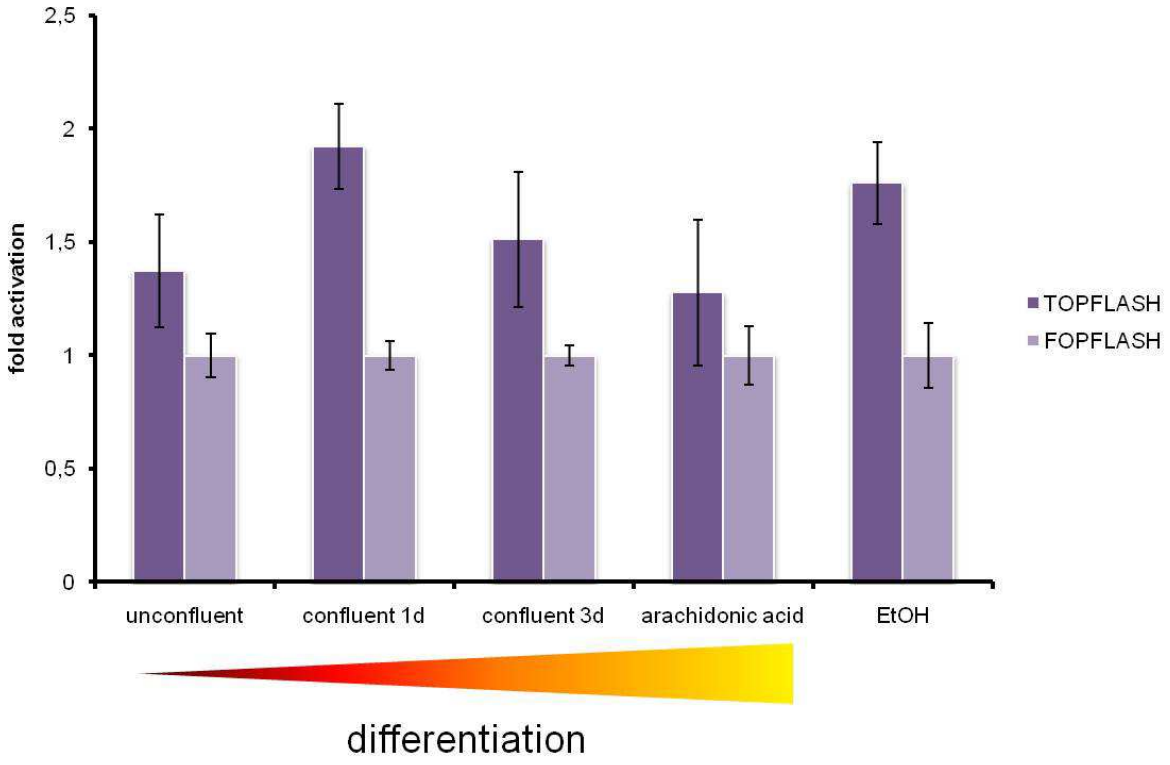


Figure 34: Correlation between Wnt pathway activity and differentiation of human sebocytes. Endogenous pathway activity was determined by TOPFLASH/FOPFLASH luciferase reporter assay under unconfluent and increasing confluence (after 1 day and 3 days), as well as after treatment with arachidonic acid and EtOH as solvent control. Results are presented as mean values ± SD of triplicates from an experiment that was repeated at least three times.

3.6.2. Inhibitors of the WNT/β-CATENIN pathway are expressed in SZ95 cells

Several Wnt ligands have been reported to be expressed during organogenesis and homeostasis of the skin (Wnt 3a, Wnt 4, Wnt 5a, Wnt 10a, Wnt 10b, Wnt 11) [347-349]. But there is a lack of data for WNT ligand expression in sebocytes.

One potential mechanism to inhibit Wnt/β-catenin signalling in human sebocytes would be by expression of endogenous inhibitors specific for this pathway. For this reason, we wanted to study if inhibitory factors are expressed during differentiation of human sebocytes. Therefore, mRNA from SZ95 cells of each differentiation state was isolated, transcribed into cDNA, and analysed for expression of selected pathway inhibitors by quantitative real-time PCR.

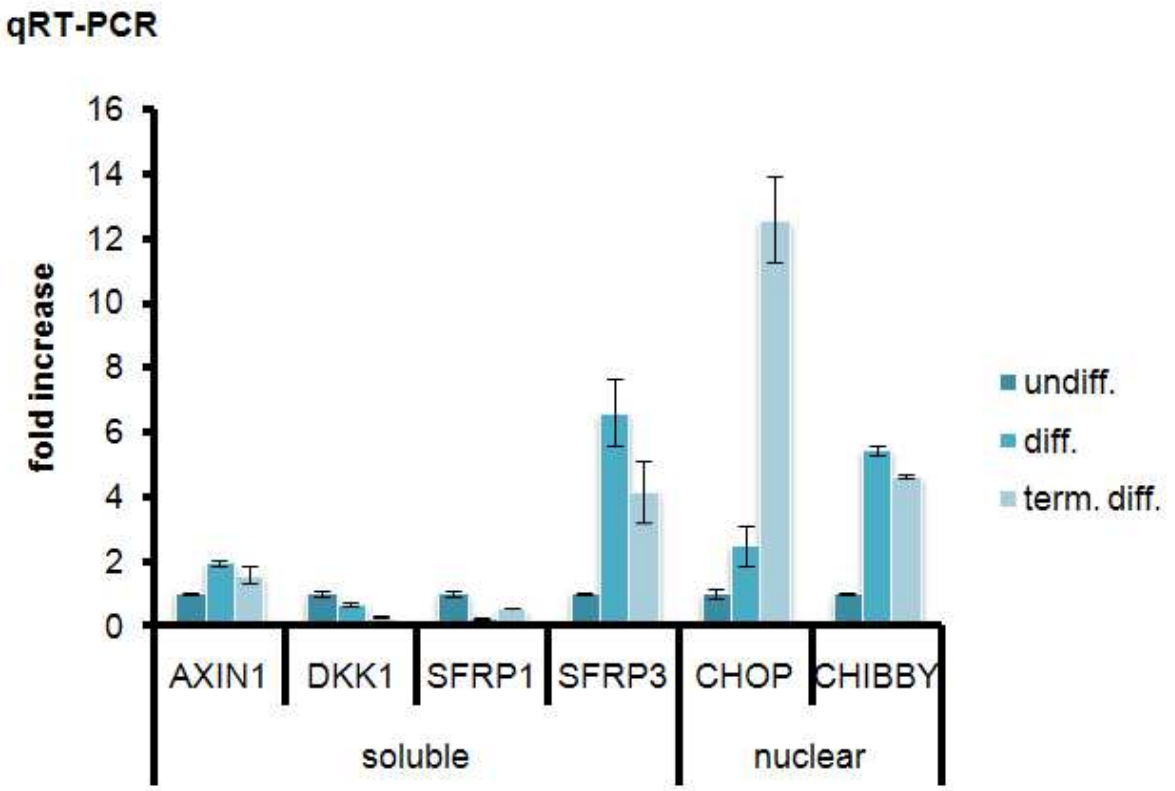


Figure 35: Expression of Wnt/β-catenin pathway inhibitors in SZ95 cells at different differentiation stages. Transcript levels of detectable secreted and nuclear inhibitors were determined by quantitative real-time PCR. Results are presented as mean values ± SD of triplicates from an experiment that was repeated at least three times.

In this context, secreted Wnt antagonists are involved in regulating Wnt signalling output. These Wnt inhibitors are divided into two main groups [350], which contain either secreted frizzled-related proteins (sFrps) binding directly to Wnt ligands or the Dickkopf (Dkk) proteins binding to Lrp5/Lrp6 [351-352]. The sFrp family comprises five sFrps [sFrp 1–5; sFrp3 is also known as Frizzled B (FrzB)] and Wnt inhibitory factor 1 (WIF1). The Dickkopf class includes Dkk1, Dkk2, Dkk3 and Dkk4 [351].

Interestingly, we found *SFRP1*, *SFRP3* and *DKK1* expressed at all differentiation stages in human sebocytes (Fig. 35). *SFRP3* was significantly up-regulated by 6.6 and 4.2-fold in differentiating and terminally differentiated cultures, respectively. This suggests that SFRP3 could play a major role in suppressing WNT/β-CATENIN signalling activity at receptor level in mature human sebocytes.

There are also nuclear factors that have been shown to block Wnt/β-catenin signalling. Chibby encodes a small nuclear coiled coil protein and was shown to inhibit the transcriptional activity of β-catenin by competing with the transcription factor Lef-1 for binding

to β-catenin [353]. Chop is a member of the CCAAT/enhancer binding protein (C/EBP) family of transcription factors and was presented to specifically inhibit the Wnt/β-catenin pathway by binding to the transcription factor Tcf [354].

Interestingly, both inhibitors were up-regulated upon differentiation in SZ95 cells (Fig. 35). Transcript levels of *CHIBBY* were increased by 5.4 and 4.6-fold in differentiating and terminally differentiated sebocytes, respectively. In addition, *CHOP* expression was up-regulated by 2.5 and even 12.6-fold at both differentiation stages, respectively.

Another mechanism to inhibit the canonical Wnt pathway is proteasomal degradation of cytoplasmic β-catenin by phosphorylation and subsequent targeting for ubiquitination (see chapter 1.4.3.).

To investigate, if the destruction complex of β-catenin plays a role in human sebocytes, we analysed the transcript levels of its key component *AXIN1* at different differentiation stages in SZ95 cells. Thereby, *AXIN1* was detectable in all sebocyte populations but displayed only minor changes in expression levels (Fig. 35). In differentiating and terminally differentiated cells these were increased by 1.9 and 1.6-fold, respectively.

These data suggest that SFRBP-3, CHIBBY, CHOP and AXIN1 represent potential factors specifically inhibiting the Wnt/β-catenin signalling activity in human sebocytes. Further experiments will be necessary to demonstrate if these inhibitors are indeed expressed during sebaceous gland development *in vivo* and to determine their functional relevance.

3.6.3. SMAD7 transcript levels are up-regulated in differentiated SZ95 cells

Next, we wanted to address the question whether WNT/β-CATENIN signalling is additionally regulated by SMAD7 in human sebocytes. Smad7 was shown to induce degradation of β-catenin and promote sebaceous gland growth in an inducible mouse model overexpressing Smad7 in the skin [241] (see chapter 1.6.2.).

To investigate the regulation of SMAD7 expression and its implication in inhibiting WNT/β-CATENIN signalling in human sebocytes, we performed qRT-PCR analysis of transcript levels at different differentiation stages in SZ95 cells.

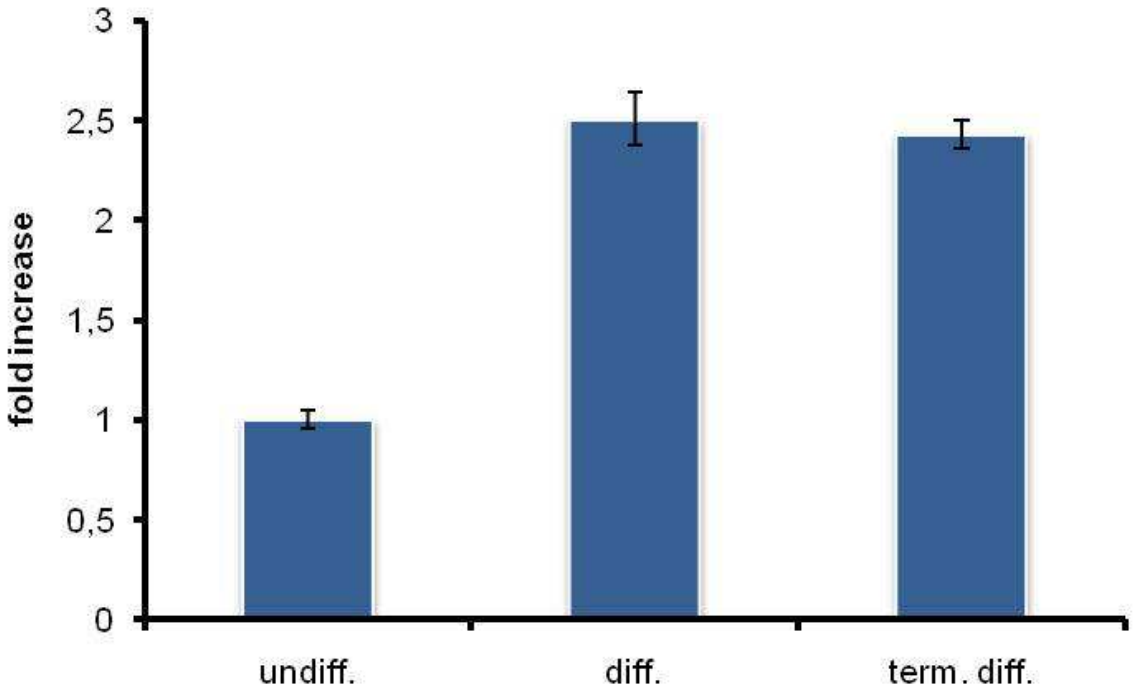


Figure 36: Expression of SMAD7 in SZ95 cells at different differentiation stages. Transcript levels were determined by quantitative real-time PCR. Results are presented as mean values ± SD of triplicates from an experiment that was repeated at least three times.

In SZ95 cells we detected an increase of *SMAD7* transcript levels by 2.5- and 2.4-fold in differentiating and terminally differentiated cultures, respectively (Fig. 36). The presence of SMAD7 mRNA in all sebocyte populations suggest an additional suppression of Wnt/β-catenin signalling by degradation of β-catenin in these cells.
In summary, pathway inhibitors as well as enhanced degradation of β-CATENIN during sebocyte differentiation may play a key role in the inhibition of canonical WNT signalling activity in human sebocytes.

3.6.4. Level of active β-CATENIN is elevated in differentiated sebocytes

Next, we wanted to know if and how endogenous β-CATENIN expression and protein levels are modulated in the process of sebocyte differentiation in accordance with abolished canonical WNT signalling activity which we previously could observe (Fig. 34).
We first determined transcript levels of β-CATENIN by quantitative real-time PCR at different differentiation stages of human sebocytes.

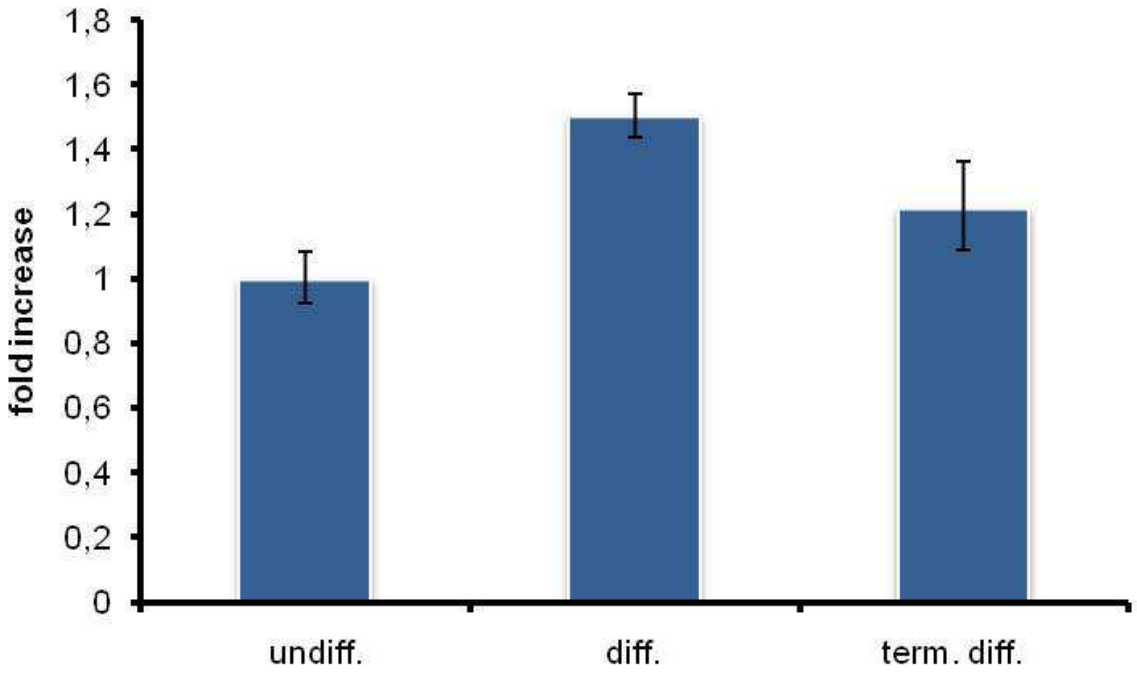


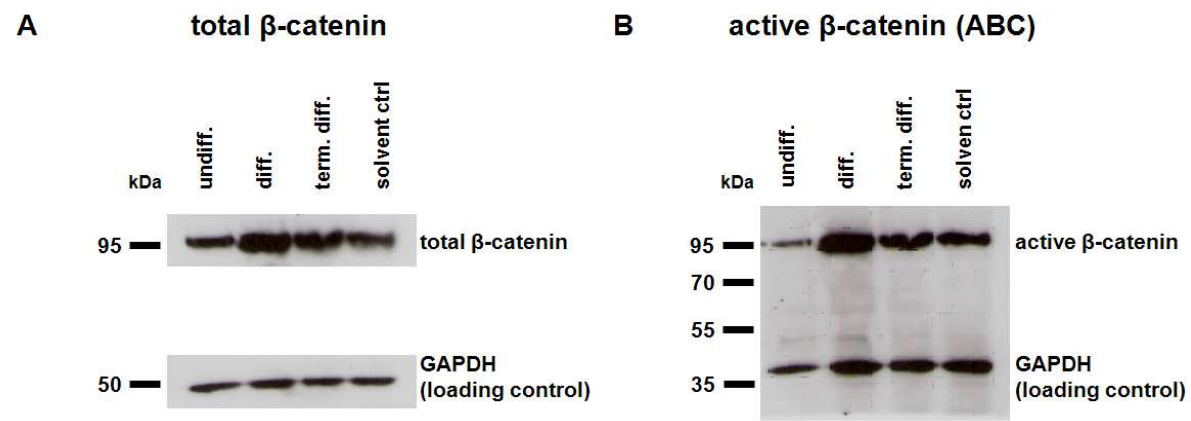
Figure 37: Expression of β-catenin in SZ95 cells at different differentiation stages. Transcript levels were determined by quantitative real-time PCR. Results are presented as mean values ± SD of triplicates from an experiment that was repeated at least three times.

Our analysis revealed no significant changes in expression levels of *β-CATENIN* during sebocyte differentiation. The transcript levels displayed only slight increases of 1.5- and 1.2-fold in differentiating and terminally differentiated cells, respectively (Fig. 37).

Then, levels of total cytoplasmic β-CATENIN protein were determined in SZ95 cells at each differentiation stage to check if β-CATENIN protein concentrations are affected by differentiation of human sebocytes. To do so, we performed Western Blot analyses with an antibody directed against full-lenght β-CATENIN and quantified the detected signals by ImageJ software.

Total amount of β-CATENIN protein (94 kDa) did also not differ significantly, and was even slightly increased by 1.6-fold in differentiating, as well as by 1.7-fold in terminally differentiated cells (Fig. 38 A).

Finally, we specifically determined active β-CATENIN protein levels in the three SZ95 populations with the same procedure, as the N-terminally dephosphorylated pool of β-catenin is crucial to transmit the Wnt signal into the nucleus [355]. Therefore, a specific antibody directed against dephosphorylated β-catenin was applied to compare its levels with total β-catenin levels in Western Blots.



Sample	fold intensity
undifferentiated	reference
differentiating	1.6x
terminal differentiated (aa)	1.7x
solvent control (EtOH)	1.3x

Sample	fold intensity
undifferentiated	reference
differentiating	3.2x
terminal differentiated (aa)	2.5x
solvent control (EtOH)	1.9x

Figure 38: Western blot analysis of β-catenin in SZ95 cells at different differentiation stages. Specific antibodies against (A) total β-catenin and (B) active/dephosphorylated β-catenin were applied. Detected protein levels were determined by ImageJ.

Surprisingly, the active form of β-CATENIN (92kDa) protein was found to be increased in differentiating human sebocytes by 3.2-fold and in terminally differentiated cells by 2.5-fold (Fig. 38 B).

These results strongly suggest that WNT/β-CATENIN pathway activation in human sebocytes can occur in sebocytes. However, this is in contrast to our previous findings that pathway is inactive in these cells (Fig. 34).

So, the question arises what is the underlying mechanism to block the pool of active β-CATENIN and to prevent induction of WNT signal activation in human sebocytes.

3.6.5. GLI2 and GLI3 overexpression increase level of active β-CATENIN but decrease Wnt/β-catenin signalling activity

Previous studies suggest that β-catenin and Gli transcription factors can form a complex and interact directly on the protein level [227, 356]. In particular, Gli3 was shown to inhibit Wnt/β-catenin signalling activity. To test, if this interaction could be the underlying mechanism to inhibit WNT/β-CATENIN signalling in human sebocytes, we investigated the potential role of the GLI transcription factors in modulating the phosphorylation state of β-CATENIN in human

sebocytes. Therefore, we co-transfected unconfluent SZ95 cells with GLI1, GLI2 and GLI3 expression constructs and checked the lysates for protein levels of β-catenin protein after an incubation period of 48 hours.

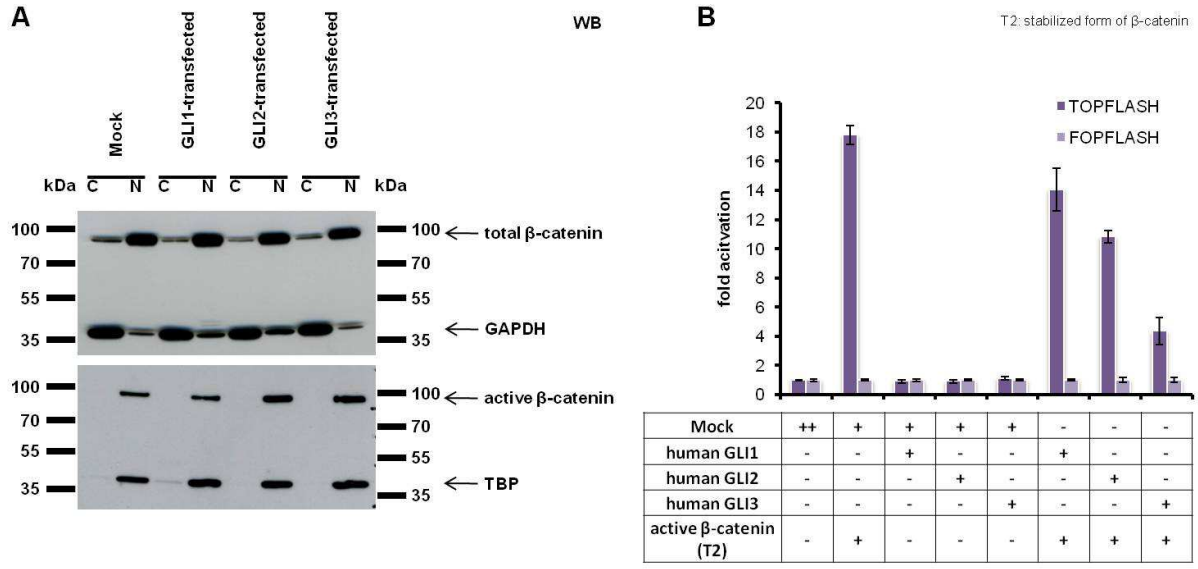


Figure 39: Regulation of active β-catenin and Wnt/β-catenin pathway activity by overexpression of GLI transcription factors. (A) Cell fractionation of SZ95 cells revealed accumulation of active β-catenin in the nucleus when overexpressing GLI2 or GLI3. GAPDH and TBP served as cytosolic and nuclear loading controls, respectively. (B) GLI2 and in particular GLI3 expression decrease Wnt/β-catenin reporter activity. Results are presented as mean values ± SD of triplicates from an experiment that was repeated at least three times. C: cytosolic fraction; N: nuclear fraction; T2: stabilised form of β-catenin.

Strikingly, we could show for the first time that GLI2 and GLI3 overexpression result in a significant increase in protein levels of active β-CATENIN in the nucleus as demonstrated after cell fractionation of SZ95 cells (Fig. 39 A, bottom). On the other hand, no changes in total β-CATENIN protein levels were detected upon transfection of all three GLI transcription factors in comparison to mock transfected human sebocytes (Fig. 39 A, top).

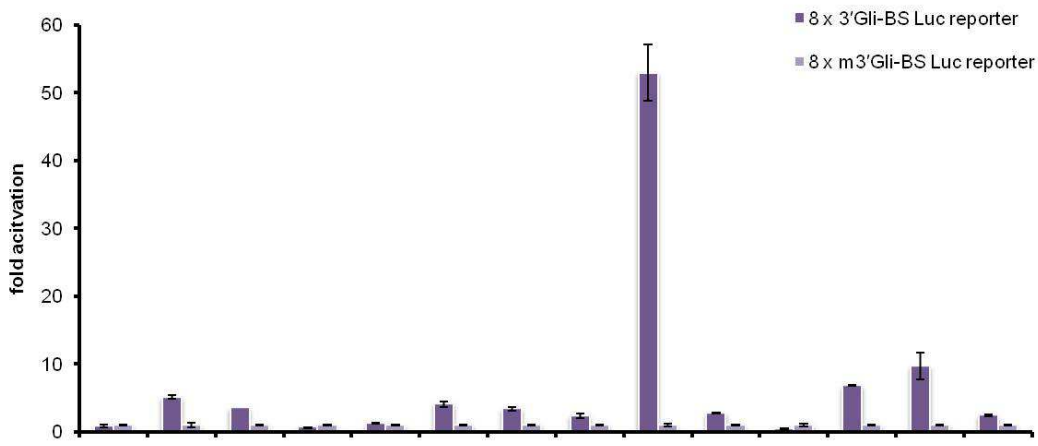
The presence of increased levels of dephosphorylated β-CATENIN levels suggests that WNT/β-CATENIN pathway is activated in GLI2- and GLI3-transfected cells.

Hence, to monitor the impact of each GLI transcription factor on pathway activation and inhibition, SZ95 cells were either co-transfected with mock or with a non-degradable and hence constitutive active form of β-CATENIN (T2). The latter served to induce activation of the TOPFLASH luciferase reporter and to detect possible inhibitory effects on it by co-transfected GLI transcription factors.

Importantly, GLI1, GLI2 and GLI3 alone did not influence the Wnt reporter activity (Fig. 39 B). This finding show that activation of the WNT/β-CATENIN signalling cascade is not induced by GLI2 and GLI3 through accumulation or stabilisation of active variants of β-CATENIN.
Interestingly, GLI2 and, in particular, GLI3 significantly decreased β-CATENIN-dependent Wnt/β-catenin reporter activity by 39.3% and 75.3%, respectively (Fig. 39 B). This result unveils a new aspect of β-CATENIN regulation by both GLI transcription factors.
Thus, the question arises whether GLI2 and GLI3 directly interact with β-CATENIN, thereby preserving it in its active state and blocking subsequent WNT/β-CATENIN pathway activation. We also previously showed that GLI2 induces expression of *GLI3* in SZ95 cells (Fig. 19). As consequence, GLI3 instead of GLI2 may be the key factor to inhibit active β-CATENIN in GLI2-transfected human sebocytes.
Further studies will be necessary to resolve the underlying mechanism in more detail.

3.6.6. Addition of stabilised β- CATENIN enhances GLI1 signalling activity

The important finding that GLI2 and GLI3 expression impede WNT/β-CATENIN signalling in human sebocytes prompted us to investigate if β-CATENIN expression has vice versa an impact on Hedgehog signalling activity. Therefore, undifferentiated SZ95 cells were co-transfected with GLI1, GLI2, GLI3 and mock or the constitutive active form of β-catenin (T2) expression constructs for Gli luciferase reporter assays.
As shown before (Fig. 18), GLI1 and GLI2 alone induced Gli luciferase reporter activity by 5.1 and 3.5-fold, respectively, whereas GLI3 decreased the activity to 0.6-fold compared to the mock control (0.9-fold) (Fig. 40, left part). Transfection of T2 alone did not significantly alter Gli reporter activity which did not exceed 1.3-fold activation under this condition.
Analogously to our previous experiments (Fig. 18), combination of two different GLI transcription factors displayed lower activation capacity of the Gli luciferase reporter by 4.1- (GLI1+GLI2), 3.4- (GLI1+GLI3) and 2.3-fold (GLI2+GLI3), respectively (Fig. 40, central part).
Strikingly, combined expression of GLI1 and T2 caused a 53-fold activation of the Gli reporter (Fig. 40, central part). Thus, GLI1 activation capacity was dramatically enhanced by the presence of high concentrations of active β-catenin.



Mock	+++	++	++	++	++	+	+	+	+	+	+	-	-	-
human GLI1	-	+	-	-	-	+	+	-	+	-	-	+	+	-
human GLI2	-	-	+	-	-	+	-	+	-	+	-	+	-	+
human GLI3	-	-	-	+	-	-	+	+	-	-	+	-	+	+
active β-catenin (T2)	-	-	-	-	+	-	-	-	+	+	+	+	+	+

Figure 40: Regulation of Gli luciferase reporter by constitutive active β-catenin and combined expression with GLI transcription factors. GLI1 signalling activity was significantly enhanced by upon β-catenin expression. Results are presented as mean values ± SD of triplicates from an experiment that was repeated at least three times.

In contrast, no significant changes in luciferase activity levels could be detected after co-transfection of GLI2 (2.8-fold) or GLI3 (0.5-fold) with T2 when compared to transfecting GLI2 (3.5-fold) and GLI3 (0.6-fold) alone (Fig. 40, central part).

Interestingly, upon combined expression of GLI1 and GLI2 with T2 or GLI1 and GLI3 with T2, the activation levels of the luciferase reporter were increased by 6.8- and even 9.7-fold, respectively (Fig. 40, right part). Thus, T2 supported activation of the luciferase reporter when compared to co-transfection of GLI1 and GLI2 (4.1-fold) or GLI1 and GLI3 (3.4-fold) without T2. However, activation of the reporter was significantly lower than combined expression of GLI1 and T2 only (53-fold). One explanation for this observation could be that all three GLI transcription factors compete to bind to the Gli luciferase reporter. But only GLI1 activation capacity is enhanced in presence of active β-catenin, and hence competition for the binding sites by the GLI transcription factors leads to a decreased total signal output despite co-transfection of T2.

On the other hand, when GLI2 was replaced by the inhibitor GLI3 in the presence of GLI1 and T2, the reporter activity reached an unexpected higher activation level (9.7-fold compared to 6.8-fold) (Fig. 40, right part). The reason for this may be that GLI3 can interact

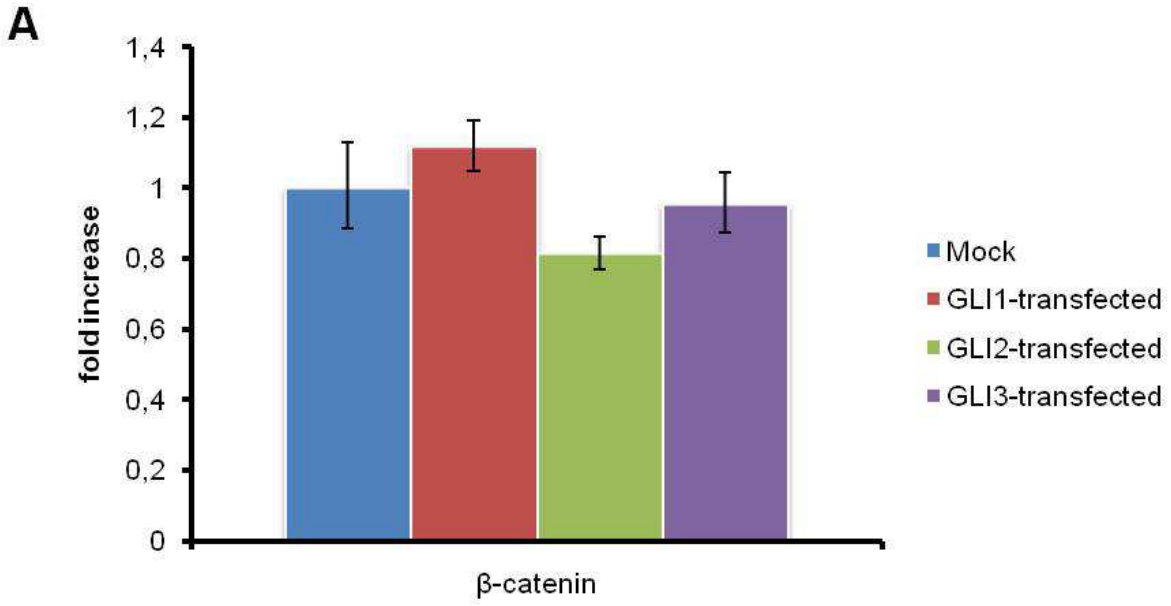
with β-catenin, as suggested by our previous experiments (Fig. 39). This interaction may impede the inhibitory effect of GLI3 on the Gli luciferase reporter in addition to the enhancement of activation potential of GLI1 by T2.

Furthermore, the combined expression of GLI2 and GLI3 with T2 led to a 2.4-fold increase of the Gli luciferase reporter (Fig. 40, right part) which was comparable to the activation capacity of co-expression of GLI2 and GLI3 (2.3-fold) (Fig. 40, central part). In this case, stabilised β-catenin had no visible effect on the reporter activity.

Our results indicate a complex network of mutual regulation between the GLI transcription factors and β-CATENIN which are the main effectors of the Hedgehog and canonical Wnt pathways, respectively. As a consequence, Hedgehog signalling is strengthened on expenses of Wnt signalling activity.

3.6.7. β-CATENIN transcription is not regulated by GLI transcription factors nor vice versa

To confirm, that our previous observations are not based on mutual transcriptional regulation by β-CATENIN and GLI transcription factors, we analysed transcript levels of β-CATENIN and GLI factors as well as additional key Hedgehog pathway components by quantitative real-time PCR in GLI-1, GLI2-, GLI3- and T2-transfected cells, respectively.



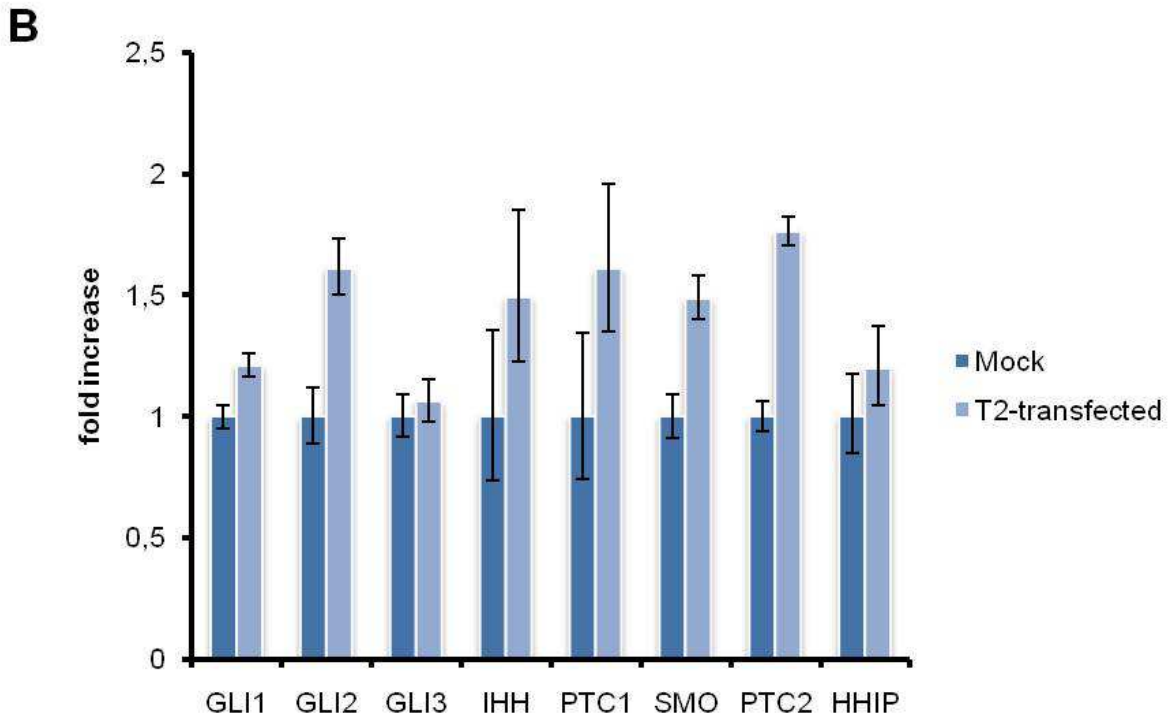


Figure 41: Expression of (A) β-catenin and (B) Hedgehog components in SZ95 cells after overexpressing GLI1, GLI2, GLI3 or stabilised β-catenin, respectively. Transcript levels were determined by quantitative real-time PCR. Mock transfected cells served as reference population. Results are presented as mean values ± SD of triplicates from an experiment that was repeated at least three times.

Upon overexpressing the GLI transcription factors, no changes were detected in transcript levels of endogenous β-CATENIN (Fig. 41 A). These findings are well in line with our previous data showing that total β-CATENIN protein levels are not altered in transfected sebocytes (Fig. 39).

Furthermore, there were no significant changes in expression levels of *GLI1*, *GLI2*, *GLI3*, *IHH*, *PTC1*, *SMO*, *PTC2* or *HHIP* in SZ95 cells transfected with T2 (Fig. 41 B), thus excluding a role of β-CATENIN signalling in regulating Hedgehog pathway components in human sebocytes.

These observations confirmed our previous data that interaction between the Hedgehog and canonical Wnt pathways most likely occurs on the protein level in human sebocytes. Therefore, future studies should focus on protein-protein interaction between β-CATENIN and the GLI transcription factors serving as interconnection point for both signalling cascades.

In summary, the previous findings propose an important interaction between the Gli transcription factors and β-catenin to regulate the Hedgehog and Wnt pathways. Further studies are necessary to unveil the underlying molecular mechanism in more detail.

4. Discussion

4.1. What is the biological function of Hedgehog signalling in human sebocytes?

Morphogenesis of skin-derived organs, such as hair, teeth and whiskers depends on signalling between mesenchyme and epithelium. Gene-deletion studies have demonstrated the requirement of Hh signalling pathway in these processes [21, 35-36]. In particular, these studies have mainly focussed on the function of Shh, whose deletion leads to lack of hair follicles and associated sebaceous glands in mice [21, 35]. Moreover, other studies imply a direct role of Hedgehog signalling in the promotion of sebaceous gland growth. For example, skin-specific overactivation by Smo expression results in increase of number and size of sebaceous glands [112].

Previous work by our group and others has demonstrated that enhanced sebaceous differentiation is accompanied by increased expression of *Ihh* and not *Shh* in sebaceous glands of the skin of transgenic mice [43, 241]. A positive correlation between sebaceous differentiation and *IHH* expression as well as its target gene *PTC1* has also been observed in the human sebocyte cell line SZ95 [43].

This let us assume that most likely IHH plays an important role in regulation of sebaceous gland homeostasis, while SHH is required for establishing the pilosebaceous unit and cycling of hair follicle during skin homeostasis [109-111]. This is further supported by the observation that Shh is able to compensate for loss of Ihh but not vice versa in the skin, thereby demonstrating redundant functions of these ligands [44].

But the underlying molecular mechanism of how the Hedgehog pathway regulates sebocyte proliferation and sebaceous differentiation still remained unveiled. This prompted us to analyse the biological function of IHH pathway in human sebocytes in detail by this thesis. In particular, we distinguished between different sebaceous stages of differentiation to compare Hedgehog pathway activity and to identify potentially Hedgehog signal transmitting and receiving cells for the first time by using an *in vitro* model sharing the physiological characteristics of sebaceous glands *in vivo* [319-321]. This approach has the advantages that each differentiation stage of sebocytes can be precisely isolated allowing us high flexibility for a magnitude of descriptive and functional assays.

4.1.1. Paracrine vs. autocrine Indian Hedgehog signalling in human sebocytes

In this thesis, we showed that Hedgehog pathway is only activated in differentiating sebocytes but not in undifferentiated and terminally differentiated populations. By analyzing the expression pattern of key Hedgehog pathway components we found that positive regulators as *IHH*, *SMO* and *GLI2* as well as the target genes *PCT1* and *GLI1* were up-regulated in differentiating sebocytes, thus confirming pathway activation at this stage.
Terminally differentiated cells displayed the strongest increase of the ligand *IHH*, and to a similar extent also of its receptor *PTC1*. Additionally, transcript levels of negative regulators *PTC2*, *HHIP* and transcriptional repressor *GLI3* were found up-regulated in these cells. Importantly, despite high expression of *IHH* and the target genes *PTC1* and *PTC2* no Hedgehog signalling activity was detected. The reason for this observation could reside in recruitment of endogenous pathway inhibitors. These would prevent potential autocrine pathway activation through endogenous IHH production. This is supported by the fact that PTC1 efficiently suppresses and overcomes pathway activation through SMO in a ratio of 1:250 [68]. Under these circumstances, GLI activators would be continuously processed to repressor form and/or degraded by proteasomes due to stabilisation of the cytoplasmic complex in the off-state of the Hedgehog pathway in these cells [94, 96-97].
Noteworthy, we only detected low levels of SHH in mature sebocytes and no DHH at all differentiation states, thus identifying IHH as the major ligand which plays a role in the regulation of sebaceous gland homeostasis by activation of the Hh pathway [43, 241].
Based on these findings, mature sebocytes may represent a major source of Hedgehog signals in the sebaceous gland, hence inducing pathway activation through IHH by a paracrine mechanism in neighbouring, less differentiated and/or undifferentiated cells. Following this, *GLI1* expression could be induced in Hedgehog target cells. Therefore, we proposed the following model (Fig. 42):

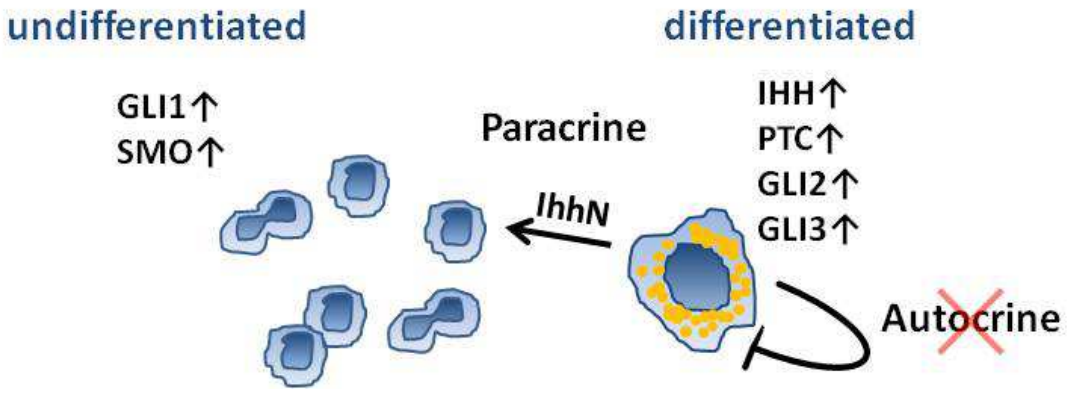


Figure 42: Model of Indian Hedgehog signalling in human sebocytes (I). Undifferentiated sebocytes are susceptible for the paracrine Hedgehog signal. Differentiated sebocyte produce and transmit the Indian Hedgehog ligand, but suppress autocrine Hedgehog pathway activation by up-regulating endogenous pathway inhibitors.

A similar paracrine feed-back regulation between differentiated and undifferentiated cells by Ihh signalling has also been shown to play a major role during development of the endochondral skeleton [357] and colonic epithelial differentiation [228]. During osteoblast differentiation, pre-and early hypertrophic chondrocytes transmit Ihh signals to immature chondrocytes, thereby inducing proliferation [357]. In colonic epithelium, terminally differentiated absorptive colonocytes were shown to activate differentiation program and inhibit proliferation of precursor cells by Ihh pathway [228, 358].
The sebaceous gland also consist of two main cell population where undifferentiated cells reside in the peripheral zone and are mitotically active, while differentiated sebocytes settle in the maturation and necrosis zone (Fig. 4) [267-268]. Thereby, the rate of immature sebocytes recruited for proliferation and consequently for differentiation would depend on the amount of secretion of Hh ligands, and hence on terminally differentiated cells. This could represent a key process in the regeneration of the sebaceous gland, as holocrine secretion demands continuous replenishment by new cells.

4.1.2. Effects of Hedgehog pathway activation and inhibition in human sebocytes

We further addressed the question what is the biological function of Ihh signalling in human sebocytes. In particular, we wanted to investigate the individual role of each GLI transcription factor by overexpression and inhibition experiments as there are three in mammals.
First Gli knock-out studies showed that Gli1-deficient mice are phenotypically normal suggesting that Gli2 is able to compensate for the lack of activator Gli1 [359]. On the other hand, mice lacking functional Gli2 or Gli3 display severe developmental malformations [359]. However, this phenotype can be rescued by replacing the Gli2 locus by Gli1 knock-in, demonstrating that Gli1 can compensate for loss-of-function of Gli2 [100]. Indeed, Gli proteins have partially redundant and partially distinct functions by regulating distinct set of .target genes. Thus, their combinatorial functions need to be tightly co-ordinated depending of affected tissue and species. For example, while Gli1 and Gli2 were shown to induce motor neurons in the spinal cord of frogs, Gli3 represses this function [360]. However, Gli1 induces

floorplate differentiation in the same species, whereas Gli2 and Gli3 inhibit this function [360]. In contrast, Gli2 but not Gli1 is primarily involved in induction of floor-plate development in mice [361-362].

Therefore, to analyze potential differences and overlapping functions of the GLI transcription factors and the impact of Hh pathway modulation in human sebocytes, we performed GLI overexpression and inhibition experiments.

There is accumulating evidence that Hh signalling plays a role in the development of other tissues by regulating proliferation and differentiation processes. On the one hand, GLI1 and GLI2 have been proposed to play a crucial role in promoting proliferation in haematopoiesis [363], central nervous system [364] skin [365] and lung [366]. On the other hand, a range of tumours are induced by over-active Hh pathway activity. For example, mutations of the components PTC (loss-of-function) [125-128] and SMO (gain-of-function) [129-130] result in familial and sporadic BCCs of the skin, respectively. Furthermore, Hh ligand-dependent overactivation was found in colon, pancreatic and ovarian carcinoma [367]. Thereby, cancerous epithelial cells express Hh ligand and may abnormally activate the Hh signalling pathway in adjacent stromal cells. In turn, these cells may then provide a growth-promoting microenvironment for the tumour [367].

With regard to the Gli transcription factors, skin-specific overexpression of Gli1 and, in particular, Gli2 was sufficient to induce BCC and promote proliferation in mouse skin and human keratinocytes, too [368-369]. Analyses of target gene expression after induction of GLI1 and GLI2 in HaCaT keratinocytes illustrated the proliferation enhancing and differentiation-opposing effects of these two oncogenic transcription factors [101]. Additionally, amplification of GLI1 gene has been attributed to development of glioblastoma [138-141]. Additionally, reduction of Gli1 [370] and Gli2 [371-372] function was shown to reduce proliferation or even lead to regression in growth of prostate as well as BCC-like tumours and melanoma, respectively.

In line with these observations, overexpression of the transcriptional activators GLI1 and GLI2, but not repressor GLI3, induced proliferation in undifferentiated sebocytes. Thus, we demonstrated the capacity of active Hedgehog pathway to promote growth in this type of cells. Interestingly, level of proliferation rate did not correlate with level of pathway activation. We previously showed that GLI1 has the highest capacity to activate Hedgehog pathway compared to GLI2. However, highest induction of proliferation was achieved by GLI2 and not by GLI1. This suggests that GLI1 and GLI2 have a different activation pattern of target genes.

On the one hand, this could be caused by structural differences in the N-terminal domain of GLI transcription factors. Thereby, GLI1 lacks the repressor domain found in the N-terminus

of GLI2 like GLI3 and affecting transcriptional outcome of these factors [373]. This has been also exemplified by skin-specific overexpression of a constitutively active *Gli2* mutant lacking the repressor domain (*Gli2ΔN2*). This led to development of several types of skin tumours, including trichoblastomas, cyclindromas, trichoepitheliomas, and BCCs similarly to Gli1 induction [368]. By contrast, transgenic mice overexpressing full-length Gli2 developed only BCC in the skin under same conditions [368].

Other groups also reported that GLI transcription factors display overlapping and also distinct DNA binding specificities of target genes [101, 368]. Interestingly, overexpression of *Gli2ΔN2* resulted in an increase of the Hedgehog target genes *Gli1*, *Ptc1* and *Ptc2* compared to overexpression of wild-type Gli2 [368]. Target gene profiles of both transcription factors also revealed differences for a small set of activated genes by GLI1 and constitutively active *Gli2ΔN2* [101]. Additionally, repression of a range of target genes occurred predominantly in response to GLI2 but not to GLI1 [101].

Accordingly, we detected major differences in the potential of GLI transcription factors to modulate expression of components and activity of the Hedgehog pathway. More precisely, GLI2 seems to play a master role as positive regulator of the Hedgehog pathway. Only GLI2 induced expression of the ligand *IHH* in human sebocytes. Thus, we provided first evidence that Hh expression can be regulated by Hedgehog pathway in these cells. In addition to this, GLI2 also increased transcript levels of transcriptional activator *GLI1*. On the other hand, ectopic expression of GLI2 did not co-localise with proliferating cells, although it induced the strongest proliferative effect. Moreover, GLI2 up-regulated endogenous levels of repressor *PTC1* and interestingly *GLI3* which could potentially counteract pathway activation. Thereby, we identified GLI3 as potential target gene of the hedgehog pathway, too.

Compared to GLI2 overexpression, total Hedgehog pathway activity was higher upon GLI1 expression. This resulted in the highest increase in expression of target genes *PTC1* and specifically of both target genes *PTC2* and *HHIP*. Importantly, Transfected GLI1 expression constructs could be detected in proliferating cells. Hence, GLI1 and not GLI2 would represent the promoter of proliferation in human sebocytes.

These findings suggest that despite promoting proliferation GLI1 and GLI2 have distinct functions and expression patterns in human sebocytes. This is also in line with previous work auf our group, where Gli1 is activated in sebocyte progenitors, whereas Ihh is expressed in differentiated sebocytes of normal human and mouse sebaceous glands and in sebocyte tumours [43]. Thus, expression of Ihh could depend on Gli2 expression in differentiated sebocytes and consequently induce proliferation and Gli1 expression in undifferentiated cells by a paracrine mechanism. Additionally, Gli2 would recruit pathway inhibitors like Ptc1 and

Gli3 to inhibit autocrine activation of Hedgehog pathway in differentiated cells. Thus, we could extend our previously proposed model as follows (Fig. 43):

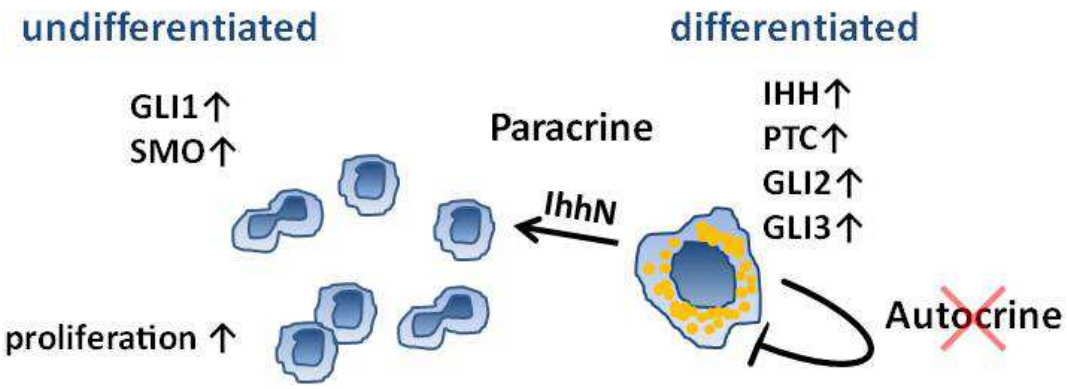


Figure 43: Model of Indian Hedgehog signalling in human sebocytes (II). Undifferentiated sebocytes are susceptible for the paracrine Hedgehog signal, which promotes proliferation. Differentiated sebocyte produce and transmit the Indian Hedgehog ligand, but suppress autocrine Hedgehog pathway activation by up-regulating pathway inhibitors.

In contrast to the transcriptional activators GLI1 and GLI2, GLI3 did neither up-regulate transcripts levels of key Hedgehog pathway components, nor affect the proliferation rate in human sebocytes. Instead, GLI3 displayed a mutually exclusive localisation with proliferative cells like GLI2. In this case, all negative effects indicated that GLI3 acts as repressor in human sebocytes. Thus, overexpression of this transcription factor should already provide us information on the impact of Hh pathway inhibition in these cells.

As far as sebaceous differentiation markers are concerned, GLI2 and in particular GLI3 were shown to induce expression of *MC5R* and *KRT6a*. Remarkably, the extent of up-regulation of these genes is more pronounced in sebocytes overexpressing GLI3 than in ones overexpressing GLI2. In a previous study, Li-Hong Gu and Pierre A. Coulombe showed modest transactivation of *Krt6a* expression by Gli2 in mice [253] but without demonstrating the underlying molecular mechanism. Thus, the question arises whether MC5R and KRT6a transcription is directly promoted by both transcription factors or just by GLI3 as a consequence of its overexpression or induction by GLI2. On the other hand, GLI2 may also act as repressor like GLI3 in human sebocytes. Inhibition of Hedgehog target genes could be necessary for induction of sebaceous differentiation, thereby suggesting that GLI3 is more effective than GLI2 as repressor and that GLI2 plays a dual role as both, activator and repressor. However, the importance of the *in vivo* function of GLI2 as repressor still remains a matter of debate. Genetic evidence indicates that the mouse Gli2 acts as a primary activator of Hh target genes *in vivo*, but it may also function as a weak repressor in Hh

signaling [96, 100, 362, 374-377]. On the one hand, Pan and colleagues confirmed that Gli2 is proteolytically processed into a transcriptional repressor *in vivo* [96]. On the other hand, Gli2 repressor function could be only shown under experimental but not physiological conditions [368, 376-379].

Furthermore, we applied specific inhibitors to abolished endogenous Hedgehog signalling on the level of SMO receptor and GLI transcription factors. Inhibition of SMO by cyclopamine resulted not only in decreased proliferation but also in increased differentiation in human sebocytes as shown in this thesis and previous work of our group [43]. In comparison to this, pathway inhibition on GLI transcription factor level by GANT61 also decreased proliferation rate, but did not significantly induce differentiation in human sebocytes, as demonstrated by unchanged amounts of cytoplasmic lipid droplets. In this case, difference may rely on the different nature of the inhibitor's impact on the pathway. Cyclopamine blocks SMO activity, so that the cytoplasmic complex is stabilised, and GLI transcription factors are consequently degraded (GLI1 and GLI2) or processed to generate GLI repressors (GLI3) [380]. Consequently, GLI repressors are present and functional by binding to and repress their target genes. In contrast to cyclopamine, GANT61 directly affect the GLI transcription factors, thereby impeding their capacity to bind to their target genes without affecting up-stream pathway activity [345]. In this instance, target genes are not regulated anymore, positively and negatively, by the GLI transcription factors. This would suggest that the presence of functional GLI repressors is required to promote sebocyte differentiation and the associated lipid droplet formation.

In summary, the present data suggest that GLI transcription factors preferentially activate a distinct set of potential target genes with distinct effects on proliferation and differentiation.

For the first time, our results strengthened the hypothesis that GLI2 recruits GLI3 to block the Hedgehog pathway and promote differentiation in maturing sebocytes confirmed by up-regulation of the molecular differentiation markers KRT6a [253] and MC5R [305]. Simultaneously, GLI2 induces the production of IHH ligand in differentiated sebocytes to transmit pro proliferative signals to undifferentiated cell neighbour cells. These cells would then express *GLI1* upon pathway activation to accelerate their proliferation.

Importantly, we showed a direct interdependence of ligand IHH, GLI2 and GLI3 expression for the first time. Therefore, this mechanism could also apply for other tissue and cell types.

Consequently, we proposed the following model of mechanism of Indian hedgehog signalling in sebocytes (Fig. 44):

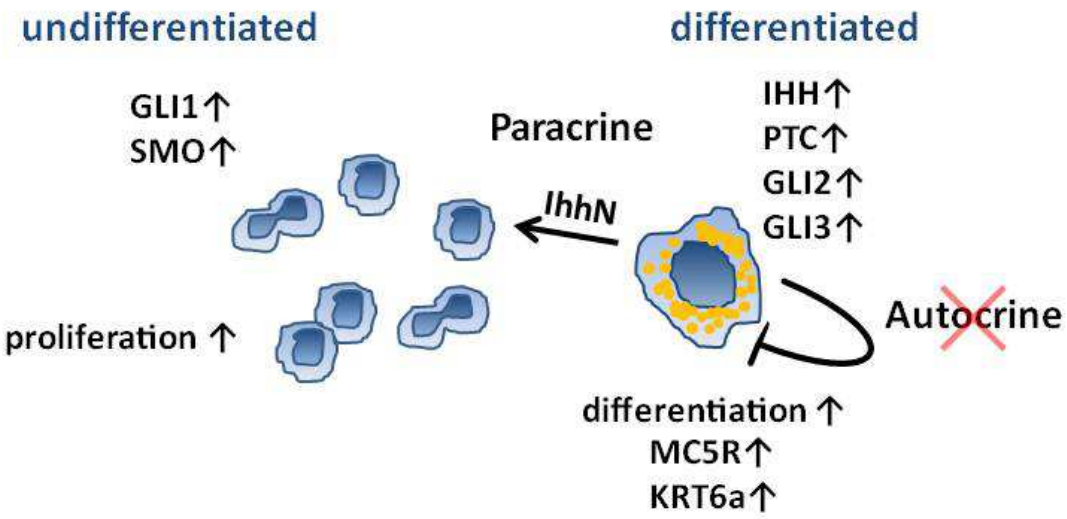


Figure 44: Model of Indian Hedgehog signalling in human sebocytes (III). Undifferentiated sebocytes are susceptible for the paracrine Hedgehog signal, which promotes proliferation. Differentiated sebocyte produce and transmit the Indian Hedgehog ligand, but suppress autocrine Hedgehog pathway activation by up-regulating pathway inhibitors. As consequence, differentiation is promoted in these cells as indicated by up-regulation of sebaceous differentiation markers.

4.2. New aspects of transcriptional regulation by the lipid metabolism in human sebocytes

As mentioned in the introduction, the lipid metabolism has been shown to be important for sebaceous gland development and differentiation (see chapter 1.7.3). Moreover, the main function of sebaceous gland is to form and accumulate lipid droplets. Thereby, differentiation of sebocytes culminates in the holocrine release of sebum.

In the present work, arachidonic acid treatment was applied to induce terminal differentiation in human sebocytes which could be observed by increased cell volume, nuclear fragmentation and, in particular, by accumulation of lipid droplets.

As a consequence, our findings revealed an additional crucial regulatory aspect of the arachidonic acid cascade on gene expression during sebocyte differentiation. Thereby, induced differentiation was accompanied by increased expression levels of the Hedgehog signalling components *IHH*, *SHH*, *PTC1*, *PTC2*, *HHIP*, *SMO* and *GLI3*, of the molecular markers *MC5R* and *ADFP*, as well as of the Wnt pathway regulatory factors *SFRP3*, *CHIBBY*, *CHOP* and *SMAD7*. These genes have not been discussed yet with regard to regulation by lipid metabolism.

As a natural constituent of membrane phospholipids, arachidonic acid undergoes signal-mediated hydrolysis, whereby the degradation products are released into the intracellular space to be, on the one hand, metabolised to second messenger molecules or, on the other hand, to stimulate third messenger systems. Among these the most important generated messengers belong to the metabolite families of prostaglandins, thromboxanes, prostacyclin and leukotrienes [381-382], which regulate various processes like pain, inflammation, blood coagulation and vasoconstriction in the body.

For a range of genes involved in fatty acid and glucose metabolism it has been shown that polyunsaturated fatty acids (PUFA) modulate their expression by binding to at least four transcription factor families including three members of the superfamily of steroid and thyroid hormone nuclear receptors, namely PPAR, LXR and hepatic nuclear factor 4 (HNF-4α) and SREBP [383]. In turn, these factors bind to specific regulatory promoter sequences sensitive to PUFAs, among which are PUFA responsive elements (PUFA-RE) [384-386] and PPAR responsive elements (PPRE) [387-388]. Interestingly, PPAR, LXR and SREBP have also been shown to play a critical role in the regulation of sebaceous gland function (see chapter 1.7.3.).

In this context, previous studies have shown that arachidonic acid acts on the transcriptional level to modulate mRNA stability and, thus, half-life of genes like *GLUT4* and *SCD1* [309, 389]. More precisely, accumulation of arachidonic acid was found to have a negative effect on Scd1 expression [279, 307-309]. This effect is of particular interest as modulation of Scd1 affects the size, and thus, the physiology of sebaceous glands [307].

Thus, the question arises whether the above mentioned regulated genes of this study are directly regulated by arachidonic acid metabolites and/or indirectly as consequence of the differentiation process affecting other regulatory pathways. The link between the fatty acid metabolism and particularly the regulation of Hedgehog and/or Wnt pathway activities in sebocytes needs to be elucidated. Whether these pathways are up- or downstream of the fatty acid metabolism or act in concert with its signalling cascades needs further profound investigation.

Noteworthy, the sebum contains several derivatives of arachidonic acid or structurally similar substrates like sebaleic acid, the major polyunsaturated fatty acid in human sebum and skin surface lipids [390]. Accordingly, a potential role in gene regulation could be attributed to the sebum, when mature sebocytes undergo holocrine secretion and cell death releasing these fatty acids to their close environment. Thereby, lipid production and differentiation could be affected in sebocytes in the close proximity and/or the physiology of cells located on the way from the sebaceous gland ducts to the exit of the hair shaft channel on the skin surface,

which get in contact with released fatty acids by responding through PUFA-RE and/or PPRE stimulated pathways.

4.3. What is the underlying mechanism of Wnt pathway repression in sebocytes?

In addition to the Hedgehog pathway, the canonical Wnt/β-catenin pathway has been previously shown to play a central role during the development and homeostasis of the pilosebaceous unit (see chapter 1.5.). Acting like a switch, increased Wnt signalling induces *de novo* hair formation, while inhibition of this pathway results in increased sebaceous differentiation at the expense of hair follicle formation. Thereby, Hedgehog signalling was located downstream of Wnt signalling [22, 246].

Several Wnt ligands have been shown to be expressed in mouse and human hair follicles, for instance Wnt-3a, Wnt-5a, Wnt-10b and Wnt-11, which trigger Wnt pathway activity during hair cycles but a direct association with sebaceous gland biology has not been shown [24, 209, 391]. Based on the observation that Wnt pathway inhibition is required for normal sebaceous gland development, we addressed the question, how the canonical Wnt pathway is regulated in sebaceous glands. Therefore, we focused on potential mechanisms of the pathway repression in human sebocytes by analysing several known inhibitors for their expression at different differentiation states for the first time. Among these, the Wnt ligand inhibitor *SFRP3* and the nuclear inhibitors *CHIBBY* and *CHOP* were found to be up-regulated during the differentiation process. Thus, this not only suggests that the initiation of the Wnt pathway is impeded by direct ligand inhibition, but also the transcription of potential target genes through nuclear inhibitors. Interestingly, other detected cytoplasmic and secreted inhibitors were down-regulated at the same time (e.g. *AXIN1*, *DKK1*, *and SFRP1*). Therefore, SFRP3, CHIBBY and CHOP may compensate for the vanishing presence of other down-regulated inhibitors in differentiating sebocytes. Thereby, endogenous pathway inhibition would be preserved which ensures sebaceous differentiation without affecting the close environment of the sebaceous gland.

When analysing amounts of β-CATENIN in human sebocytes at different stages, total mRNA and protein levels remain stable during differentiation. These observations suggest that expression of β-CATENIN is not modulated during sebocyte maturation. However, non-phosphorylated, transcriptionally active β-CATENIN is found increased in differentiating SZ95

populations, but not Wnt signalling activity. Therefore, the present pool of active β-CATENIN is not able to induce Wnt signal activation in these cells.

One obvious mechanism to prevent β-catenin activity is its degradation. A previous study in mouse skin identified Smad7 as inductor of β-catenin degradation resulting in the inhibition of canonical Wnt signalling [241]. Transcript levels of *SMAD7* were also found up-regulated in differentiated SZ95 cells, thus suggesting that SMAD7 induces β-catenin degradation and Wnt pathway inhibition at this stage in human sebocytes. But protein levels of total and active β-CATENIN were not decreasing and/or did not show degradation during sebocyte differentiation. Thus, suppression of β-CATENIN signalling must be mainly achieved by action of the previously mentioned nuclear factors CHIBBY and CHOP or by an alternative mechanism.

Further mechanisms to block active β-CATENIN and potential functions exerted by this pool of β-CATENIN will be discussed below.

4.4. How is β-catenin regulated in sebocytes?

We searched for potential levels of interactions between Hedgehog and Wnt pathways like it has been presented for other cellular systems (see chapter 1.6). However, our expression analyses showed no influence of overexpressed GLI1, GLI2 and GLI3 on *β-CATENIN* transcription level and vice versa in human sebocytes.

An alternative mechanism of how β-catenin activity is affected by Hedgehog pathway has been suggested by F. Ulloa and colleagues. Investigating the consequences of SHH deficiency on neural tube development in mouse embryos they found Wnt responsive genes like *Axin2* to be decreased [227]. This led to their hypothesis that Wnt signalling is decreased in the absence of Shh signalling. By overexpressing a construct coding the repressor form of Gli3 (Gli3R) they prevented ß-catenin mediated reporter gene expression in several cell lines and during development *of Xenopus* embryos [227]. The authors demonstrated by co-immunoprecipitation that there was a direct interaction between Gli3R and the carboxy-terminal domain of ß-catenin which includes the transactivation domain.

We investigated if this mechanism of β-catenin inhibition by Gli3 is also true for human sebocytes. Therefore, we analysed the effect of different combinations of co-transfected stabilised β-catenin and human GLI expression constructs on Wnt responsive reporter constructs concomitantly with determination of the endogenous ratio of active versus total β-CATENIN protein levels. Strikingly, these assays confirmed an inhibitory effect of GLI3 on β-

CATENIN induction of the TOPFLASH reporter construct. Moreover, these experiments also unveiled a similar role for GLI2 for the first time. Like GLI3, addition of GLI2 also resulted in a remarkable decrease of the β-CATENIN-sensitive reporter output.

If only the repressor form of GLI3 is able to bind to β-CATENIN, and hence to inhibit its function to activate target gene transcription, a similar mechanism applies to GLI2. By this way, a fraction of GLI2 could be proteolytically processed to generate a shortened variant containing the repressor domain which is capable of binding to β-CATENIN. Other studies have confirmed phosphorylation of Gli2 by PKA required for processing and suggested an important role of Gli2 processing during Hh-regulated mouse embryonic patterning *in vitro* and *in vivo*, respectively [97, 377]. Furthermore, Gli2 was shown to be partially processed and, as consequence, to also act as repressor in addition to activator functions in *Drosophila* [392]. Interestingly, Gli3 repressor activity is regulated by Hh, but not Gli2 repressor activity.

Alternatively, GLI2 was shown to induce the expression of *GLI3*, which may lead to an increased presence of GLI3 repressor and, only then, subsequent inhibition of β-CATENIN.

Surprisingly, as stated before for differentiated sebocytes, protein levels of active β-CATENIN were found increased in SZ95 cells transfected with GLI2 and GLI3 expression constructs, whereby cell fractionation analysis allocated this accumulation to the nucleus. These data suggest that the active form of β-CATENIN is prevented from initiating transcription of target genes by interacting with the repressor forms of GLI transcription factors, analogously to the effect of the nuclear repressor CHIBBY [353].

Other studies also reported similar effects for a Kruppel-like transcription factor, namely Gli-similar 2 (Glis2), which exhibits different functions dependent on gene and associated promoter sequence as an activator or repressor. It has been shown that Glis2 and in particular its repressor form is able to directly bind to β-catenin and to p120 which is known to be part of adherens junction containing β-catenin, too [393-394]. By binding to β-catenin, Glis2 represses Tcf-mediated transcriptional activation. Owing to the similarity of Glis2 and Gli1-3 transcription factors, one could envision an analogous mechanism for GLI2 and GLI3 [395].

4.5. Does WNT/β-CATENIN signalling affect GLI function in sebocytes?

We also addressed the question whether β-CATENIN interferes with Hedgehog signal transduction. Indeed, co-expression of GLI1 and stabilised β-catenin displayed a strong induction of the Gli reporter constructs but without mutually affecting transcript levels. Our

results are in line with previous findings by O. Meada and colleagues [396]. They showed that β-catenin enhances the Hedgehog signal through GLI1 without increasing the amount of expression and/or changing intracellular distribution of GLI1 in various human cancer cell lines. Moreover, β-catenin had a similar effect on GLI2-mediated Hh pathway activity but to a smaller degree [396]. However, they reported no complex formation between GLI1 and β-catenin.

By contrast, the activity of the Gli reporter construct was not changed when GLI2 or GLI3 were co-expressed with stabilised β-catenin compared to cells only transfected with GLI2 or GLI3 in our experiments. These results suggest, that the potential fraction of GLI2 and/or GLI3 interfering with β-CATENIN (see chapter 4.4.) has no influence on the Hedgehog pathway. Furthermore, addition of GLI2 or GLI3 resulted in a significant decrease of the enhancing effect of stabilised β-catenin on the activation of the Gli reporter by GLI1. These observations might be indicative for competing direct and/or indirect interactions of the GLI transcription factors with β-CATENIN, whereby GLI2 and GLI3 are favoured and limit the inducing effect of β-CATENIN on Wnt signalling as well as on Hedgehog signalling.

However, it is also conceivable that β-CATENIN counteracts the inhibitory effects of a potential GLI2 repressor variant itself, of GLI2 due to subsequent recruitment of GLI3 repressor and/or of GLI3 repressor which results in the observed increase of the Gli reporter activity compared to co-expression of GLI1 and GLI2 as well as of GLI1 and GLI3 without the presence of stabilised β-catenin.

During our expression analyses only low endogenous levels of GLI1 were detectable in undifferentiated sebocytes which would explain why addition of exogenous β-CATENIN alone only resulted in a weak induction of the Gli luciferase reporter.

4.6. Proposed model of GLI and β-CATENIN interference

One important aspect is that β-CATENIN may act as transcriptional co-factor by binding to GLI1 and enhancing its function as transcriptional GLI activator in human sebocytes. A similar role has been previously described in human cancer cell lines overexpressing both factors [396]. Thus, not only *GLI1* expression alone but also β-CATENIN protein levels would have a significant impact on the Hedgehog signal response. But there is still a lack of evidence of a direct protein-protein interaction between GLI1 and β-CATENIN.

GLI2 and GLI3 might also play a role as inhibitors of the Wnt pathway by impeding the transcriptional activation capacity of β-CATENIN potentially through direct binding as

demonstrated by the work of Ulloa and colleagues [227]. The question still remains whether GLI2 directly influences β-CATENIN activity or indirectly by induction of *GLI3* expression, because there is also no evidence that GLI2 can bind to β-CATENIN, too.

GLI2 and GLI3 transcription factors could also serve to stabilise and store active β-CATENIN, as our experiments showed that this variant accumulates in the nucleus of cells overexpressing one of these transcription factors.

In this context, cadherins were shown to antagonise the accumulation of β-catenin in the cytoplasm by attaching it to the membrane as structural component of adherens junctions. Regarding this, the decreased cadherin expression leads to increased Wnt signalling in fly and mouse development [149-150]. Conversely, the overexpression of cadherins results in a reduction of Wnt signalling activity [151]. Importantly, conformational changes of β-CATENIN have been observed to direct binding preferences towards either cadherins or members of the Tcf/Lef transcription factor family [152]. A similar mechanism could be implicated in the regulation of β-CATENIN variants by GLI2 and/or GLI3 in the nucleus.

Other molecules which bind both, Gli transcription factors and β-catenin, could also contribute to the cross-link of Wnt and Hedgehog pathways and the observed effects. For example Su(Fu), Pka, CkI and Gsk3β (see chapters 1.1.4, 1.4.3 and 1.6.1) are all involved in regulation of Gli transcription factors and β-catenin activity by direct physical interaction and modification. Therefore, a direct binding of Gli transcription factors to β-catenin might not be necessary and modulation of activity occurs through recruitment other factors.

To summarise we propose the following general model of Hedgehog and Wnt pathway interaction on protein level: Active Hedgehog pathway leads to expression and stabilisation of the transcription factor Gli1 whose capacities as transcriptional activator is enhanced in presence of β-catenin. In turn, decreased Hedgehog signalling would promote Gli repressor formation and thereby not only inhibit Hedgehog target gene activation but also canonical Wnt pathway activation by interference of Gli repressors with β-catenin. As a result, active β-catenin accumulates in the nucleus, but the function or fate of this pool still remains unclear. Considering that expression and total protein levels of β-CATENIN remain stable throughout sebocyte maturation, modulation and distribution of distinct β-CATENIN pools by GLI transcription factors may represent a key regulatory feature not only of Hh but also of Wnt targets.

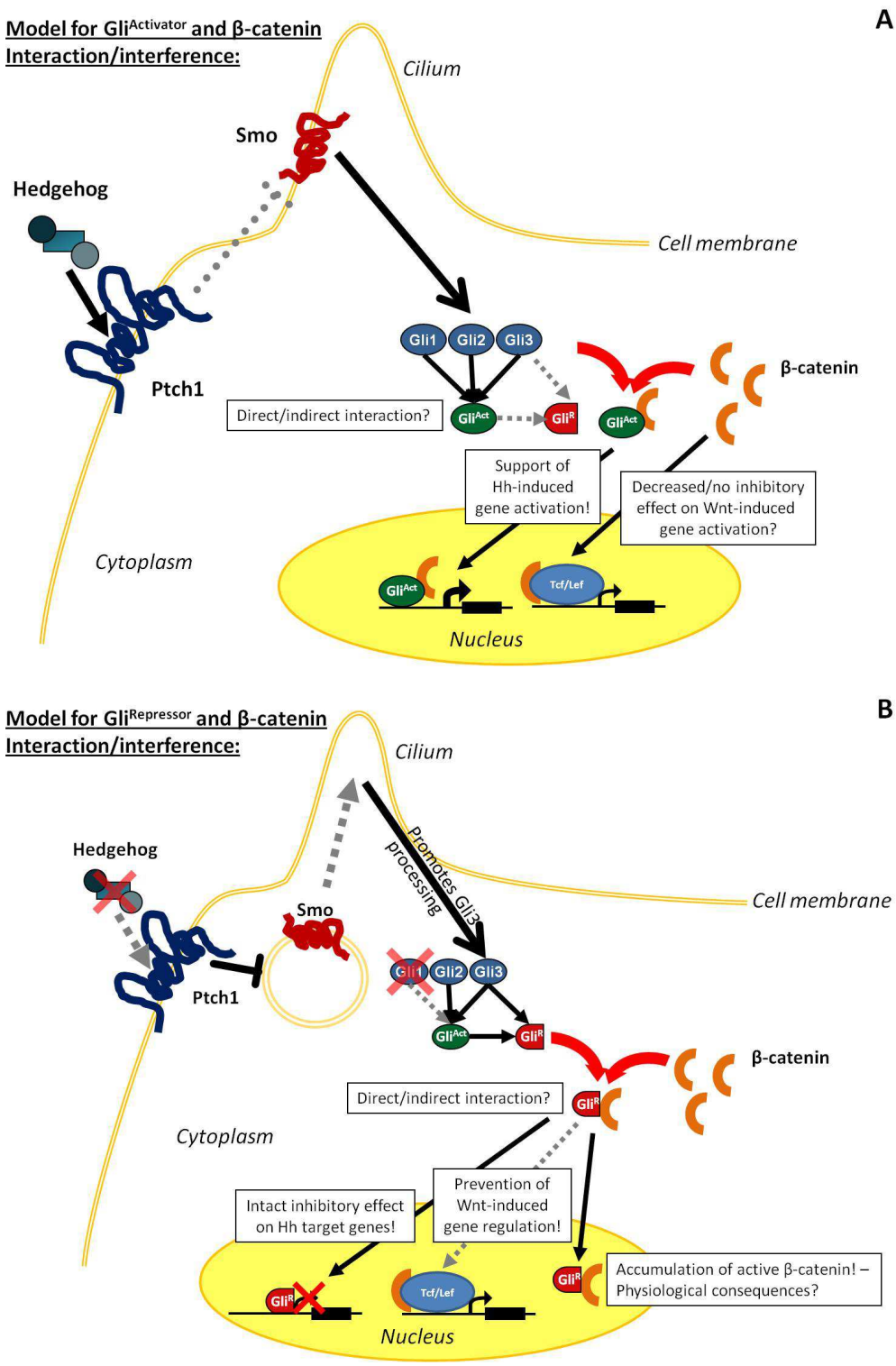


Figure 45: Model for (A) GLI^{Activator} and (B) GLI^{Repressor} interaction with β-CATENIN. (A) β-CATENIN enhances transcriptional GLI^{Activator} activity possibly as transcriptional co-factor. (B) GLI^{Repressor} prevents β-CATENIN from activating Wnt target genes through members of the TCF/LEF family and promotes accumulation of active β-CATENIN in the nucleus.

Taken together, this interaction would constitute a novel and important regulation point in several processes and disorders resulting from misregulation of one or both factors or pathways. New therapeutics could be designed to target both, Wnt and Hedgehog signalling pathways.

4.7. Outlook

This thesis investigated the molecular mechanism and role of Indian Hedgehog signalling in human sebocytes. The results demonstrated a pro-proliferative function of the active Indian Hedgehog pathway in human sebocytes. Conversely, pathway inhibition reduced this effect significantly and promoted the expression of sebaceous differentiation markers suggesting that an inactivated Hedgehog pathway is necessary for correct maturation of this type of cells. Thereby, we also presented that expression and distribution of GLI transcription factors are differentially regulated during sebaceous differentiation. Furthermore, overexpression experiments of each GLI transcription factor revealed not only overlapping, but also specific functions like regulation of proliferation rate and expression levels of pathway components as well as differentiation markers.

The results presented here are based on analyses of transcripts levels in combination with reporter assays as functional read-out on protein level. Hence, the question still remains open how Hedgehog pathway proteins are presented and distributed in human sebocytes in a time-dependent manner during differentiation. As consequence, the identity of Hh signal transmitting and receiving cells has not been clearly shown, yet.

To address this, specific antibodies (in particular for Hh ligands and Gli transcription factors) are required to perform co-localisation analyses with components of the Hh pathway and differentiation markers (e.g. lipid droplets and molecular markers).

Additionally, fluorescence-activated cell sorting (FACS) could be applied for isolation of sebocytes in dependence on sebaceous differentiation markers for subsequent profound analysis of Hedgehog pathway transmission and activation on expression (e.g. gene and protein analyses) and functional level (e.g. proliferation and reporter assays).

To further investigate the differences in GLI functionalities, FACS would help to isolate GLI1-, GLI2-, GLI3-overexpressing as well as GLI1-, GLI2- and GLI3-deficient sebocytes and to further determine the individual role of these transcription factors during sebaceous gland homeostasis. In this context, alternative methods would be necessary to generate GLI1-, GLI2- and GLI3-deficient sebocytes, since it was not possible to suppress GLI expression by

siRNA and shRNA methods in our hands (see chapter 3.4.). Recently, a new technique for gene editing, knock-out and knock-in, previously described in gene therapy, is commercially available for the research cell culture using synthetic zinc finger nucleases (ZNFs), which are specifically designed for the customer needs. Introducing a complete knock-out would definitively circumvent not only low gene silencing efficiency observed by the GLI knock-down assays, but also decrease the risk of off-target effects [397-398]. Alternatively, an inducible knockout model specifically directed against Gli1, Gli2 and Gli3 in sebaceous gland of mice would represent an effective tool do perform these analyses.

We also described that the lipid metabolism plays a crucial role in the regulation of key Hedgehog pathway components and Wnt inhibitors when we treated sebocytes with arachidonic acid.

Hence, profound promoter sequence analyses of the presented regulated genes in human sebocytes would be necessary to dissect the mechanism which underlies their regulation by the lipid metabolism. Thereby, known fatty acid response elements (e.g. PUFA-RE and PPRE) could be searched for. This is of particular interest, as sebaceous glands represent a lipid-enriched microenvironment where gene regulation by fatty acid could play a major physiological role.

Moreover, we demonstrated that β-catenin and Gli proteins mutually interfere during induction of specific reporter constructs suggesting that the outcomes of Hh and Wnt pathway activity are modulated by direct or indirect interactions between these factors.

The question whether a direct interaction or an indirect effect through other factors underlies the interference between Gli and β-catenin function also needs to be answered. Therefore, co-immunoprecipitation experiments with these factors have to be performed. Moreover, site-directed mutagenesis of potential binding domains would provide information about the mechanism of interaction and physiological role of GLI-dependent regulation of β-CATENIN in human sebocytes and in other tissues, too.

Benefiting from these data, new *in vivo* models could be generated, which positively or negatively affect the Hedgehog pathway and its interaction with the Wnt pathway. These would not only greatly extend our knowledge of the underlying molecular mechanisms during sebaceous gland development and homeostasis but also of their implication in other tissues. Thereby, potential targets for the treatment of skin disorders like acne and skin cancers could be identified and give rise to new therapies.

5. References

1. **Nusslein-Volhard, C. and E. Wieschaus, *Mutations affecting segment number and polarity in Drosophila.* Nature, 1980. 287(5785): p. 795-801.**
2. **Wada, H. and K. Makabe, *Genome duplications of early vertebrates as a possible chronicle of the evolutionary history of the neural crest.* Int J Biol Sci, 2006. 2(3): p. 133-41.**
3. **Echelard, Y., et al., *Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity.* Cell, 1993. 75(7): p. 1417-30.**
4. **Roelink, H., et al., *Floor plate and motor neuron induction by vhh-1, a vertebrate homolog of hedgehog expressed by the notochord.* Cell, 1994. 76(4): p. 761-75.**
5. **Jessell, T.M., *Neuronal specification in the spinal cord: inductive signals and transcriptional codes.* Nat Rev Genet, 2000. 1(1): p. 20-9.**
6. **Roelink, H., et al., *Floor plate and motor neuron induction by different concentrations of the amino-terminal cleavage product of sonic hedgehog autoproteolysis.* Cell, 1995. 81(3): p. 445-55.**
7. **Marti, E., et al., *Distribution of Sonic hedgehog peptides in the developing chick and mouse embryo.* Development, 1995. 121(8): p. 2537-47.**
8. **Placzek, M. and J. Briscoe, *The floor plate: multiple cells, multiple signals.* Nat Rev Neurosci, 2005. 6(3): p. 230-40.**
9. **Sampath, K., et al., *Functional differences among Xenopus nodal-related genes in left-right axis determination.* Development, 1997. 124(17): p. 3293-302.**
10. **Pagan-Westphal, S.M. and C.J. Tabin, *The transfer of left-right positional information during chick embryogenesis.* Cell, 1998. 93(1): p. 25-35.**
11. **Schilling, T.F., J.P. Concordet, and P.W. Ingham, *Regulation of left-right asymmetries in the zebrafish by Shh and BMP4.* Dev Biol, 1999. 210(2): p. 277-87.**
12. **Watanabe, Y. and H. Nakamura, *Control of chick tectum territory along dorsoventral axis by Sonic hedgehog.* Development, 2000. 127(5): p. 1131-40.**
13. **Meyer, N.P. and H. Roelink, *The amino-terminal region of Gli3 antagonizes the Shh response and acts in dorsoventral fate specification in the developing spinal cord.* Dev Biol, 2003. 257(2): p. 343-55.**
14. **Pepicelli, C.V., P.M. Lewis, and A.P. McMahon, *Sonic hedgehog regulates branching morphogenesis in the mammalian lung.* Curr Biol, 1998. 8(19): p. 1083-6.**
15. **Bellusci, S., et al., *Involvement of Sonic hedgehog (Shh) in mouse embryonic lung growth and morphogenesis.* Development, 1997. 124(1): p. 53-63.**
16. **Urase, K., et al., *Spatial expression of Sonic hedgehog in the lung epithelium during branching morphogenesis.* Biochem Biophys Res Commun, 1996. 225(1): p. 161-6.**
17. **Chuang, P.T., T. Kawcak, and A.P. McMahon, *Feedback control of mammalian Hedgehog signaling by the Hedgehog-binding protein, Hip1, modulates Fgf signaling during branching morphogenesis of the lung.* Genes Dev, 2003. 17(3): p. 342-7.**
18. **Dahmane, N., et al., *Activation of the transcription factor Gli1 and the Sonic hedgehog signalling pathway in skin tumours.* Nature, 1997. 389(6653): p. 876-81.**
19. **Oro, A.E., et al., *Basal cell carcinomas in mice overexpressing sonic hedgehog.* Science, 1997. 276(5313): p. 817-21.**

20. **Morgan, B.A., et al., *Stage-specific effects of sonic hedgehog expression in the epidermis.* Dev Biol, 1998. 201(1): p. 1-12.**
21. **St-Jacques, B., et al., *Sonic hedgehog signaling is essential for hair development.* Curr Biol, 1998. 8(19): p. 1058-68.**
22. **Silva-Vargas, V., et al., *Beta-catenin and Hedgehog signal strength can specify number and location of hair follicles in adult epidermis without recruitment of bulge stem cells.* Dev Cell, 2005. 9(1): p. 121-31.**
23. **Iseki, S., et al., *Sonic hedgehog is expressed in epithelial cells during development of whisker, hair, and tooth.* Biochem Biophys Res Commun, 1996. 218(3): p. 688-93.**
24. **Reddy, S., et al., *Characterization of Wnt gene expression in developing and postnatal hair follicles and identification of Wnt5a as a target of Sonic hedgehog in hair follicle morphogenesis.* Mech Dev, 2001. 107(1-2): p. 69-82.**
25. **Riddle, R.D., et al., *Sonic hedgehog mediates the polarizing activity of the ZPA.* Cell, 1993. 75(7): p. 1401-16.**
26. **Chang, D.T., et al., *Products, genetic linkage and limb patterning activity of a murine hedgehog gene.* Development, 1994. 120(11): p. 3339-53.**
27. **Johnson, R.L., et al., *Ectopic expression of Sonic hedgehog alters dorsal-ventral patterning of somites.* Cell, 1994. 79(7): p. 1165-73.**
28. **Hardcastle, Z., et al., *The Shh signalling pathway in tooth development: defects in Gli2 and Gli3 mutants.* Development, 1998. 125(15): p. 2803-11.**
29. **Gritli-Linde, A., et al., *Shh signaling within the dental epithelium is necessary for cell proliferation, growth and polarization.* Development, 2002. 129(23): p. 5323-37.**
30. **Vortkamp, A., et al., *Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein.* Science, 1996. 273(5275): p. 613-22.**
31. **Lanske, B., et al., *PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth.* Science, 1996. 273(5275): p. 663-6.**
32. **Litingtung, Y., et al., *Sonic hedgehog is essential to foregut development.* Nat Genet, 1998. 20(1): p. 58-61.**
33. **Hebrok, M., et al., *Regulation of pancreas development by hedgehog signaling.* Development, 2000. 127(22): p. 4905-13.**
34. **Tsukui, T., et al., *Multiple left-right asymmetry defects in Shh(-/-) mutant mice unveil a convergence of the shh and retinoic acid pathways in the control of Lefty-1.* Proc Natl Acad Sci U S A, 1999. 96(20): p. 11376-81.**
35. **Chiang, C., et al., *Essential role for Sonic hedgehog during hair follicle morphogenesis.* Dev Biol, 1999. 205(1): p. 1-9.**
36. **Dassule, H.R., et al., *Sonic hedgehog regulates growth and morphogenesis of the tooth.* Development, 2000. 127(22): p. 4775-85.**
37. **Bitgood, M.J., L. Shen, and A.P. McMahon, *Sertoli cell signaling by Desert hedgehog regulates the male germline.* Curr Biol, 1996. 6(3): p. 298-304.**
38. **Wijgerde, M., et al., *Hedgehog signaling in mouse ovary: Indian hedgehog and desert hedgehog from granulosa cells induce target gene expression in developing theca cells.* Endocrinology, 2005. 146(8): p. 3558-66.**
39. **Yao, H.H., W. Whoriskey, and B. Capel, *Desert Hedgehog/Patched 1 signaling specifies fetal Leydig cell fate in testis organogenesis.* Genes Dev, 2002. 16(11): p. 1433-40.**
40. **St-Jacques, B., M. Hammerschmidt, and A.P. McMahon, *Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation.* Genes Dev, 1999. 13(16): p. 2072-86.**
41. **Dyer, M.A., et al., *Indian hedgehog activates hematopoiesis and vasculogenesis and can respecify prospective neurectodermal cell fate in the mouse embryo.* Development, 2001. 128(10): p. 1717-30.**

42. **van den Brink, G.R., *Hedgehog signaling in development and homeostasis of the gastrointestinal tract.* Physiol Rev, 2007. 87(4): p. 1343-75.**

43. **Niemann, C., et al., *Indian hedgehog and beta-catenin signaling: role in the sebaceous lineage of normal and neoplastic mammalian epidermis.* Proc Natl Acad Sci U S A, 2003. 100 Suppl 1: p. 11873-80.**

44. **Gallego, M.I., et al., *Differential requirements for shh in mammary tissue and hair follicle morphogenesis.* Dev Biol, 2002. 249(1): p. 131-9.**

45. **Hatsell, S.J. and P. Cowin, *Gli3-mediated repression of Hedgehog targets is required for normal mammary development.* Development, 2006. 133(18): p. 3661-70.**

46. **Long, F., et al., *Genetic manipulation of hedgehog signaling in the endochondral skeleton reveals a direct role in the regulation of chondrocyte proliferation.* Development, 2001. 128(24): p. 5099-108.**

47. **Dakubo, G.D., et al., *Indian hedgehog signaling from endothelial cells is required for sclera and retinal pigment epithelium development in the mouse eye.* Dev Biol, 2008. 320(1): p. 242-55.**

48. **Razzaque, M.S., et al., *Conditional deletion of Indian hedgehog from collagen type 2alpha1-expressing cells results in abnormal endochondral bone formation.* J Pathol, 2005. 207(4): p. 453-61.**

49. **Burke, R., et al., *Dispatched, a novel sterol-sensing domain protein dedicated to the release of cholesterol-modified hedgehog from signaling cells.* Cell, 1999. 99(7): p. 803-15.**

50. **Amanai, K. and J. Jiang, *Distinct roles of Central missing and Dispatched in sending the Hedgehog signal.* Development, 2001. 128(24): p. 5119-27.**

51. **Chamoun, Z., et al., *Skinny hedgehog, an acyltransferase required for palmitoylation and activity of the hedgehog signal.* Science, 2001. 293(5537): p. 2080-4.**

52. **Ingham, P.W. and A.P. McMahon, *Hedgehog signaling in animal development: paradigms and principles.* Genes Dev, 2001. 15(23): p. 3059-87.**

53. **Caspary, T., et al., *Mouse Dispatched homolog1 is required for long-range, but not juxtacrine, Hh signaling.* Curr Biol, 2002. 12(18): p. 1628-32.**

54. **Dai, P., et al., *Ski is involved in transcriptional regulation by the repressor and full-length forms of Gli3.* Genes Dev, 2002. 16(22): p. 2843-8.**

55. **Ma, Y., et al., *Hedgehog-mediated patterning of the mammalian embryo requires transporter-like function of dispatched.* Cell, 2002. 111(1): p. 63-75.**

56. **Lee, J.D., et al., *An acylatable residue of Hedgehog is differentially required in Drosophila and mouse limb development.* Dev Biol, 2001. 233(1): p. 122-36.**

57. **Porter, J.A., K.E. Young, and P.A. Beachy, *Cholesterol modification of hedgehog signaling proteins in animal development.* Science, 1996. 274(5285): p. 255-9.**

58. **Zhu, A.J. and M.P. Scott, *Incredible journey: how do developmental signals travel through tissue?* Genes Dev, 2004. 18(24): p. 2985-97.**

59. **The, I., Y. Bellaiche, and N. Perrimon, *Hedgehog movement is regulated through tout velu-dependent synthesis of a heparan sulfate proteoglycan.* Mol Cell, 1999. 4(4): p. 633-9.**

60. **Chuang, P.T. and A.P. McMahon, *Vertebrate Hedgehog signalling modulated by induction of a Hedgehog-binding protein.* Nature, 1999. 397(6720): p. 617-21.**

61. **Rahnama, F., R. Toftgard, and P.G. Zaphiropoulos, *Distinct roles of PTCH2 splice variants in Hedgehog signalling.* Biochem J, 2004. 378(Pt 2): p. 325-34.**

62. **Hahn, H., et al., *A mammalian patched homolog is expressed in target tissues of sonic hedgehog and maps to a region associated with developmental abnormalities.* J Biol Chem, 1996. 271(21): p. 12125-8.**

63. **Carpenter, D., et al., *Characterization of two patched receptors for the vertebrate hedgehog protein family.* Proc Natl Acad Sci U S A, 1998. 95(23): p. 13630-4.**

64. **Lee, Y., et al., *Patched2 modulates tumorigenesis in patched1 heterozygous mice.* Cancer Res, 2006. 66(14): p. 6964-71.**

65. **Casali, A. and G. Struhl, *Reading the Hedgehog morphogen gradient by measuring the ratio of bound to unbound Patched protein.* Nature, 2004. 431(7004): p. 76-80.**

66. **Murakami, S., et al., *Crystal structure of bacterial multidrug efflux transporter AcrB.* Nature, 2002. 419(6907): p. 587-93.**

67. **Lu, X., S. Liu, and T.B. Kornberg, *The C-terminal tail of the Hedgehog receptor Patched regulates both localization and turnover.* Genes Dev, 2006. 20(18): p. 2539-51.**

68. **Taipale, J., et al., *Patched acts catalytically to suppress the activity of Smoothened.* Nature, 2002. 418(6900): p. 892-7.**

69. **Stone, D.M., et al., *The tumour-suppressor gene patched encodes a candidate receptor for Sonic hedgehog.* Nature, 1996. 384(6605): p. 129-34.**

70. **Denef, N., et al., *Hedgehog induces opposite changes in turnover and subcellular localization of patched and smoothened.* Cell, 2000. 102(4): p. 521-31.**

71. **Strutt, H., et al., *Mutations in the sterol-sensing domain of Patched suggest a role for vesicular trafficking in Smoothened regulation.* Curr Biol, 2001. 11(8): p. 608-13.**

72. **Martin, V., et al., *The sterol-sensing domain of Patched protein seems to control Smoothened activity through Patched vesicular trafficking.* Curr Biol, 2001. 11(8): p. 601-7.**

73. **Incardona, J.P., J. Gruenberg, and H. Roelink, *Sonic hedgehog induces the segregation of patched and smoothened in endosomes.* Curr Biol, 2002. 12(12): p. 983-95.**

74. **Zhu, A.J., et al., *Altered localization of Drosophila Smoothened protein activates Hedgehog signal transduction.* Genes Dev, 2003. 17(10): p. 1240-52.**

75. **Nakano, Y., et al., *Functional domains and sub-cellular distribution of the Hedgehog transducing protein Smoothened in Drosophila.* Mech Dev, 2004. 121(6): p. 507-18.**

76. **Caspary, T., C.E. Larkins, and K.V. Anderson, *The graded response to Sonic Hedgehog depends on cilia architecture.* Dev Cell, 2007. 12(5): p. 767-78.**

77. **Huangfu, D. and K.V. Anderson, *Signaling from Smo to Ci/Gli: conservation and divergence of Hedgehog pathways from Drosophila to vertebrates.* Development, 2006. 133(1): p. 3-14.**

78. **Huangfu, D., et al., *Hedgehog signalling in the mouse requires intraflagellar transport proteins.* Nature, 2003. 426(6962): p. 83-7.**

79. **Park, T.J., S.L. Haigo, and J.B. Wallingford, *Ciliogenesis defects in embryos lacking inturned or fuzzy function are associated with failure of planar cell polarity and Hedgehog signaling.* Nat Genet, 2006. 38(3): p. 303-11.**

80. **Vierkotten, J., et al., *Ftm is a novel basal body protein of cilia involved in Shh signalling.* Development, 2007. 134(14): p. 2569-77.**

81. **Rosenbaum, J.L. and G.B. Witman, *Intraflagellar transport.* Nat Rev Mol Cell Biol, 2002. 3(11): p. 813-25.**

82. **Corbit, K.C., et al., *Vertebrate Smoothened functions at the primary cilium.* Nature, 2005. 437(7061): p. 1018-21.**

83. **Rohatgi, R., L. Milenkovic, and M.P. Scott, *Patched1 regulates hedgehog signaling at the primary cilium.* Science, 2007. 317(5836): p. 372-6.**

84. **Lin, X., et al., *Disruption of gastrulation and heparan sulfate biosynthesis in EXT1-deficient mice.* Dev Biol, 2000. 224(2): p. 299-311.**

85. **Lum, L., et al., *Identification of Hedgehog pathway components by RNAi in Drosophila cultured cells.* Science, 2003. 299(5615): p. 2039-45.**

86. **Bellaiche, Y., I. The, and N. Perrimon, *Tout-velu is a Drosophila homologue of the putative tumour suppressor EXT-1 and is needed for Hh diffusion.* Nature, 1998. 394(6688): p. 85-8.**

87. **Bornemann, D.J., et al., *Abrogation of heparan sulfate synthesis in Drosophila disrupts the Wingless, Hedgehog and Decapentaplegic signaling pathways.* Development, 2004. 131(9): p. 1927-38.**

88. **Han, C., et al., *Distinct and collaborative roles of Drosophila EXT family proteins in morphogen signalling and gradient formation.* Development, 2004. 131(7): p. 1563-75.**

89. **Koziel, L., et al., *Ext1-dependent heparan sulfate regulates the range of Ihh signaling during endochondral ossification.* Dev Cell, 2004. 6(6): p. 801-13.**

90. **Allen, B.L., T. Tenzen, and A.P. McMahon, *The Hedgehog-binding proteins Gas1 and Cdo cooperate to positively regulate Shh signaling during mouse development.* Genes Dev, 2007. 21(10): p. 1244-57.**

91. **Tenzen, T., et al., *The cell surface membrane proteins Cdo and Boc are components and targets of the Hedgehog signaling pathway and feedback network in mice.* Dev Cell, 2006. 10(5): p. 647-56.**

92. **Zhang, W., et al., *Cdo functions at multiple points in the Sonic Hedgehog pathway, and Cdo-deficient mice accurately model human holoprosencephaly.* Dev Cell, 2006. 10(5): p. 657-65.**

93. **McLellan, J.S., et al., *The mode of Hedgehog binding to Ihog homologues is not conserved across different phyla.* Nature, 2008. 455(7215): p. 979-83.**

94. **Merchant, M., et al., *Suppressor of fused regulates Gli activity through a dual binding mechanism.* Mol Cell Biol, 2004. 24(19): p. 8627-41.**

95. **Liem, K.F., Jr., et al., *Mouse Kif7/Costal2 is a cilia-associated protein that regulates Sonic hedgehog signaling.* Proc Natl Acad Sci U S A, 2009. 106(32): p. 13377-82.**

96. **Pan, Y., et al., *Sonic hedgehog signaling regulates Gli2 transcriptional activity by suppressing its processing and degradation.* Mol Cell Biol, 2006. 26(9): p. 3365-77.**

97. **Wang, B., J.F. Fallon, and P.A. Beachy, *Hedgehog-regulated processing of Gli3 produces an anterior/posterior repressor gradient in the developing vertebrate limb.* Cell, 2000. 100(4): p. 423-34.**

98. **Jacob, J. and J. Briscoe, *Gli proteins and the control of spinal-cord patterning.* EMBO Rep, 2003. 4(8): p. 761-5.**

99. **Ruiz i Altaba, A., C. Mas, and B. Stecca, *The Gli code: an information nexus regulating cell fate, stemness and cancer.* Trends Cell Biol, 2007. 17(9): p. 438-47.**

100. **Bai, C.B. and A.L. Joyner, *Gli1 can rescue the in vivo function of Gli2.* Development, 2001. 128(24): p. 5161-72.**

101. **Eichberger, T., et al., *Overlapping and distinct transcriptional regulator properties of the GLI1 and GLI2 oncogenes.* Genomics, 2006. 87(5): p. 616-32.**

102. **Roessler, E., et al., *A previously unidentified amino-terminal domain regulates transcriptional activity of wild-type and disease-associated human GLI2.* Hum Mol Genet, 2005. 14(15): p. 2181-8.**

103. **Litingtung, Y., et al., *Shh and Gli3 are dispensable for limb skeleton formation but regulate digit number and identity.* Nature, 2002. 418(6901): p. 979-83.**

104. **Paces-Fessy, M., et al., *The negative regulator of Gli, Suppressor of fused (Sufu), interacts with SAP18, Galectin3 and other nuclear proteins.* Biochem J, 2004. 378(Pt 2): p. 353-62.**

105. **Jia, J., et al., *Shaggy/GSK3 antagonizes Hedgehog signalling by regulating Cubitus interruptus.* Nature, 2002. 416(6880): p. 548-52.**

106. **Wang, B. and Y. Li, *Evidence for the direct involvement of {beta}TrCP in Gli3 protein processing.* Proc Natl Acad Sci U S A, 2006. 103(1): p. 33-8.**

107. **Huntzicker, E.G., et al., *Dual degradation signals control Gli protein stability and tumor formation.* Genes Dev, 2006. 20(3): p. 276-81.**

108. **Karlsson, L., C. Bondjers, and C. Betsholtz, *Roles for PDGF-A and sonic hedgehog in development of mesenchymal components of the hair follicle.* Development, 1999. 126(12): p. 2611-21.**

109. **Oro, A.E. and K. Higgins, *Hair cycle regulation of Hedgehog signal reception.* Dev Biol, 2003. 255(2): p. 238-48.**

110. **Sato, N., P.L. Leopold, and R.G. Crystal, *Induction of the hair growth phase in postnatal mice by localized transient expression of Sonic hedgehog.* J Clin Invest, 1999. 104(7): p. 855-64.**

111. **Michno, K., et al., *Shh expression is required for embryonic hair follicle but not mammary gland development.* Dev Biol, 2003. 264(1): p. 153-65.**

112. **Allen, M., et al., *Hedgehog signaling regulates sebaceous gland development.* Am J Pathol, 2003. 163(6): p. 2173-8.**

113. **Ming, J.E., et al., *Mutations in PATCHED-1, the receptor for SONIC HEDGEHOG, are associated with holoprosencephaly.* Hum Genet, 2002. 110(4): p. 297-301.**

114. **Belloni, E., et al., *Identification of Sonic hedgehog as a candidate gene responsible for holoprosencephaly.* Nat Genet, 1996. 14(3): p. 353-6.**

115. **Roessler, E., et al., *Mutations in the human Sonic Hedgehog gene cause holoprosencephaly.* Nat Genet, 1996. 14(3): p. 357-60.**

116. **Brown, L.Y., et al., *Holoprosencephaly due to mutations in ZIC2: alanine tract expansion mutations may be caused by parental somatic recombination.* Hum Mol Genet, 2001. 10(8): p. 791-6.**

117. **Radhakrishna, U., et al., *Mutation in GLI3 in postaxial polydactyly type A.* Nat Genet, 1997. 17(3): p. 269-71.**

118. **Kang, S., et al., *GLI3 frameshift mutations cause autosomal dominant Pallister-Hall syndrome.* Nat Genet, 1997. 15(3): p. 266-8.**

119. **Kim, P.C., R. Mo, and C. Hui Cc, *Murine models of VACTERL syndrome: Role of sonic hedgehog signaling pathway.* J Pediatr Surg, 2001. 36(2): p. 381-4.**

120. **Shefer, S., et al., *Markedly inhibited 7-dehydrocholesterol-delta 7-reductase activity in liver microsomes from Smith-Lemli-Opitz homozygotes.* J Clin Invest, 1995. 96(4): p. 1779-85.**

121. **Incardona, J.P., et al., *Receptor-mediated endocytosis of soluble and membrane-tethered Sonic hedgehog by Patched-1.* Proc Natl Acad Sci U S A, 2000. 97(22): p. 12044-9.**

122. **Kelley, R.L., et al., *Holoprosencephaly in RSH/Smith-Lemli-Opitz syndrome: does abnormal cholesterol metabolism affect the function of Sonic Hedgehog?* Am J Med Genet, 1996. 66(4): p. 478-84.**

123. **Gorlin, R.J. and R.W. Goltz, *Multiple nevoid basal-cell epithelioma, jaw cysts and bifid rib. A syndrome.* N Engl J Med, 1960. 262: p. 908-12.**

124. **Howell, J.B. and M.R. Caro, *The basal-cell nevus: its relationship to multiple cutaneous cancers and associated anomalies of development.* AMA Arch Derm, 1959. 79(1): p. 67-77; discussion 77-80.**

125. **Hahn, H., et al., *Mutations of the human homolog of Drosophila patched in the nevoid basal cell carcinoma syndrome.* Cell, 1996. 85(6): p. 841-51.**

126. Goodrich, L.V., et al., *Conservation of the hedgehog/patched signaling pathway from flies to mice: induction of a mouse patched gene by Hedgehog.* Genes Dev, 1996. 10(3): p. 301-12.

127. Chidambaram, A., et al., *Mutations in the human homologue of the Drosophila patched gene in Caucasian and African-American nevoid basal cell carcinoma syndrome patients.* Cancer Res, 1996. 56(20): p. 4599-601.

128. Wicking, C., et al., *Most germ-line mutations in the nevoid basal cell carcinoma syndrome lead to a premature termination of the PATCHED protein, and no genotype-phenotype correlations are evident.* Am J Hum Genet, 1997. 60(1): p. 21-6.

129. Xie, J., et al., *Activating Smoothened mutations in sporadic basal-cell carcinoma.* Nature, 1998. 391(6662): p. 90-2.

130. Reifenberger, J., et al., *Somatic mutations in the PTCH, SMOH, SUFUH and TP53 genes in sporadic basal cell carcinomas.* Br J Dermatol, 2005. 152(1): p. 43-51.

131. Pietsch, T., et al., *Medulloblastomas of the desmoplastic variant carry mutations of the human homologue of Drosophila patched.* Cancer Res, 1997. 57(11): p. 2085-8.

132. Raffel, C., et al., *Sporadic medulloblastomas contain PTCH mutations.* Cancer Res, 1997. 57(5): p. 842-5.

133. Wolter, M., et al., *Mutations in the human homologue of the Drosophila segment polarity gene patched (PTCH) in sporadic basal cell carcinomas of the skin and primitive neuroectodermal tumors of the central nervous system.* Cancer Res, 1997. 57(13): p. 2581-5.

134. Maesawa, C., et al., *Mutations in the human homologue of the Drosophila patched gene in esophageal squamous cell carcinoma.* Genes Chromosomes Cancer, 1998. 21(3): p. 276-9.

135. McGarvey, T.W., et al., *PTCH gene mutations in invasive transitional cell carcinoma of the bladder.* Oncogene, 1998. 17(9): p. 1167-72.

136. Tostar, U., et al., *Deregulation of the hedgehog signalling pathway: a possible role for the PTCH and SUFU genes in human rhabdomyoma and rhabdomyosarcoma development.* J Pathol, 2006. 208(1): p. 17-25.

137. Wen, P.Y. and S. Kesari, *Malignant gliomas in adults.* N Engl J Med, 2008. 359(5): p. 492-507.

138. Kinzler, K.W., et al., *Identification of an amplified, highly expressed gene in a human glioma.* Science, 1987. 236(4797): p. 70-3.

139. Collins, V.P., *Gene amplification in human gliomas.* Glia, 1995. 15(3): p. 289-96.

140. Kinzler, K.W., et al., *The GLI gene is a member of the Kruppel family of zinc finger proteins.* Nature, 1988. 332(6162): p. 371-4.

141. Bigner, S.H., et al., *Gene amplification in malignant human gliomas: clinical and histopathologic aspects.* J Neuropathol Exp Neurol, 1988. 47(3): p. 191-205.

142. Thorpe, C.J., A. Schlesinger, and B. Bowerman, *Wnt signalling in Caenorhabditis elegans: regulating repressors and polarizing the cytoskeleton.* Trends Cell Biol, 2000. 10(1): p. 10-7.

143. Kuhl, M., et al., *The Wnt/Ca2+ pathway: a new vertebrate Wnt signaling pathway takes shape.* Trends Genet, 2000. 16(7): p. 279-83.

144. Nusse, R., et al., *A new nomenclature for int-1 and related genes: the Wnt gene family.* Cell, 1991. 64(2): p. 231.

145. Nusse, R. and H.E. Varmus, *Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome.* Cell, 1982. 31(1): p. 99-109.

146. Rijsewijk, F., et al., *The Drosophila homolog of the mouse mammary oncogene int-1 is identical to the segment polarity gene wingless.* Cell, 1987. 50(4): p. 649-57.

147. Cabrera, C.V., et al., *Phenocopies induced with antisense RNA identify the wingless gene.* Cell, 1987. 50(4): p. 659-63.

148. Huber, A.H., W.J. Nelson, and W.I. Weis, *Three-dimensional structure of the armadillo repeat region of beta-catenin.* Cell, 1997. 90(5): p. 871-82.

149. Cox, R.T., et al., *Membrane-tethered Drosophila Armadillo cannot transduce Wingless signal on its own.* Development, 1999. 126(6): p. 1327-35.

150. Ciruna, B. and J. Rossant, *FGF signaling regulates mesoderm cell fate specification and morphogenetic movement at the primitive streak.* Dev Cell, 2001. 1(1): p. 37-49.

151. Heasman, J., et al., *Overexpression of cadherins and underexpression of beta-catenin inhibit dorsal mesoderm induction in early Xenopus embryos.* Cell, 1994. 79(5): p. 791-803.

152. Gottardi, C.J. and B.M. Gumbiner, *Distinct molecular forms of beta-catenin are targeted to adhesive or transcriptional complexes.* J Cell Biol, 2004. 167(2): p. 339-49.

153. Ben-Ze'ev, A., *The dual role of cytoskeletal anchor proteins in cell adhesion and signal transduction.* Ann N Y Acad Sci, 1999. 886: p. 37-47.

154. Burrus, L.W. and A.P. McMahon, *Biochemical analysis of murine Wnt proteins reveals both shared and distinct properties.* Exp Cell Res, 1995. 220(2): p. 363-73.

155. Kadowaki, T., et al., *The segment polarity gene porcupine encodes a putative multitransmembrane protein involved in Wingless processing.* Genes Dev, 1996. 10(24): p. 3116-28.

156. Komekado, H., et al., *Glycosylation and palmitoylation of Wnt-3a are coupled to produce an active form of Wnt-3a.* Genes Cells, 2007. 12(4): p. 521-34.

157. Kurayoshi, M., et al., *Post-translational palmitoylation and glycosylation of Wnt-5a are necessary for its signalling.* Biochem J, 2007. 402(3): p. 515-23.

158. Mason, J.O., J. Kitajewski, and H.E. Varmus, *Mutational analysis of mouse Wnt-1 identifies two temperature-sensitive alleles and attributes of Wnt-1 protein essential for transformation of a mammary cell line.* Mol Biol Cell, 1992. 3(5): p. 521-33.

159. Smolich, B.D., et al., *Wnt family proteins are secreted and associated with the cell surface.* Mol Biol Cell, 1993. 4(12): p. 1267-75.

160. Tanaka, K., Y. Kitagawa, and T. Kadowaki, *Drosophila segment polarity gene product porcupine stimulates the posttranslational N-glycosylation of wingless in the endoplasmic reticulum.* J Biol Chem, 2002. 277(15): p. 12816-23.

161. Willert, K., et al., *Wnt proteins are lipid-modified and can act as stem cell growth factors.* Nature, 2003. 423(6938): p. 448-52.

162. Takada, R., et al., *Monounsaturated fatty acid modification of Wnt protein: its role in Wnt secretion.* Dev Cell, 2006. 11(6): p. 791-801.

163. Galli, L.M., et al., *Porcupine-mediated lipid-modification regulates the activity and distribution of Wnt proteins in the chick neural tube.* Development, 2007. 134(18): p. 3339-48.

164. Banziger, C., et al., *Wntless, a conserved membrane protein dedicated to the secretion of Wnt proteins from signaling cells.* Cell, 2006. 125(3): p. 509-22.

165. Belenkaya, T.Y., et al., *The retromer complex influences Wnt secretion by recycling wntless from endosomes to the trans-Golgi network.* Dev Cell, 2008. 14(1): p. 120-31.

166. Franch-Marro, X., et al., *Wingless secretion requires endosome-to-Golgi retrieval of Wntless/Evi/Sprinter by the retromer complex.* Nat Cell Biol, 2008. 10(2): p. 170-7.

167. Port, F., et al., *Wingless secretion promotes and requires retromer-dependent cycling of Wntless.* Nat Cell Biol, 2008. 10(2): p. 178-85.

168. Yang, P.T., et al., *Wnt signaling requires retromer-dependent recycling of MIG-14/Wntless in Wnt-producing cells.* Dev Cell, 2008. 14(1): p. 140-7.

169. Seaman, M.N., *Recycle your receptors with retromer.* Trends Cell Biol, 2005. 15(2): p. 68-75.

170. van den Heuvel, M., et al., *Mutations in the segment polarity genes wingless and porcupine impair secretion of the wingless protein.* EMBO J, 1993. 12(13): p. 5293-302.

171. Cadigan, K.M., *Regulating morphogen gradients in the Drosophila wing.* Semin Cell Dev Biol, 2002. 13(2): p. 83-90.

172. Strigini, M. and S.M. Cohen, *Formation of morphogen gradients in the Drosophila wing.* Semin Cell Dev Biol, 1999. 10(3): p. 335-44.

173. Blair, S.S., *Cell signaling: wingless and glypicans together again.* Curr Biol, 2005. 15(3): p. R92-4.

174. Franch-Marro, X., et al., *Glypicans shunt the Wingless signal between local signalling and further transport.* Development, 2005. 132(4): p. 659-66.

175. Gallet, A., L. Staccini-Lavenant, and P.P. Therond, *Cellular trafficking of the glypican Dally-like is required for full-strength Hedgehog signaling and wingless transcytosis.* Dev Cell, 2008. 14(5): p. 712-25.

176. Tamai, K., et al., *A mechanism for Wnt coreceptor activation.* Mol Cell, 2004. 13(1): p. 149-56.

177. Zeng, X., et al., *A dual-kinase mechanism for Wnt co-receptor phosphorylation and activation.* Nature, 2005. 438(7069): p. 873-7.

178. Daniels, D.L. and W.I. Weis, *Beta-catenin directly displaces Groucho/TLE repressors from Tcf/Lef in Wnt-mediated transcription activation.* Nat Struct Mol Biol, 2005. 12(4): p. 364-71.

179. Cavallo, R.A., et al., *Drosophila Tcf and Groucho interact to repress Wingless signalling activity.* Nature, 1998. 395(6702): p. 604-8.

180. Roose, J., et al., *The Xenopus Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors.* Nature, 1998. 395(6702): p. 608-12.

181. Cadigan, K.M. and Y.I. Liu, *Wnt signaling: complexity at the surface.* J Cell Sci, 2006. 119(Pt 3): p. 395-402.

182. Gordon, M.D. and R. Nusse, *Wnt signaling: multiple pathways, multiple receptors, and multiple transcription factors.* J Biol Chem, 2006. 281(32): p. 22429-33.

183. He, X., et al., *LDL receptor-related proteins 5 and 6 in Wnt/beta-catenin signaling: arrows point the way.* Development, 2004. 131(8): p. 1663-77.

184. Cong, F., L. Schweizer, and H. Varmus, *Wnt signals across the plasma membrane to activate the beta-catenin pathway by forming oligomers containing its receptors, Frizzled and LRP.* Development, 2004. 131(20): p. 5103-15.

185. Holmen, S.L., et al., *Wnt-independent activation of beta-catenin mediated by a Dkk1-Fz5 fusion protein.* Biochem Biophys Res Commun, 2005. 328(2): p. 533-9.

186. Tolwinski, N.S., et al., *Wg/Wnt signal can be transmitted through arrow/LRP5,6 and Axin independently of Zw3/Gsk3beta activity.* Dev Cell, 2003. 4(3): p. 407-18.

187. Tamai, K., et al., *LDL-receptor-related proteins in Wnt signal transduction.* Nature, 2000. 407(6803): p. 530-5.

188. **Davidson, G., et al., *Casein kinase 1 gamma couples Wnt receptor activation to cytoplasmic signal transduction.* Nature, 2005. 438(7069): p. 867-72.**

189. **MacDonald, B.T., et al., *Wnt signal amplification via activity, cooperativity, and regulation of multiple intracellular PPPSP motifs in the Wnt co-receptor LRP6.* J Biol Chem, 2008. 283(23): p. 16115-23.**

190. **Bilic, J., et al., *Wnt induces LRP6 signalosomes and promotes dishevelled-dependent LRP6 phosphorylation.* Science, 2007. 316(5831): p. 1619-22.**

191. **Wallingford, J.B. and R. Habas, *The developmental biology of Dishevelled: an enigmatic protein governing cell fate and cell polarity.* Development, 2005. 132(20): p. 4421-36.**

192. **Wharton, K.A., Jr., *Runnin' with the Dvl: proteins that associate with Dsh/Dvl and their significance to Wnt signal transduction.* Dev Biol, 2003. 253(1): p. 1-17.**

193. **Molenaar, M., et al., *XTcf-3 transcription factor mediates beta-catenin-induced axis formation in Xenopus embryos.* Cell, 1996. 86(3): p. 391-9.**

194. **Behrens, J., et al., *Functional interaction of beta-catenin with the transcription factor LEF-1.* Nature, 1996. 382(6592): p. 638-42.**

195. **Sharpe, C., N. Lawrence, and A. Martinez Arias, *Wnt signalling: a theme with nuclear variations.* Bioessays, 2001. 23(4): p. 311-8.**

196. **Brembeck, F.H., et al., *Essential role of BCL9-2 in the switch between beta-catenin's adhesive and transcriptional functions.* Genes Dev, 2004. 18(18): p. 2225-30.**

197. **Kramps, T., et al., *Wnt/wingless signaling requires BCL9/legless-mediated recruitment of pygopus to the nuclear beta-catenin-TCF complex.* Cell, 2002. 109(1): p. 47-60.**

198. **Belenkaya, T.Y., et al., *pygopus Encodes a nuclear protein essential for wingless/Wnt signaling.* Development, 2002. 129(17): p. 4089-101.**

199. **Parker, D.S., J. Jemison, and K.M. Cadigan, *Pygopus, a nuclear PHD-finger protein required for Wingless signaling in Drosophila.* Development, 2002. 129(11): p. 2565-76.**

200. **Thompson, B., et al., *A new nuclear component of the Wnt signalling pathway.* Nat Cell Biol, 2002. 4(5): p. 367-73.**

201. **de la Roche, M. and M. Bienz, *Wingless-independent association of Pygopus with dTCF target genes.* Curr Biol, 2007. 17(6): p. 556-61.**

202. **Hecht, A., et al., *The p300/CBP acetyltransferases function as transcriptional coactivators of beta-catenin in vertebrates.* EMBO J, 2000. 19(8): p. 1839-50.**

203. **Mosimann, C., G. Hausmann, and K. Basler, *Parafibromin/Hyrax activates Wnt/Wg target gene transcription by direct association with beta-catenin/Armadillo.* Cell, 2006. 125(2): p. 327-41.**

204. **Wolf, D., et al., *Acetylation of beta-catenin by CREB-binding protein (CBP).* J Biol Chem, 2002. 277(28): p. 25562-7.**

205. **Arce, L., N.N. Yokoyama, and M.L. Waterman, *Diversity of LEF/TCF action in development and disease.* Oncogene, 2006. 25(57): p. 7492-504.**

206. **Hurlstone, A. and H. Clevers, *T-cell factors: turn-ons and turn-offs.* EMBO J, 2002. 21(10): p. 2303-11.**

207. **Tetsu, O. and F. McCormick, *Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells.* Nature, 1999. 398(6726): p. 422-6.**

208. **He, T.C., et al., *Identification of c-MYC as a target of the APC pathway.* Science, 1998. 281(5382): p. 1509-12.**

209. **Widelitz, R.B., *Wnt signaling in skin organogenesis.* Organogenesis, 2008. 4(2): p. 123-33.**

210. **Gat, U., et al., *De Novo hair follicle morphogenesis and hair tumors in mice expressing a truncated beta-catenin in skin.* Cell, 1998. 95(5): p. 605-14.**

211. Alonso, L. and E. Fuchs, *Stem cells in the skin: waste not, Wnt not.* Genes Dev, 2003. 17(10): p. 1189-200.

212. Zhang, Y., et al., *Activation of beta-catenin signaling programs embryonic epidermis to hair follicle fate.* Development, 2008. 135(12): p. 2161-72.

213. Suzuki, K., et al., *Embryonic hair follicle fate change by augmented beta-catenin through Shh and Bmp signaling.* Development, 2009. 136(3): p. 367-72.

214. Lowry, W.E., et al., *Defining the impact of beta-catenin/Tcf transactivation on epithelial stem cells.* Genes Dev, 2005. 19(13): p. 1596-611.

215. van Genderen, C., et al., *Development of several organs that require inductive epithelial-mesenchymal interactions is impaired in LEF-1-deficient mice.* Genes Dev, 1994. 8(22): p. 2691-703.

216. Huelsken, J., et al., *beta-Catenin controls hair follicle morphogenesis and stem cell differentiation in the skin.* Cell, 2001. 105(4): p. 533-45.

217. Niemann, C., et al., *Expression of DeltaNLef1 in mouse epidermis results in differentiation of hair follicles into squamous epidermal cysts and formation of skin tumours.* Development, 2002. 129(1): p. 95-109.

218. Jamora, C., et al., *Links between signal transduction, transcription and adhesion in epithelial bud development.* Nature, 2003. 422(6929): p. 317-22.

219. Merrill, B.J., et al., *Tcf3 and Lef1 regulate lineage differentiation of multipotent stem cells in skin.* Genes Dev, 2001. 15(13): p. 1688-705.

220. DasGupta, R., H. Rhee, and E. Fuchs, *A developmental conundrum: a stabilized form of beta-catenin lacking the transcriptional activation domain triggers features of hair cell fate in epidermal cells and epidermal cell fate in hair follicle cells.* J Cell Biol, 2002. 158(2): p. 331-44.

221. Chan, E.F., et al., *A common human skin tumour is caused by activating mutations in beta-catenin.* Nat Genet, 1999. 21(4): p. 410-3.

222. Lo Celso, C., D.M. Prowse, and F.M. Watt, *Transient activation of beta-catenin signalling in adult mouse epidermis is sufficient to induce new hair follicles but continuous activation is required to maintain hair follicle tumours.* Development, 2004. 131(8): p. 1787-99.

223. Niemann, C., et al., *Dual role of inactivating Lef1 mutations in epidermis: tumor promotion and specification of tumor type.* Cancer Res, 2007. 67(7): p. 2916-21.

224. Takeda, H., et al., *Human sebaceous tumors harbor inactivating mutations in LEF1.* Nat Med, 2006. 12(4): p. 395-7.

225. Ingham, P.W., *Segment polarity genes and cell patterning within the Drosophila body segment.* Curr Opin Genet Dev, 1991. 1(2): p. 261-7.

226. Alvarez-Medina, R., et al., *Wnt canonical pathway restricts graded Shh/Gli patterning activity through the regulation of Gli3 expression.* Development, 2008. 135(2): p. 237-47.

227. Ulloa, F., N. Itasaki, and J. Briscoe, *Inhibitory Gli3 activity negatively regulates Wnt/beta-catenin signaling.* Curr Biol, 2007. 17(6): p. 545-50.

228. van den Brink, G.R., et al., *Indian Hedgehog is an antagonist of Wnt signaling in colonic epithelial cell differentiation.* Nat Genet, 2004. 36(3): p. 277-82.

229. Varnat, F., G. Zacchetti, and A. Ruiz i Altaba, *Hedgehog pathway activity is required for the lethality and intestinal phenotypes of mice with hyperactive Wnt signaling.* Mech Dev, 2010. 127(1-2): p. 73-81.

230. Winston, J.T., et al., *The SCFbeta-TRCP-ubiquitin ligase complex associates specifically with phosphorylated destruction motifs in IkappaBalpha and beta-catenin and stimulates IkappaBalpha ubiquitination in vitro.* Genes Dev, 1999. 13(3): p. 270-83.

231. Maniatis, T., *A ubiquitin ligase complex essential for the NF-kappaB, Wnt/Wingless, and Hedgehog signaling pathways.* Genes Dev, 1999. 13(5): p. 505-10.

232. **Price, M.A. and D. Kalderon, *Proteolysis of the Hedgehog signaling effector Cubitus interruptus requires phosphorylation by Glycogen Synthase Kinase 3 and Casein Kinase 1.* Cell, 2002. 108(6): p. 823-35.**

233. **Lum, L., et al., *Hedgehog signal transduction via Smoothened association with a cytoplasmic complex scaffolded by the atypical kinesin, Costal-2.* Mol Cell, 2003. 12(5): p. 1261-74.**

234. **Kalderon, D., *Similarities between the Hedgehog and Wnt signaling pathways.* Trends Cell Biol, 2002. 12(11): p. 523-31.**

235. **Meng, X., et al., *Suppressor of fused negatively regulates beta-catenin signaling.* J Biol Chem, 2001. 276(43): p. 40113-9.**

236. **Taylor, M.D., et al., *Failure of a medulloblastoma-derived mutant of SUFU to suppress WNT signaling.* Oncogene, 2004. 23(26): p. 4577-83.**

237. **Ding, Q., et al., *Mouse suppressor of fused is a negative regulator of sonic hedgehog signaling and alters the subcellular distribution of Gli1.* Curr Biol, 1999. 9(19): p. 1119-22.**

238. **Takemaru, K.I. and R.T. Moon, *The transcriptional coactivator CBP interacts with beta-catenin to activate gene expression.* J Cell Biol, 2000. 149(2): p. 249-54.**

239. **Akimaru, H., et al., *Drosophila CBP is a co-activator of cubitus interruptus in hedgehog signalling.* Nature, 1997. 386(6626): p. 735-8.**

240. **Dai, P., et al., *Sonic Hedgehog-induced activation of the Gli1 promoter is mediated by GLI3.* J Biol Chem, 1999. 274(12): p. 8143-52.**

241. **Han, G., et al., *Smad7-induced beta-catenin degradation alters epidermal appendage development.* Dev Cell, 2006. 11(3): p. 301-12.**

242. **Watt, F.M., *Unexpected Hedgehog-Wnt interactions in epithelial differentiation.* Trends Mol Med, 2004. 10(12): p. 577-80.**

243. **Niemann, C., *Differentiation of the sebaceous gland.* Dermatoendocrinol, 2009. 1(2): p. 64-7.**

244. **Hammerschmidt, B. and T. Schlake, *Localization of Shh expression by Wnt and Eda affects axial polarity and shape of hairs.* Dev Biol, 2007. 305(1): p. 246-61.**

245. **Mullor, J.L., et al., *Wnt signals are targets and mediators of Gli function.* Curr Biol, 2001. 11(10): p. 769-73.**

246. **Yang, S.H., et al., *Pathological responses to oncogenic Hedgehog signaling in skin are dependent on canonical Wnt/beta3-catenin signaling.* Nat Genet, 2008. 40(9): p. 1130-5.**

247. **Thody, A.J. and S. Shuster, *Control and function of sebaceous glands.* Physiol Rev, 1989. 69(2): p. 383-416.**

248. **Zouboulis, C.C., *Acne and sebaceous gland function.* Clin Dermatol, 2004. 22(5): p. 360-6.**

249. **Zouboulis, C.C., *[The sebaceous gland].* Hautarzt, 2010. 61(6): p. 467-8, 4704, 476-7.**

250. **Arnold, I. and F.M. Watt, *c-Myc activation in transgenic mouse epidermis results in mobilization of stem cells and differentiation of their progeny.* Curr Biol, 2001. 11(8): p. 558-68.**

251. **Koster, M.I., K.A. Huntzinger, and D.R. Roop, *Epidermal differentiation: transgenic/knockout mouse models reveal genes involved in stem cell fate decisions and commitment to differentiation.* J Investig Dermatol Symp Proc, 2002. 7(1): p. 41-5.**

252. **Waikel, R.L., et al., *Deregulated expression of c-Myc depletes epidermal stem cells.* Nat Genet, 2001. 28(2): p. 165-8.**

253. **Gu, L.H. and P.A. Coulombe, *Hedgehog signaling, keratin 6 induction, and sebaceous gland morphogenesis: implications for pachyonychia congenita and related conditions.* Am J Pathol, 2008. 173(3): p. 752-61.**

254. Oshima, H., et al., *Morphogenesis and renewal of hair follicles from adult multipotent stem cells.* Cell, 2001. 104(2): p. 233-45.
255. Cotsarelis, G., *Epithelial stem cells: a folliculocentric view.* J Invest Dermatol, 2006. 126(7): p. 1459-68.
256. Lyle, S., et al., *Human hair follicle bulge cells are biochemically distinct and possess an epithelial stem cell phenotype.* J Investig Dermatol Symp Proc, 1999. 4(3): p. 296-301.
257. Lyle, S., et al., *The C8/144B monoclonal antibody recognizes cytokeratin 15 and defines the location of human hair follicle stem cells.* J Cell Sci, 1998. 111 (Pt 21): p. 3179-88.
258. Liu, Y., et al., *Keratin 15 promoter targets putative epithelial stem cells in the hair follicle bulge.* J Invest Dermatol, 2003. 121(5): p. 963-8.
259. Ito, M., et al., *Stem cells in the hair follicle bulge contribute to wound repair but not to homeostasis of the epidermis.* Nat Med, 2005. 11(12): p. 1351-4.
260. Bieniek, R., et al., *Sebaceous tumours contain a subpopulation of cells expressing the keratin 15 stem cell marker.* Br J Dermatol, 2007. 156(2): p. 378-80.
261. Misago, N. and Y. Narisawa, *Cytokeratin 15 expression in neoplasms with sebaceous differentiation.* J Cutan Pathol, 2006. 33(9): p. 634-41.
262. Snippert, H.J., et al., *Lgr6 marks stem cells in the hair follicle that generate all cell lineages of the skin.* Science, 2010. 327(5971): p. 1385-9.
263. Jensen, K.B., et al., *Lrig1 expression defines a distinct multipotent stem cell population in mammalian epidermis.* Cell Stem Cell, 2009. 4(5): p. 427-39.
264. Horsley, V., et al., *Blimp1 defines a progenitor population that governs cellular input to the sebaceous gland.* Cell, 2006. 126(3): p. 597-609.
265. Magnusdottir, E., et al., *Epidermal terminal differentiation depends on B lymphocyte-induced maturation protein-1.* Proc Natl Acad Sci U S A, 2007. 104(38): p. 14988-93.
266. Sellheyer, K. and D. Krahl, *Blimp-1: a marker of terminal differentiation but not of sebocytic progenitor cells.* J Cutan Pathol, 2010. 37(3): p. 362-70.
267. Niizuma, K., *An electron microscopic study of lipid droplets in the normal human sebaceous gland.* J Dermatol, 1981. 8(5): p. 391-400.
268. Smith, K.R. and D.M. Thiboutot, *Thematic review series: skin lipids. Sebaceous gland lipids: friend or foe?* J Lipid Res, 2008. 49(2): p. 271-81.
269. Schneider, M.R. and R. Paus, *Sebocytes, multifaceted epithelial cells: lipid production and holocrine secretion.* Int J Biochem Cell Biol, 2010. 42(2): p. 181-5.
270. Picardo, M., et al., *Sebaceous gland lipids.* Dermatoendocrinol, 2009. 1(2): p. 68-71.
271. Zouboulis, C.C., et al., *Frontiers in sebaceous gland biology and pathology.* Exp Dermatol, 2008. 17(6): p. 542-51.
272. Thiele, J.J., S.U. Weber, and L. Packer, *Sebaceous gland secretion is a major physiologic route of vitamin E delivery to skin.* J Invest Dermatol, 1999. 113(6): p. 1006-10.
273. Kligman, A.M., *The Uses of Sebum.* Br J Dermatol, 1963. 75: p. 307-19.
274. Georgel, P., et al., *A toll-like receptor 2-responsive lipid effector pathway protects mammals against skin infections with gram-positive bacteria.* Infect Immun, 2005. 73(8): p. 4512-21.
275. Fluhr, J.W., et al., *Glycerol regulates stratum corneum hydration in sebaceous gland deficient (asebia) mice.* J Invest Dermatol, 2003. 120(5): p. 728-37.
276. Gates, A.H. and M. Karasek, *Hereditary Absence of Sebaceous Glands in the Mouse.* Science, 1965. 148(3676): p. 1471-1473.

277. Zheng, Y., et al., *Scd1 is expressed in sebaceous glands and is disrupted in the asebia mouse.* Nat Genet, 1999. 23(3): p. 268-70.

278. Chen, H.C., et al., *Leptin modulates the effects of acyl CoA:diacylglycerol acyltransferase deficiency on murine fur and sebaceous glands.* J Clin Invest, 2002. 109(2): p. 175-81.

279. Binczek, E., et al., *Obesity resistance of the stearoyl-CoA desaturase-deficient (scd1-/-) mouse results from disruption of the epidermal lipid barrier and adaptive thermoregulation.* Biol Chem, 2007. 388(4): p. 405-18.

280. Josefowicz, W.J. and M.H. Hardy, *The expression of the gene asebia in the laboratory mouse. I. Epidermis and dermis.* Genet Res, 1978. 31(1): p. 53-65.

281. Choudhry, R., et al., *Localization of androgen receptors in human skin by immunohistochemistry: implications for the hormonal regulation of hair growth, sebaceous glands and sweat glands.* J Endocrinol, 1992. 133(3): p. 467-75.

282. Liang, T., et al., *Immunocytochemical localization of androgen receptors in human skin using monoclonal antibodies against the androgen receptor.* J Invest Dermatol, 1993. 100(5): p. 663-6.

283. Mooradian, A.D., J.E. Morley, and S.G. Korenman, *Biological actions of androgens.* Endocr Rev, 1987. 8(1): p. 1-28.

284. Dijkstra, A.C., et al., *Is increased 5 alpha-reductase activity a primary phenomenon in androgen-dependent skin disorders?* J Invest Dermatol, 1987. 89(1): p. 87-92.

285. Rosignoli, C., et al., *Involvement of the SREBP pathway in the mode of action of androgens in sebaceous glands in vivo.* Exp Dermatol, 2003. 12(4): p. 480-9.

286. Fimmel, S., et al., *Inhibition of the androgen receptor by antisense oligonucleotides regulates the biological activity of androgens in SZ95 sebocytes.* Horm Metab Res, 2007. 39(2): p. 149-56.

287. Imperato-McGinley, J., et al., *The androgen control of sebum production. Studies of subjects with dihydrotestosterone deficiency and complete androgen insensitivity.* J Clin Endocrinol Metab, 1993. 76(2): p. 524-8.

288. Strauss, J.S., A.M. Kligman, and P.E. Pochi, *The effect of androgens and estrogens on human sebaceous glands.* J Invest Dermatol, 1962. 39: p. 139-55.

289. Hodgins, M.B., J.B. Hay, and J.B. Donnelly, *Human skin androgen metabolism and preliminary evidence for its control by two forms of 17 beta-hydroxysteroid oxidoreductase.* J Endocrinol, 1982. 93(3): p. 403-13.

290. Sawaya, M.E. and V.H. Price, *Different levels of 5alpha-reductase type I and II, aromatase, and androgen receptor in hair follicles of women and men with androgenetic alopecia.* J Invest Dermatol, 1997. 109(3): p. 296-300.

291. Nelson, A.M., et al., *13-cis Retinoic acid induces apoptosis and cell cycle arrest in human SEB-1 sebocytes.* J Invest Dermatol, 2006. 126(10): p. 2178-89.

292. Tsukada, M., et al., *13-cis retinoic acid exerts its specific activity on human sebocytes through selective intracellular isomerization to all-trans retinoic acid and binding to retinoid acid receptors.* J Invest Dermatol, 2000. 115(2): p. 321-7.

293. Hommel, L., et al., *Sebum excretion rate in subjects treated with oral all-trans-retinoic acid.* Dermatology, 1996. 193(2): p. 127-30.

294. Russell, L.E., et al., *Characterization of liver X receptor expression and function in human skin and the pilosebaceous unit.* Exp Dermatol, 2007. 16(10): p. 844-52.

295. Hong, I., et al., *LXRalpha enhances lipid synthesis in SZ95 sebocytes.* J Invest Dermatol, 2008. 128(5): p. 1266-72.

296. Downie, M.M., et al., *Peroxisome proliferator-activated receptor and farnesoid X receptor ligands differentially regulate sebaceous differentiation in human sebaceous gland organ cultures in vitro.* Br J Dermatol, 2004. 151(4): p. 766-75.

297. Di-Poi, N., et al., *Functions of peroxisome proliferator-activated receptors (PPAR) in skin homeostasis.* Lipids, 2004. 39(11): p. 1093-9.

298. Rosen, E.D., et al., *PPAR gamma is required for the differentiation of adipose tissue in vivo and in vitro.* Mol Cell, 1999. 4(4): p. 611-7.

299. Deplewski, D. and R.L. Rosenfield, *Growth hormone and insulin-like growth factors have different effects on sebaceous cell growth and differentiation.* Endocrinology, 1999. 140(9): p. 4089-94.

300. Smith, T.M., et al., *IGF-1 induces SREBP-1 expression and lipogenesis in SEB-1 sebocytes via activation of the phosphoinositide 3-kinase/Akt pathway.* J Invest Dermatol, 2008. 128(5): p. 1286-93.

301. Rosenfield, R.L., P.P. Wu, and N. Ciletti, *Sebaceous epithelial cell differentiation requires cyclic adenosine monophosphate generation.* In Vitro Cell Dev Biol Anim, 2002. 38(1): p. 54-7.

302. Getting, S.J., *Targeting melanocortin receptors as potential novel therapeutics.* Pharmacol Ther, 2006. 111(1): p. 1-15.

303. Hatta, N., et al., *Expression, candidate gene, and population studies of the melanocortin 5 receptor.* J Invest Dermatol, 2001. 116(4): p. 564-70.

304. Thiboutot, D., et al., *The melanocortin 5 receptor is expressed in human sebaceous glands and rat preputial cells.* J Invest Dermatol, 2000. 115(4): p. 614-9.

305. Zhang, L., et al., *Melanocortin-5 receptor: a marker of human sebocyte differentiation.* Peptides, 2006. 27(2): p. 413-20.

306. Chen, W., et al., *Exocrine gland dysfunction in MC5-R-deficient mice: evidence for coordinated regulation of exocrine gland function by melanocortin peptides.* Cell, 1997. 91(6): p. 789-98.

307. Miyazaki, M., W.C. Man, and J.M. Ntambi, *Targeted disruption of stearoyl-CoA desaturase1 gene in mice causes atrophy of sebaceous and meibomian glands and depletion of wax esters in the eyelid.* J Nutr, 2001. 131(9): p. 2260-8.

308. Parimoo, S., et al., *Identification of a novel SCD gene and expression of the SCD gene family in mouse skin.* J Investig Dermatol Symp Proc, 1999. 4(3): p. 320-2.

309. Sessler, A.M., et al., *Regulation of stearoyl-CoA desaturase 1 mRNA stability by polyunsaturated fatty acids in 3T3-L1 adipocytes.* J Biol Chem, 1996. 271(47): p. 29854-8.

310. Thiboutot, D.M., *An overview of acne and its treatment.* Cutis, 1996. 57(1 Suppl): p. 8-12.

311. Parry, M.E. and G.R. Sharpe, *Seborrhoeic dermatitis is not caused by an altered immune response to Malassezia yeast.* Br J Dermatol, 1998. 139(2): p. 254-63.

312. Schwartz, R.A., C.A. Janusz, and C.K. Janniger, *Seborrheic dermatitis: an overview.* Am Fam Physician, 2006. 74(1): p. 125-30.

313. Troy, J.L. and A.B. Ackerman, *Sebaceoma. A distinctive benign neoplasm of adnexal epithelium differentiating toward sebaceous cells.* Am J Dermatopathol, 1984. 6(1): p. 7-13.

314. Song, A., et al., *Sebaceous cell carcinoma of the ocular adnexa: clinical presentations, histopathology, and outcomes.* Ophthal Plast Reconstr Surg, 2008. 24(3): p. 194-200.

315. Nelson, B.R., et al., *Sebaceous carcinoma.* J Am Acad Dermatol, 1995. 33(1): p. 1-15; quiz 16-8.

316. Shields, J.A., et al., *Sebaceous carcinoma of the ocular region: a review.* Surv Ophthalmol, 2005. 50(2): p. 103-22.

317. Zouboulis, C.C. and A. Boschnakow, *Chronological ageing and photoageing of the human sebaceous gland.* Clin Exp Dermatol, 2001. 26(7): p. 600-7.

318. Zouboulis, C.C., et al., *Establishment and characterization of an immortalized human sebaceous gland cell line (SZ95).* J Invest Dermatol, 1999. 113(6): p. 1011-20.

319. Zouboulis, C.C., et al., *Progressive differentiation of human sebocytes in vitro is characterized by increasing cell size and altering antigen expression and is regulated by culture duration and retinoids.* Exp Dermatol, 1994. 3(4): p. 151-60.

320. Zouboulis, C.C., et al., *Culture of human sebocytes and markers of sebocytic differentiation in vitro.* Skin Pharmacol, 1991. 4(2): p. 74-83.

321. Xia, L.Q., et al., *Isolation of human sebaceous glands and cultivation of sebaceous gland-derived cells as an in vitro model.* J Invest Dermatol, 1989. 93(3): p. 315-21.

322. Fimmel, S., et al., *Responsiveness to androgens and effectiveness of antisense oligonucleotides against the androgen receptor on human epidermal keratinocytes is dependent on the age of the donor and the location of cell origin.* Horm Metab Res, 2007. 39(2): p. 157-65.

323. Chen, W., et al., *Expression of peroxisome proliferator-activated receptor and CCAAT/enhancer binding protein transcription factors in cultured human sebocytes.* J Invest Dermatol, 2003. 121(3): p. 441-7.

324. Zhang, Q., et al., *Involvement of PPARgamma in oxidative stress-mediated prostaglandin E(2) production in SZ95 human sebaceous gland cells.* J Invest Dermatol, 2006. 126(1): p. 42-8.

325. Henke, D., R. Danilowicz, and T. Eling, *Arachidonic acid metabolism by isolated epidermal basal and differentiated keratinocytes from the hairless mouse.* Biochim Biophys Acta, 1986. 876(2): p. 271-9.

326. Keeney, D.S., et al., *A keratinocyte-specific epoxygenase, CYP2B12, metabolizes arachidonic acid with unusual selectivity, producing a single major epoxyeicosatrienoic acid.* J Biol Chem, 1998. 273(15): p. 9279-84.

327. Wrobel, A., et al., *Differentiation and apoptosis in human immortalized sebocytes.* J Invest Dermatol, 2003. 120(2): p. 175-81.

328. Zouboulis, C.C., *Sebaceous glands and the prostaglandin pathway--key stones of an exciting mosaic.* J Invest Dermatol, 2005. 125(5): p. x-xi.

329. Pappas, A., M. Anthonavage, and J.S. Gordon, *Metabolic fate and selective utilization of major fatty acids in human sebaceous gland.* J Invest Dermatol, 2002. 118(1): p. 164-71.

330. van de Wetering, M., et al., *Armadillo coactivates transcription driven by the product of the Drosophila segment polarity gene dTCF.* Cell, 1997. 88(6): p. 789-99.

331. Sasaki, H., et al., *A binding site for Gli proteins is essential for HNF-3beta floor plate enhancer activity in transgenics and can respond to Shh in vitro.* Development, 1997. 124(7): p. 1313-22.

332. Funayama, N., et al., *Embryonic axis induction by the armadillo repeat domain of beta-catenin: evidence for intracellular signaling.* J Cell Biol, 1995. 128(5): p. 959-68.

333. Zhu, A.J. and F.M. Watt, *beta-catenin signalling modulates proliferative potential of human epidermal keratinocytes independently of intercellular adhesion.* Development, 1999. 126(10): p. 2285-98.

334. Rosenfield, R.L., et al., *Rat preputial sebocyte differentiation involves peroxisome proliferator-activated receptors.* J Invest Dermatol, 1999. 112(2): p. 226-32.

335. Leibovitz, A., et al., *Classification of human colorectal adenocarcinoma cell lines.* Cancer Res, 1976. 36(12): p. 4562-9.

336. Taipale, J., et al., *Effects of oncogenic mutations in Smoothened and Patched can be reversed by cyclopamine.* Nature, 2000. 406(6799): p. 1005-9.

337. Bohm, M., et al., *Evidence for expression of melanocortin-1 receptor in human sebocytes in vitro and in situ.* J Invest Dermatol, 2002. 118(3): p. 533-9.

338. Jiang, H.P. and G. Serrero, *Isolation and characterization of a full-length cDNA coding for an adipose differentiation-related protein.* Proc Natl Acad Sci U S A, 1992. 89(17): p. 7856-60.

339. Heid, H.W., et al., *Adipophilin is a specific marker of lipid accumulation in diverse cell types and diseases.* Cell Tissue Res, 1998. 294(2): p. 309-21.

340. Robenek, H., et al., *Adipophilin-enriched domains in the ER membrane are sites of lipid droplet biogenesis.* J Cell Sci, 2006. 119(Pt 20): p. 4215-24.

341. Chang, B.H., et al., *Protection against fatty liver but normal adipogenesis in mice lacking adipose differentiation-related protein.* Mol Cell Biol, 2006. 26(3): p. 1063-76.

342. Chatel, G., et al., *Hedgehog signaling pathway is inactive in colorectal cancer cell lines.* Int J Cancer, 2007. 121(12): p. 2622-7.

343. Hsu, Y.C., H.A. Pasolli, and E. Fuchs, *Dynamics between stem cells, niche, and progeny in the hair follicle.* Cell, 2011. 144(1): p. 92-105.

344. Tomic-Canic, M., et al., *Epidermal signal transduction and transcription factor activation in activated keratinocytes.* J Dermatol Sci, 1998. 17(3): p. 167-81.

345. Lauth, M., et al., *Inhibition of GLI-mediated transcription and tumor cell growth by small-molecule antagonists.* Proc Natl Acad Sci U S A, 2007. 104(20): p. 8455-60.

346. Greenspan, P., E.P. Mayer, and S.D. Fowler, *Nile red: a selective fluorescent stain for intracellular lipid droplets.* J Cell Biol, 1985. 100(3): p. 965-73.

347. Fathke, C., et al., *Wnt signaling induces epithelial differentiation during cutaneous wound healing.* BMC Cell Biol, 2006. 7: p. 4.

348. Saitoh, A., et al., *Characterization of Wnt gene expression in murine skin: possible involvement of epidermis-derived Wnt-4 in cutaneous epithelial-mesenchymal interactions.* Exp Cell Res, 1998. 243(1): p. 150-60.

349. Okuse, T., et al., *Differential expression and localization of WNTs in an animal model of skin wound healing.* Wound Repair Regen, 2005. 13(5): p. 491-7.

350. van Amerongen, R. and R. Nusse, *Towards an integrated view of Wnt signaling in development.* Development, 2009. 136(19): p. 3205-14.

351. Mao, B., et al., *Kremen proteins are Dickkopf receptors that regulate Wnt/beta-catenin signalling.* Nature, 2002. 417(6889): p. 664-7.

352. Rothbacher, U. and P. Lemaire, *Creme de la Kremen of Wnt signalling inhibition.* Nat Cell Biol, 2002. 4(7): p. E172-3.

353. Takemaru, K., et al., *Chibby, a nuclear beta-catenin-associated antagonist of the Wnt/Wingless pathway.* Nature, 2003. 422(6934): p. 905-9.

354. Horndasch, M., et al., *The C/EBP homologous protein CHOP (GADD153) is an inhibitor of Wnt/TCF signals.* Oncogene, 2006. 25(24): p. 3397-407.

355. Staal, F.J., et al., *Wnt signaling is required for thymocyte development and activates Tcf-1 mediated transcription.* Eur J Immunol, 2001. 31(1): p. 285-93.

356. Liao, X., et al., *Aberrant activation of hedgehog signaling pathway contributes to endometrial carcinogenesis through beta-catenin.* Mod Pathol, 2009. 22(6): p. 839-47.

357. McMahon, A.P., P.W. Ingham, and C.J. Tabin, *Developmental roles and clinical significance of hedgehog signaling.* Curr Top Dev Biol, 2003. 53: p. 1-114.

358. Ramalho-Santos, M., D.A. Melton, and A.P. McMahon, *Hedgehog signals regulate multiple aspects of gastrointestinal development.* Development, 2000. 127(12): p. 2763-72.

359. Park, H.L., et al., *Mouse Gli1 mutants are viable but have defects in SHH signaling in combination with a Gli2 mutation.* Development, 2000. 127(8): p. 1593-605.

360. Ruiz i Altaba, A., *Combinatorial Gli gene function in floor plate and neuronal inductions by Sonic hedgehog.* Development, 1998. 125(12): p. 2203-12.

361. Motoyama, J., et al., *Differential requirement for Gli2 and Gli3 in ventral neural cell fate specification.* Dev Biol, 2003. 259(1): p. 150-61.

362. Matise, M.P., et al., *Gli2 is required for induction of floor plate and adjacent cells, but not most ventral neurons in the mouse central nervous system.* Development, 1998. 125(15): p. 2759-70.

363. Merchant, A., et al., *Gli1 regulates the proliferation and differentiation of HSCs and myeloid progenitors.* Blood, 2010. 115(12): p. 2391-6.

364. Vestergaard, J., et al., *GLI1 is involved in cell cycle regulation and proliferation of NT2 embryonal carcinoma stem cells.* DNA Cell Biol, 2008. 27(5): p. 251-6.

365. Mill, P., et al., *Sonic hedgehog-dependent activation of Gli2 is essential for embryonic hair follicle development.* Genes Dev, 2003. 17(2): p. 282-94.

366. Rutter, M., et al., *Gli2 influences proliferation in the developing lung through regulation of cyclin expression.* Am J Respir Cell Mol Biol, 2010. 42(5): p. 615-25.

367. Yauch, R.L., et al., *A paracrine requirement for hedgehog signalling in cancer.* Nature, 2008. 455(7211): p. 406-10.

368. Sheng, H., et al., *Dissecting the oncogenic potential of Gli2: deletion of an NH(2)-terminal fragment alters skin tumor phenotype.* Cancer Res, 2002. 62(18): p. 5308-16.

369. Regl, G., et al., *Human GLI2 and GLI1 are part of a positive feedback mechanism in Basal Cell Carcinoma.* Oncogene, 2002. 21(36): p. 5529-39.

370. Sanchez, P., et al., *Inhibition of prostate cancer proliferation by interference with SONIC HEDGEHOG-GLI1 signaling.* Proc Natl Acad Sci U S A, 2004. 101(34): p. 12561-6.

371. Hutchin, M.E., et al., *Sustained Hedgehog signaling is required for basal cell carcinoma proliferation and survival: conditional skin tumorigenesis recapitulates the hair growth cycle.* Genes Dev, 2005. 19(2): p. 214-23.

372. Alexaki, V.I., et al., *GLI2-mediated melanoma invasion and metastasis.* J Natl Cancer Inst, 2010. 102(15): p. 1148-59.

373. Sasaki, H., et al., *Regulation of Gli2 and Gli3 activities by an amino-terminal repression domain: implication of Gli2 and Gli3 as primary mediators of Shh signaling.* Development, 1999. 126(17): p. 3915-24.

374. Ding, Q., et al., *Diminished Sonic hedgehog signaling and lack of floor plate differentiation in Gli2 mutant mice.* Development, 1998. 125(14): p. 2533-43.

375. McDermott, A., et al., *Gli2 and Gli3 have redundant and context-dependent function in skeletal muscle formation.* Development, 2005. 132(2): p. 345-57.

376. Buttitta, L., et al., *Interplays of Gli2 and Gli3 and their requirement in mediating Shh-dependent sclerotome induction.* Development, 2003. 130(25): p. 6233-43.

377. Pan, Y., C. Wang, and B. Wang, *Phosphorylation of Gli2 by protein kinase A is required for Gli2 processing and degradation and the Sonic Hedgehog-regulated mouse development.* Dev Biol, 2009. 326(1): p. 177-89.

378. Komada, M., et al., *Expression of Fgf15 is regulated by both activator and repressor forms of Gli2 in vitro.* Biochem Biophys Res Commun, 2008. 369(2): p. 350-6.

379. Kesper, D.A., L. Didt-Koziel, and A. Vortkamp, *Gli2 activator function in preosteoblasts is sufficient to mediate Ihh-dependent osteoblast differentiation, whereas the repressor function of Gli2 is dispensable for endochondral ossification.* Dev Dyn, 2010. 239(6): p. 1818-26.

380. Hu, M.C., et al., *GLI3-dependent transcriptional repression of Gli1, Gli2 and kidney patterning genes disrupts renal morphogenesis.* Development, 2006. 133(3): p. 569-78.

381. Henneberry, A.L., M.M. Wright, and C.R. McMaster, *The major sites of cellular phospholipid synthesis and molecular determinants of Fatty Acid and lipid head group specificity.* Mol Biol Cell, 2002. 13(9): p. 3148-61.

382. Cook, J.A., et al., *Prostaglandins, thromboxanes, leukotrienes, and cytochrome P-450 metabolites of arachidonic acid.* New Horiz, 1993. 1(1): p. 60-9.

383. Pegorier, J.P., C. Le May, and J. Girard, *Control of gene expression by fatty acids.* J Nutr, 2004. 134(9): p. 2444S-2449S.

384. Jump, D.B., et al., *Polyunsaturated fatty acids inhibit S14 gene transcription in rat liver and cultured hepatocytes.* Proc Natl Acad Sci U S A, 1993. 90(18): p. 8454-8.

385. Liimatta, M., et al., *Dietary polyunsaturated fatty acids interfere with the insulin/glucose activation of L-type pyruvate kinase gene transcription.* Mol Endocrinol, 1994. 8(9): p. 1147-53.

386. Waters, K.M., C.W. Miller, and J.M. Ntambi, *Localization of a polyunsaturated fatty acid response region in stearoyl-CoA desaturase gene 1.* Biochim Biophys Acta, 1997. 1349(1): p. 33-42.

387. Long, S.D. and P.H. Pekala, *Regulation of GLUT4 gene expression by arachidonic acid. Evidence for multiple pathways, one of which requires oxidation to prostaglandin E2.* J Biol Chem, 1996. 271(2): p. 1138-44.

388. Gottlicher, M., et al., *Fatty acids activate a chimera of the clofibric acid-activated receptor and the glucocorticoid receptor.* Proc Natl Acad Sci U S A, 1992. 89(10): p. 4653-7.

389. Tebbey, P.W., et al., *Arachidonic acid down-regulates the insulin-dependent glucose transporter gene (GLUT4) in 3T3-L1 adipocytes by inhibiting transcription and enhancing mRNA turnover.* J Biol Chem, 1994. 269(1): p. 639-44.

390. Cossette, C., et al., *Human neutrophils convert the sebum-derived polyunsaturated fatty acid Sebaleic acid to a potent granulocyte chemoattractant.* J Biol Chem, 2008. 283(17): p. 11234-43.

391. Ouji, Y., et al., *Wnt-10b, uniquely among Wnts, promotes epithelial differentiation and shaft growth.* Biochem Biophys Res Commun, 2008. 367(2): p. 299-304.

392. Aza-Blanc, P., et al., *Expression of the vertebrate Gli proteins in Drosophila reveals a distribution of activator and repressor activities.* Development, 2000. 127(19): p. 4293-301.

393. Kim, Y.S., H.S. Kang, and A.M. Jetten, *The Kruppel-like zinc finger protein Glis2 functions as a negative modulator of the Wnt/beta-catenin signaling pathway.* FEBS Lett, 2007. 581(5): p. 858-64.

394. Hosking, C.R., et al., *The transcriptional repressor Glis2 is a novel binding partner for p120 catenin.* Mol Biol Cell, 2007. 18(5): p. 1918-27.

395. Zhang, F., et al., *Characterization of Glis2, a novel gene encoding a Gli-related, Kruppel-like transcription factor with transactivation and repressor functions. Roles in kidney development and neurogenesis.* J Biol Chem, 2002. 277(12): p. 10139-49.

396. Maeda, O., et al., *Enhancement of GLI1-transcriptional activity by beta-catenin in human cancer cells.* Oncol Rep, 2006. 16(1): p. 91-6.

397. Urnov, F.D., et al., *Highly efficient endogenous human gene correction using designed zinc-finger nucleases.* Nature, 2005. 435(7042): p. 646-51.

398. Porteus, M.H., *Mammalian gene targeting with designed zinc finger nucleases.* Mol Ther, 2006. 13(2): p. 438-46.

6. Danksagung

Mein besonderer Dank gilt meiner Betreuerin Dr. Catherin Niemann, die mir die Möglichkeit gab, an diesem interessanten Projekt zu arbeiten und mir während der gesamten Promotionszeit menschlich und fachlich immer zur Seite stand.

Außerdem möchte ich mich bei Prof. Dr. A. A. Noegel und Prof. Dr. S. Roth bedanken, die mich als Tutoren auf diesem Weg begleitet und unterstützt haben. Von den Gesprächen mit ihnen und ihren Erfahrungen konnte ich sehr profitieren.

Ein großer Dank gilt auch Dr. D. Grosskopf-Kroiher für ihre leidenschaftliche Unterstützung und Betreuung des IPMM-Programms des ZMMK und der positiven Ausstrahlung, die sie dabei stets verbreitet, sowie dem gesamten IPMM-Komitee.

Einen herzlichen Dank richte ich an alle meine Kolleginnen und Kollegen der Arbeitsgruppe von Dr. C. Niemann, insbesondere Moni, Peter, Andy, Parisa, Daniela, Susan, Heike, Uwe und Daggi, die mich während dieser Zeit menschlich begleitet und fachlich unterstützt haben.

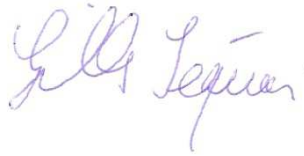
Ebenfalls bedanke ich mich bei allen Kolleginnen und Kollegen des Instituts für Pathologie, Instituts für Dermatologie, Instituts der medizinischen Biochemie und des ZMMK (insbesondere AG Niessen) an der Uniklinik Köln für ihre freundliche Unterstützung.

Ganz besonders danke ich Stephanie und Arthur für ihre Liebe und außerordentliche Geduld während der gesamten Zeit, die ich benötigte, um diese Arbeit fertigzustellen.

Schließlich danke ich auch besonders meinen Eltern und meiner Familie für ihre anhaltende Liebe, Unterstützung und ihren Glauben an mich.

7. Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie noch nicht veröffentlich worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahren nicht vornehmen werde. Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Dr. C. Niemann betreut worden.

Köln, den 27. Juli 2011 Unterschrift: 

8. Curriculum vitae

Personal data

Name:		Gilles Frédéric Brice Séquaris
Address:		Emmastraße 8 50937 Cologne Germany
Email:		gilles.sequaris@web.de
Born:		16.09.1977, Le Chesnay (France)
Nationality:		French

School:	1984-1988	Städtische Grundschule Nord, Jülich
	1988-1997	Gymnasium Zitadelle, Jülich
University:	Since 1997	Studying biology (diploma) at the University of Cologne
	2004	Diploma exam in Genetics, Zoology and Biochemistry with final grade "sehr gut"
Diploma thesis:	2004-2005	„Proteolytic cleavage and nuclear access of cadherins „ in the laboratory of Prof. C. Niessen at CMMC, University of Cologne
Employment:	2005-2009	PhD in the laboratory of Dr. C. Niemann at CMMC, University of Cologne
	Since 2007	member of the Interdisciplinary Program Molecular Medicine, University of Cologne
	Since 2010	Post-doc position in the laboratory of Prof. M. Roden at the German Diabetes Center, Heinrich-Heine University of Düsseldorf
PhD thesis:	2011	"Molecular mechanism of Indian Hedgehog signalling in human sebocytes"

Awards:	1995-1996	Großer Anerkennungspreis Bundes Umwelt Wettbewerb 1995/96 1. Platz im Regionalwettbewerb „Jugend forscht“ im Fachbereich Biologie 1996 Sonderpreis „Jugend erforscht die Umwelt“ im Rahmen des Regionalwettbewerbs „Jugend forscht“ 1996 3. Platz im Landeswettbewerb „Jugend forscht“ im Fachbereich Biologie 1996 Sonderpreis „Umwelt“ im Rahmen des Landeswettbewerb „Jugend forscht“ 1996

Publications, Talks and Posters:

Publications

Outtakes of diploma thesis in the online publication of Dr. K. Billion’s dissertation (2006): **Proteolytic Cleavage of cadherins: Functional role of the cleaved extracellular and cytoplasmic domains.** *KUPS*

Fehrenschild, D., Schettina, P., Sequaris, G., Kraus, A., Siebolts, U., Munro, P.M., Daniels, J.T., Watt, F.M., Niemann, C.: **Functional Lef1 signalling is required for corneal cell fate and lens differentiation.** (in submission)

Posters

Sequaris, G., Simion, O., Niemann, C.: **Indian Hedgehog signalling in epidermal differentiation and sebaceous tumour formation.** (International SFB 589 Symposium „Skin Diseases – Human Genetics - Animal Models“, December 2006, Cologne)

Sequaris, G., Niemann, C.: **Molecular mechanism of Indian Hedgehog signalling in human sebocytes.** (Epistem Conference, February 2008, Ghent, Belgium)

Sequaris, G., Kakanj, P., Niemann, C.: **Regulation of sebaceous gland homeostasis by Hedgehog and Lef1 signaling.** (International SFB 829 Symposium „ Cellular

Mechanisms Regulating Skin Homeostasis and Skin Diseases", May 2009, Cologne)

Sequaris, G., Szendrödi, J., Kotzka, J., Phielix, E., Knebel, E., Partke, H.-J., Müller-Wieland, D., Roden, M.: **Effects of Ectopic Lipid Accumulation in Liver and Muscle on Mitochondrial Function.** (71st Scientific Sessions of American Diabetes Association, June 2011, San Diego, USA)

Talks

Sequaris, G., Szendrödi, J., Kotzka, J., Phielix, E., Knebel, E., Partke, H.-J., Müller-Wieland, D., Roden, M.: **Der Effekt vermehrter Lipidakkumulation in der Leber auf die hepatische Mitochondrienfunktion.** *Diabetologie & Stoffwechsel* 2011; 6: S29

Köln, den 06. März 2012 Unterschrift: