The apoptosis resistance of a keratinocytic cell line and of basal cell carcinoma is mediated by the transcription factor Gli2 via cFlip upregulation

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1 Abbreviations

BCC: basal cell carcinoma
bp: basepairs
BSA: bovine serum albumin
CDS: coding sequence
CFLAR: cellular FADD-like apoptosis regulator (=cFlip)
cFlip: cellular FLICE-inhibiting protein
CD95: cluster of differentiation 95 (=Fas)
CD95L: ligand for CD95 (=FasL)
cDNA: complementary DNA
CO_2: carbondioxide
cpm: counts per minute
DD: death domain
DED: death effector domain
d(H_2O): distilled water
dIdC: deoxyinosinedeoxycytidine
DISC: death inducing signaling complex
DMEM: Dulbecco's modified Eagle medium
DNA: deoxyribonucleic acid
DR: death receptor
DTT: Dithiothreitol
E.coli: *Escherichia coli*
EDTA: Ethylendiamintetraacetate
EGFP: enhanced green fluorescent protein
EMSA: electric mobility shift assay
FACS: fluorescence activated cell sorting
FADD: Fas associated death domain
FCS: fetal calf serum
FITC: fluorescein isothiocyanate
FLICE: FADD-like interleukin-β converting enzyme
Flip: FLICE-inhibiting protein
HRP: horseradish peroxidase
kb: kilobases
KCl: potassium chloride
kDa: kiloDalton
M: molar
mRNA: messenger RNA
NaAc: Sodium Acetate
NCBI: national center for biotechnology information
NFκB: nuclear factor kappa B
oligo: oligonucleotide
PAGE: polyacrylamide gel electrophoresis
PBS: phosphate buffered saline
PCR: polymerase chain reaction
PFA: paraformaldehyde
PI: propidium iodide
PE: phycoerythrin
PTCH: Patched
RNA: ribonucleic acid
RNAi: RNA interference
rpm: rounds per minute
RT_1: room temperature
RT_2: reverse transcriptase
RT-PCR: reverse transcriptase polymerase chain reaction
SCC: squamous cell carcinoma
SDS: sodium dodecyl sulfate
siRNA: short interfering RNA
TE: Tris-EDTA
Tet: tetracycline
TNF: tumor necrosis factor
TNFR: tumor necrosis factor receptor
TR: Trail receptor
TRADD: TNFR associated death domain
Trail: tumor necrosis factor related apoptosis inducing ligand
UTR: untranslated region
UV: utraviolet light
UVA: utraviolet A
UVB: utraviolet B
V: Volts
vFlip: viral Flip
-/-: knockout

2 Summary

With regard to the fact that many basal cell carcinoma (BCC) bear mutations in a key player of the Hedgehog signal pathway (PTCH), and have thus an imbalance in the Hedgehog mediators, the Gli transcription factors, we studied the relationship between elevated Gli and oncogenicity, focusing especially on apoptosis mechanisms. It has already been shown that Bcl-2, an antagonist of the intrinsic, mitochondrial apoptosis pathway, is a transcriptional target of the primary Hedgehog signal mediator, Gli2. Our aim was to locate and define further Gli2 targets that are related to apoptosis. We made use of a human transgenic keratinocytic cell line (HaCat NHis-Gli2) that expresses high levels of Gli2 under the control of a tetracycline (tet) -controlled transactivator. It allowed us to shut on Gli2 expression by culturing the cells in a tetracycline-containing medium. We firstly screened differential gene expression between tet-on and tet-off cells using Affymetrix gene chip analysis. It turned out that besides the expected Gli2 targets, also cFlip, a potent Caspase 8 inhibitor, was significantly upregulated upon Gli2 overexpression. We confirmed this result by quantitative RT-PCR on the mRNA level and by Western blot on the protein level, and could observe a time-dependent cFlip upregulation in response to Gli2. As an enzymatically inactive structural homolog of Caspase 8, cFlip blocks the extrinsic, death-ligand induced pathway of apoptosis at the level of the death receptor complex formation.

In a next step, we assessed the apoptosis-inhibitory impact of Gli2 and the role of cFlip. Our HaCat cells NHis-Gli2 cells express the death receptors TR1 and TR2 (Trail receptors 1 and 2) and are thus susceptible to Trail-induced apoptosis (shown by FACS data and apoptosis assays based on DNA fragmentation). Indeed, when we overexpress Gli2, the cells are significantly protected against Trail-induced apoptosis. With other molecules that are related to extrinsic

apoptosis being equally expressed (Affymetrix data), we postulated that cFlip must play a considerable role in the Gli2-mediated protective effect. We therefore downregulated cFlip using RNAi technology and found that cells, although expressing high Gli2 levels, lost their protection, pointing to cFlip as a potent player in the Gli2-mediated defence against apoptosis. All apoptosis assays were done by FACS screening of DNA fragmentation (propidium iodide staining), and were confirmed using the APOPercentage™ assay (Biocolor). This assay reports a different step of the apoptotic process, as it stains apoptotic cells in situ using a dye that is taken up only by those cells that flip their membranes inside out. In order to further confirm the apoptosis data, and to prove that cFlip is the key player, we performed a Caspase 8 activity assay and could show that Trail-triggered Caspase 8 activity is significantly reduced in Gli2 overexpressing cells. Caspase 8 activity could be rescued by cFlip downregulation (RNAi) even in the Gli2 overexpressing situation. We thus identified cFlip as an important player in the Gli2-mediated apoptosis resistance in our model cell line.

We then *in silico* analysed the putative cFlip promoter region (so far undefined), and identified several clusters of potential Gli2 binding sites as defined from formerly published transcriptional targets of Gli2 (e.g. Bcl-2). We cloned these clusters into a luciferase expression reporter vector and were able to identify one cluster that reacted on elevated Gli2 levels as a promoter when transfected into our tet-inducible model cell line.

The four potential binding sites in this cluster were analyzed in a gel shift assay, and two of them clearly showed binding to Gli2. We thereby at least partially defined a cFlip promoter region or a cis-element of the cFlip gene.

In a second phase, we addressed the situation in basal cell carcinoma. We were lucky to get a collaboration with Dr. P. Häusermann from the Dermatology Unit of the University Hospital in Basel, who provided us with BCC tissue specimens. We

screened protein expression in all BCC specimens in cryosections, and found that in high Gli2 expressing tumors, cFlip was also highly coexpressed. We then used the RNAi technology on cultured pieces of BCC to downregulate Gli2 ex vivo in these tumors, and measured the expression of Gli2 and of its targets Bcl-2 and cFlip. We succeeded to downregulate Gli2 efficiently and found that also the expression of its targets was significantly lowered, confirming that cFlip is a transcriptional target of Gli2.

We then assessed the apoptosis susceptibility of BCC tissue ex vivo under native and Gli2-downregulated conditions. As Trail receptors 1 and 2 were expressed on the BCC tissues tested, we applied soluble Trail on cultured pieces of BCC. We observed a higher cell death in Gli2-downregulated BCCs compared to native tissue, which supports an anti-apoptotic impact of Gli2 via cFlip in BCC.

The results found in HaCat NHis-Gli2 and in BCCs tested point to a tumor defense mechanism, postulating that BCC can escape from the immune system, among other ways by preventing death-ligand induced apoptosis through the upregulation of the anti-apoptotic cFlip.

3 Introduction

3.1 Cancer

'Cancer' is a general term, describing all *malignant neoplasms* that are characterized by an uncontrolled cell proliferation paired with the ability of affected cells to spread either by direct growth into nearby healthy tissue, or transported to distant sites by metastasis via the bloodstream or lymphatic tissue. In most cases, a cancer is originating from one single transformed cell. Cancers can be roughly divided into 'Lymphoma' (Leukemia), 'Sarcoma' (mesenchymal origin), 'Mesothelioma' (mesothelial origin), 'Glioma' (glia cell derived), 'Germinoma' (testicle- and ovary origin) and 'Choriocarcinoma (placental origin). A 'carcinoma' is a cancer that is derived from epithelial cells and can be found in any organ. The most common kinds of cancers are carcinoma, as in breast, prostate, lung and colon cancer.

There are innumerable causes for cancer development, and therefore the number and types of cancers are large. To date, more than 100 cancer types have been described. Whatever cause is underlying the development of such a malignancy, all tumor formations have two requirements in common; i.e. an unlimited potential of the transformed cells to proliferate, paired with the inability of the host's immune system to control the malignant cells by triggering apoptosis.

3.1.1 Skin cancer

Among all cancers, skin cancers are the most common ones (50 % of all cancers in the Western population). Skin cancers can be divided into melanoma- and non melanoma skin cancers, the latter group including Squamous Cell Carcinoma

(SCC) and Basal Cell Carcinoma (BCC). Most important risk factors for developing skin cancers are UV light exposure and skin type.

3.1.1.1 Melanoma

Melanoma, the most serious form of skin cancers, is a highly metastasizing malignancy originating from melanocytes. Melanocytes are the melanin producing cells located in the superficial areas of the skin. Due to this location, the tumor, if diagnosed early, can be relatively easily removed by surgery.

3.1.1.2 Squamous Cell Carcinoma

Squamous Cell Carcinoma (SCC) is the second most dangerous skin cancer. Originating in the epidermal layer of the skin, the tumors are thus located in a skin region where they can usually successfully be excised. However, in some cases SCC can metastasize and spread to other sites of the body which makes SCC a serious disease.

3.1.1.3 Basal cell carcinoma (BCC)

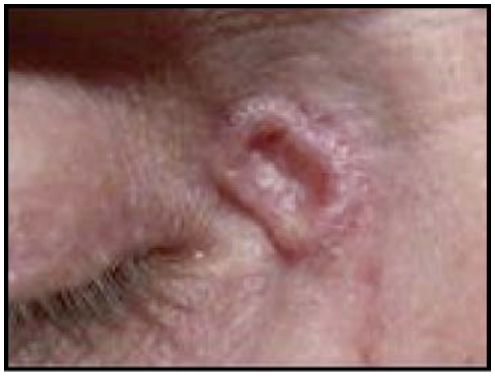
Basal cell carcinoma is by far the most frequent cancer worldwide. Incidence rates are constantly rising due to increased UV light exposure mostly because of outdoor leisure activities and because of the destruction of the ozon layer. BCC is most common in the Caucasian population and rarely affects black- and other dark skinned populations. Highest incidence rates are found among the white population in Australia [1]. The most recent figures from the American Cancer Society suggest a total of 1 million new cases for the year 2005 in the United States. The incidence of BCC is 30 percent higher in men than in women [2]. There is a pronounced geographic variation in incidence, with clear association to sun exposure (UV signature, see below) and race of the population.

Age is a further most important factor influencing incidence. Persons aged higher than 55 years have a 100-fold higher incidence of BCC than persons of age below 20 years [3].

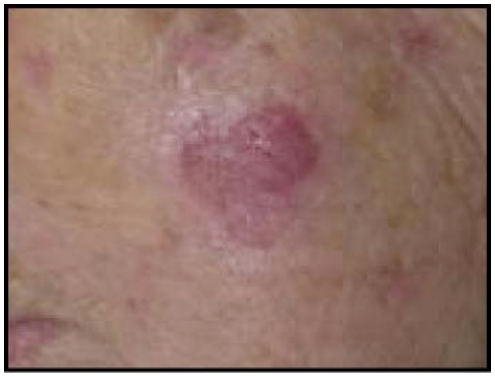
BCC ususally does not metastasize. Rates of metastasis in BCC have been estimated as 0.1 %, [4], however, this malignancy can be very aggressive due to a highly infiltrative and invasive growth pattern by direct extension into adjacent tissue, leading to a severe destruction of affected skin. Especially in the case of facial BCC, the growth characteristic can be highly disfiguring and is therefore associated with severe morbidity. It is not entirely clear why BCC usually does not metastasize, however, BCC is known to be a highly stromal-dependent tumor that requires certain components of the surrounding stroma such as SDF1α (stromal derived factor 1α) [5]. When BCC cells are displaced from the original tumor via the bloodstream or lymphatic tissue, they may lack stromal components at the new site so that growth of malignant cells at a distant site cannot occur [6].

3.1.2 Subtypes of basal cell carcinoma

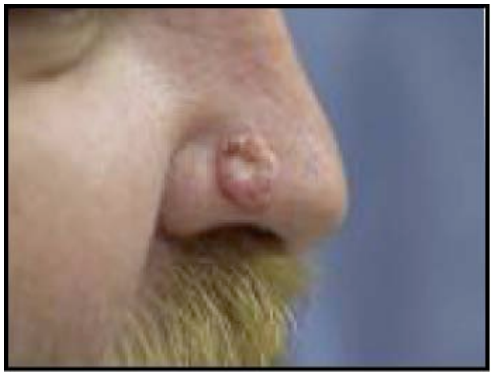
(Images from www.lloyd-derm.com)



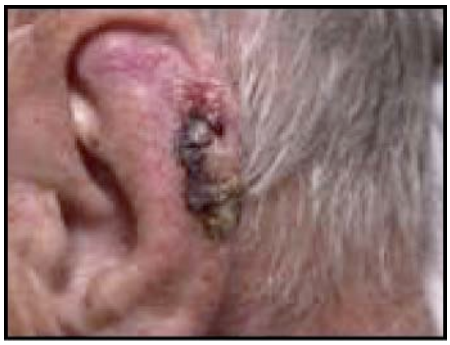
The **amorphic subtype** (sclerodermic form) appears as a scarring plaque with unclear margins and is the most difficult BCC to treat with traditional therapies (see below). Its growth pattern is mainly horizontally.



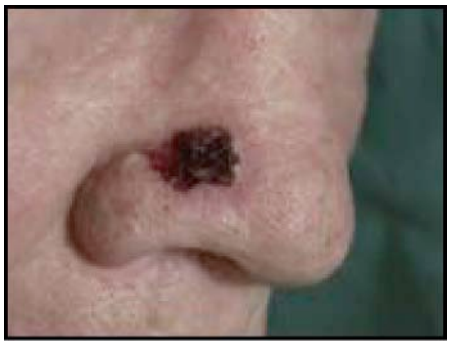
The **superficial subtype** of Basal Cell Carcinoma is most often found on the shoulders and the upper body parts. It is a slowly growing tumor, appearing as a pink or brown patch, sometimes with bumpy margins.



The most common subtype of BCC is the **nodular form**. The nodular BCC appears as a deep-rooted white firm nodule, sometimes centrally ulcerating, and it has well-defined margins.



The most dangerous and invasive form of BCC is the **sclerosing subtype**, which is a variant of the nodular form. It usually involves a large area which appears white and with difficult to define margins. The sclerosing type of BCC is highly invasive and can cause large erosions of the soft tissues within the skin, such as dermis and muscle tissue, affecting even the underlying bones.



All BCC subtypes can, if melanocytes are involved, be **pigmented**.

3.1.3 Treatment of basal cell carcinoma

Therapies of BCC can be divided into physical therapy, chemotherapy and immunostimulatory therapy. The most efficient alternatives are outlined in the following.

3.1.3.1 Physical therapies

3.1.3.1.1 Excision

The complete excision of a BCC tumor including surrounding healthy tissue (clearance distance or safety margin 2-10 mm) is the first choice treatment in most BCC cases. Recurrency rates can be relatively high (5 % up to 10 %) [7] and most probably based on incomplete excision due to too small safety margins.

3.1.3.1.2 Radiation

BCC belongs to the most radiation-sensitive cutaneous tumors, and is therefore predestined for radiation therapy, especially when the site of the tumor does not allow for excision therapy, such as eye lid or bone. Using x-ray radiation, the visible part of a BCC including a clearance distance of 0.5 - 1.5 cm is treated.

3.1.3.1.3 Cryotherapy

Cryotherapy is carried out with liquid nitrogen (at -196 °C) on smaller superficial BCCs with clear margins.

3.1.3.2 Chemotherapy and immunostimulatory agents

The treatment includes local chemo- or immunotherapies in the case of multiple BCC. **5-Fluorouracil** is a cytostatic drug acting mainly on hyperproliferating cells and is applied locally via a creme. Type-I **Interferon** is an efficient immunotherapeutic drug applied intralesionally, however, long term studies are not yet available and the drug is costly. **Imiquimod** is an effective alternative therapy lately established. This drug is locally applied on BCC via a creme. Imiquimod binds to toll-like receptors 7 and -8 and thereby triggers dendritic cells to stimulate inflammatory activities, facilitating BCC cell apoptosis. So far, no long term studies

are available. Generally, the medication therapies and improper physical therapies have shown to bear a relative high risk of relapse (10-50 %). The most efficient therapy is complete excision (remission rate 5-10 %), followed by radiation and cryotherapy (remission rate 10 %).

3.1.4 Cellular and molecular basis of BCC development

A histological view of a BCC-affected skin cryosection is shown in Figure 1.

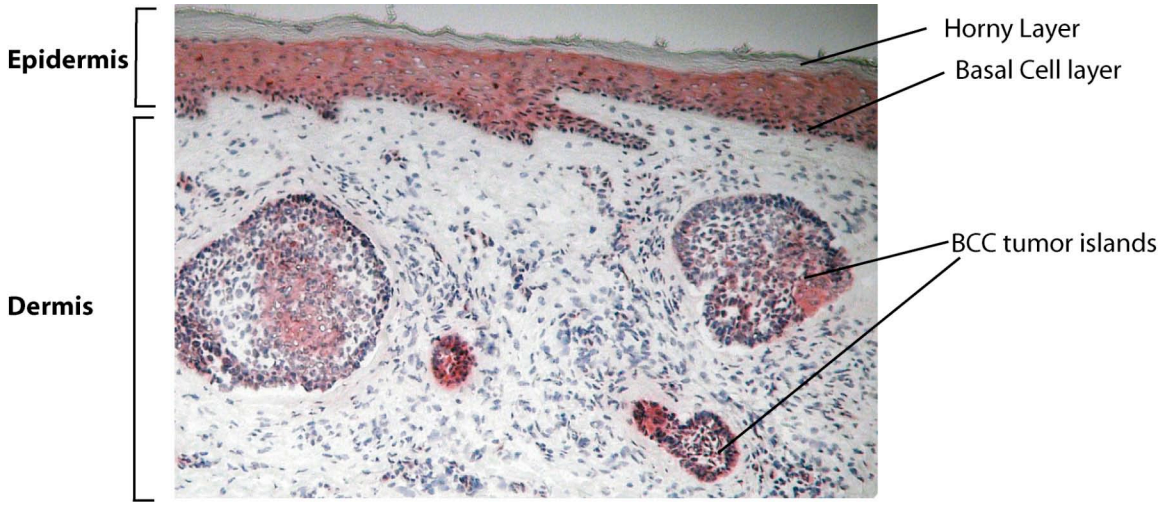


Fig 1

Histological view of a BCC-affected skin. BCC tumor islands are clearly visible as densely packaged cell clusters within the dermis (Hematoxilin nuclear staining). The red color is staining for TR2, a molecule expressed both in epidermal and BCC cells (see Results).

The origin of the cell which forms BCC is not yet entirely defined. BCC may originate from interfollicular basal cells or from keratinocytes of the hair follicle or sebaceous glands [8]. In fact, BCC share gene expression patterns with cells of the basal cell layer of the epidermis and also of the outer root sheath of hair follicles. Especially, the expression of BCC cytokeratines identical to those of hair

follicle cells, and differing from those of epidermis are strong evidence for the hair follicle origin theory of BCC, but the topic is still under debate [9]. However, the fact that primary BCC are not found on mucosae (where hair follicles are absent) is a further support for the hair follicle origin theory.

As expected by the high risk factor UV light, BCC development is highly correlated with gene mutations caused by DNA damage through UVB. The typical '**UV signature**' of UV-light mediated mutations, i.e. C (cytidine) to T (thymidine) or CC to TT transitions at dipyrimidine sites [10], are found in the two mutations that are most correlated to BCC development, i.e. mutations in the tumor suppressor gene *p53* (present in 56 % of human BCC) and mutations in the *Ptch* gene (incidence 30-40 % of human BCC) [11]. While the role of p53 as a tumor suppressor gene which regulates cell cycle and apoptosis among other cellular processes, is very well understood, and the impact of its mutation seems clear (more than 50 % of human cancers are associated with p53 mutations), it is necessary to take a closer look at the role of *Ptch* mutations in the tumor development.

Patched protein, the gene product of *Ptch*, is a key player in the **Hedgehog signaling pathway**, a pathway which is of great importance for the regulation of major developmental processes in almost every tissue formation.

3.1.4.1 Hedgehog signal pathway

The Hedgehog signaling pathway is regulating cell proliferation, pattern formation and differentiation during embryogenesis as well as apoptosis and therefore bears a high oncogenic potential if deregulated. This pathway has first been described in pattern formation of Drosophila embryogenesis. Whereas wildtype larvae express a line of bristles in the dorsal edge of every segment, heterozygous Hedgehog

mutants express the bristles without any pattern evenly distributed all over each segment, which gives the larvae a Hedgehog-like appearance. In mammals, three Hedgehog proteins are described to date, that is Ihh (indian hedgehog), Dhh (desert hedgehog) and Shh (sonic hedgehog). They more or less evoke the same functions in different tissues.

The pathway is initiated by binding of the extracellular Hedgehog signal peptide to its membrane bound receptor, which, over a signal cascade, is leading to the activation of the Hedgehog pathway mediators, the Gli transcription factors. Figure 2 shows an overview over the Hedgehog signaling, silent in the left panel and active in the right one.

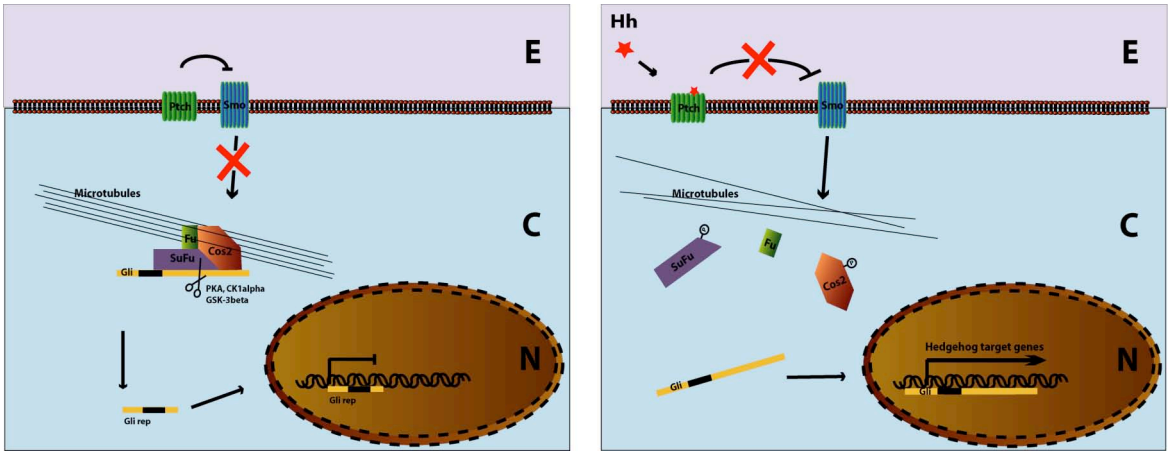


Fig 2

Overview over the Hedgehog signal pathway inactive (left) or active (right). In absence of the Hedgehog signal peptide (Hh), Smoothened (Smo) is inhibited by Patched (Ptch) action. In this situation Gli is retained in the cytosol bound to the cytoskeleton via a protein complex, and gets processed by protein kinases, giving rise to a repressive form of Gli (c-terminally truncated). When Hh binds and inactivates Ptch, Smo becomes active and signals to the protein complex, leading to hyperphosphorylation of the latter, followed by its disintegration. Full length Gli loosens its hold to the cytoskeleton and now translocates to the nucleus where it acts as a transcriptional activator. (E: Extracellular space; C: Cytosol; N: Nucleus)

In a silent Hedgehog pathway situation where the Hedgehog peptide is absent, Ptch, the transmembrane Hedghog peptide receptor is exerting an inhibitory action onto another transmembrane protein Smo, Smoothened, and hinders it to signal to the cytoplasmic section of the signaling pathway. It this situation, the Gli proteins are retained to the cytoplasm by being bound to the microtubules over a protein complex comprised of Costal2, SuFu, and an adapter protein Fused. This makes the Gli proteins available to cleavage through protein kinases PKA, CK1α and GSK-3β, resulting in a c-terminally truncated form of Gli, Gli_{rep} which translocates to the nucleus and acts as a repressor of Hedgehog target genes. Thus, with a silent Hedgehog pathway, or a gain of function mutation of the Hedgehog repressor Ptch, the Gli proteins mediate a repression of the Hedgehog target genes.

In the opposite situation, when the Hedgehog signal peptide is present and binds to and inactivates Ptch, or Ptch is inactivated due to a loss of function mutation, then Smo is no longer controlled by Ptch and gets able to exert its action on the cytoplasmic section of the pathway, leading to a hyperphosphorylation of the proteins bound to the complex with Gli. This makes full length Gli dissociate from the microtubules and it can now translocate to the nucleus where it can bind to, and induce transcription of Hedgehog target genes.

As Hedgehog target genes are known to promote cell proliferation and inhibit differentiation, it is obvious that any mutation, like a loss of function mutation in *Ptch*, that leads to an overactivation of Gli transcription factors, bears a high oncogenic potential.

3.1.4.1.1 Gli transcription factors

To date, three Gli proteins are described in mammals. They are large (>1000 amino acids) 5-finger Zinc finger transcription factors which have first been

described in Glioma cells, designating them as 'Glioma-associated oncogenes' Gli1, Gli2 and Gli3. They bind DNA target sequences with the last three zinc fingers [12], while the function of the first two zinc fingers is yet unknown. There are hints however, that Gli proteins can dimerize via the first two zinc fingers, keeping the last three zinc fingers available for DNA binding [13].

Gli proteins (the homologs of Cubitus interruptus, Ci in Drosophila), mediate transcription control by Hedgehog signaling in vertebrates. The three Gli proteins have similar biochemical properties and are most probably derived from gene duplication, which might have allowed the evolution of a context-specific action for each Gli protein [14]. While Gli1 possesses only an activation domain, Gli2 and Gli3 contain both activation and repression domains (see Figure 3). When the Hedgehog signaling is silent, i.e. in the absence of Hh peptide, Gli2 and Gli3 proteins are C terminally truncated by several protein kinases (see above, Gli_{rep}). C terminally truncated forms are localized in the nucleus and have dominant negative activity over that of full-length proteins, providing a strong repressive action on Hedgehog targets when the pathway is inactive. In the absence of truncated Gli however, the full length form of Gli2, and Gli1 itself are strong transcriptional activators of target genes, as their C terminal activation domains are then dominant over the N terminal repressor domains.

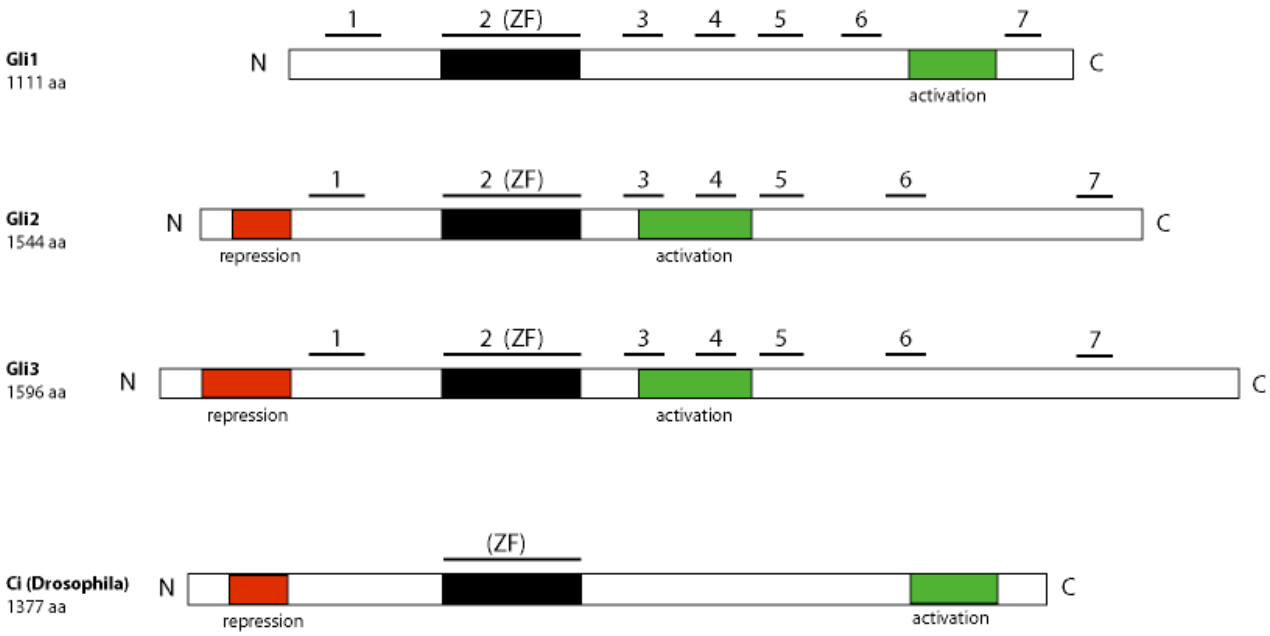


Fig 3

The mammalian Gli protein family (Gli 1– 3) in comparison to the Drosophila homolog Ci. Underlined numbers indicate conserved amino acid sequences in the three family members which presumably originated from gene duplication (see text). Activation domains are C terminally located (green) and get cut off during processing in absence of Hedgehog peptide. In the full length form, the activation domains are functionally dominant over the N-terminal repression domains (red). (ZF: zinc finger domain)

The three Gli proteins have partially redundant and partially distinct functions. They induce different target genes and presumably act in a combinatorial manner which is varying between tissues and between species. It is important to note that at least Gli2 and Gli3 also respond to other triggers besides Hedgehog, as they are expressed in tissues and regions where Hh is not expressed at all. Fibroblast Growth Factor (Fgf) for example has been shown to regulate Gli2 and Gli3 in the embryonic mesoderm [15], indicating that they network several signal pathways. Figure 4 shows an schematic overview of the interactions between the three Gli proteins.

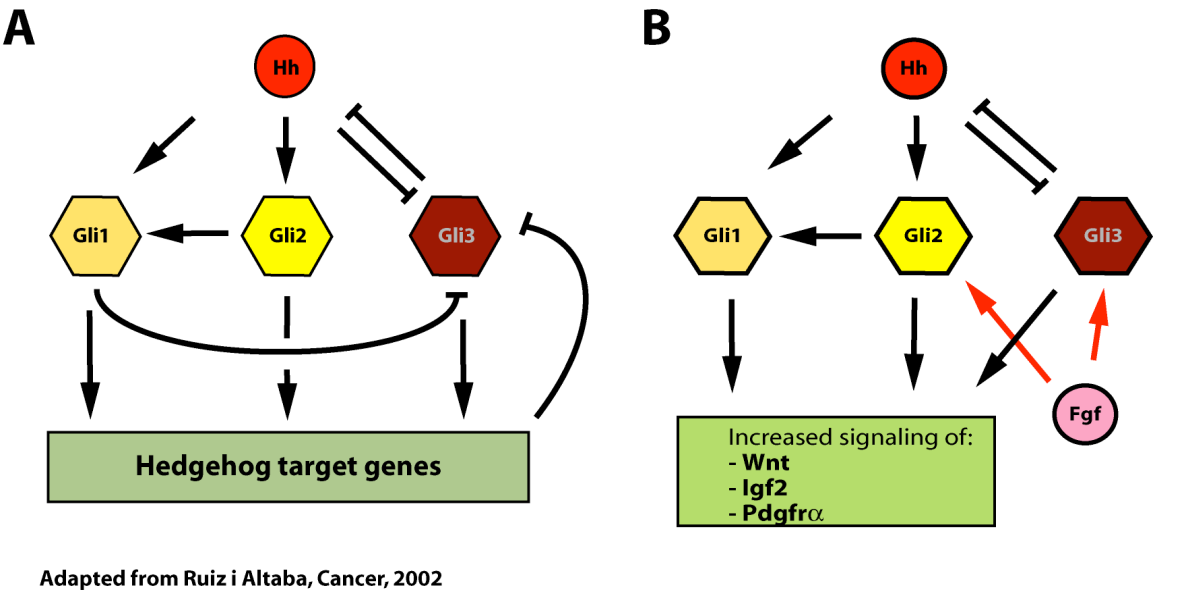


Fig 4

Interactions between the three mammalian Gli family members and influence of Hedgehog signaling on other signal pathways. A: Gli1 and Gli2 are the positive mediators of Hedgehog signaling. Gli1 is a transcriptional target of Gli2 and represses Gli3, which itself is a negative regulator of Hedgehog. Gli2 is the primary Hedgehog mediator (see text). B: Gli2 and Gli3 are transcriptional targets of Fgf, and all 3 Gli proteins regulate further signal pathways (see text).

Gli3 is a transcriptional repressor of Hedgehog genes and is itself repressed in response to Hedgehog signals [16,17]. Among other targets, Ptch is a gene that is transcriptionally repressed by Gli3 which thereby exerts a negative feedback loop of the Hedgehog signal itself. Gli1 and Gli2 are the positive mediators of Hedgehog signaling, although some of their regulatory functions are distinct, others are overlapping [18]. Gli1 is believed to transactivate through a VP16-like domain (Herpes simple virus protein-16 like) [19], while Gli3 and possibly also Gli2 are transactivating through the CBP (Creb Binding Protein) co-activator, which is the same way Drosophila Ci is acting [20,21].

In summary, Hh signaling induces the transcription of the positive Hh mediators Gli1 and Gli2 whereas it represses the transcription of the Hh antagonist Gli3. Loss-of-function mutations in Ptch lead to continuous signaling through Smoothened, and thereby result in overactivated Hedgehog signaling and Gli protein overexpression.

Hedgehog signaling is a tightly controlled pathway. Many of its target genes regulate checkpoint molecules of the pathway itself (reviewed in [22]) and thereby exert several feedback mechanisms. This is not surprising, as controlled Hedgehog activity is needed for development. On the one hand, lack of, or too low Hedgehog signaling is leading to severe developmental defects (reviewed in [23]), on the other hand, overactivation of Hedgehog signaling is highly linked to tumorigenesis.

3.1.4.1.2 Gli and cancer

Basically, the Hedgehog signaling pathway is essential for many developmental processes especially during embryogenesis. Cell proliferation and cell fate determination are controlled by Hedgehog target genes. After embryogenesis the overall activity of the pathway is diminished, however, many data show that the Hedgehog pathway remains active in some adult organs like the skin, where it regulates maintenance and proliferation of stem cells. As a consequence, uncontrolled activation of the pathway is a major cause for cancer, and many tumor types have been linked to overactivated Hedgehog signaling (reviewed in [24] and [25]). BCC, Medulloblastoma, Trichoepithelioma, Bladder carcinoma, Esophageal Squamous Cell Carcinoma, Primitive Neuroectodermal Tumor, Meningioma, Rhabdomyosarcoma and Glioblastoma are all highly correlating with loss-of-function mutations in Ptch.

With a special focus on skin cancers, overproduction of either Gli2 or Gli1 induces skin carcinogenesis [26-28] and high Gli2 expression has been shown many times to promote BCC tumorigenesis [28-31].

3.1.4.1.3 Gli2 is the primary positive transducer of Hedgehog signaling

Gli2 seems to be the primary positive transducer of Hedgehog signaling, as can be assumed from the following findings:

a) $Gli1^{-/-}$ mice are viable and have no obvious defects whereas $Gli2^{-/-}$ mice are lethally defective in the very same aspects as $Hh^{-/-}$ mice [32-34].

b) in case of experimental Hh peptide overexpression, removal of Gli2, but not of Gli1, rescues the normal phenotype [35] and

c) hair follicle development is dependent on Gli2, Gli1 is dispensable for this process [36]

3.1.4.1.4 Gli2

The *Gli2* gene can be spliced by two independent splicing processes into four isoforms Gli2 - α, β, γ and -δ, giving rise to four proteins of 133-, 131-, 88-, and 86 kDa size, respectively [37]. Gli2 is particularly highly expressed in BCC, however, the link between elevated Gli2 and tumor formation in BCC is still subject to many investigations. Gli2 promotes cell proliferation, for example by inducing Cyclin D expression, it further mediates the increase of other cell proliferating signal pathways (see above), and presumably Gli2 also interferes with apoptosis pathways by altering the balance between anti-and pro-apoptotic molecules in the cell. Regl et al. have shown that the strong apoptosis inhibitor Bcl-2 is a direct transcriptional target of Gli2 [38]. So far, no further Gli2 targets could be linked to apoptosis yet, however, we postulate that more apoptosis-related genes will be identified as Gli2 targets in the future.

3.1.4.1.5 NHis-Gli2

In order to address the question in which way Gli2 supports tumor formation in BCC, we aimed to identify Gli2 target genes that are linked to apoptosis. We made use of a cellular system that allows to experimentally induce Gli2 overexpression. We obtained the transgenic cell line NHis-Gli2 from Prof. Aberger [39] from the University of Salzburg. This HaCat keratinocytic cell line is stably transfected with two plasmids in order to render the cells inducible for transgenic Gli2 expression by tetracycline. Figure 5 shows the Tet ON system introduced in this cell line. The reverse tet controlled trans-activator (rtTA), coded on one transfected plasmid, is continously expressed upon the sp6 promoter. The reverse tet controlled trans-activator can bind P_{tet}, a fusion between a minimal promoter (RNA polymerase II promoter from Herpes simplex VP16) and an array of several tetO sequences (tetracycline resistance operon sequences from *E.coli*), only in the presence of tetracycline and thereby stimulates the onset of transcription of Gli2β. In the absence of tetracycline, the rtTA is unable to bind P_{tet}, and no or very few Gli2 is expressed.

In our transgenic cell line NHis-Gli2, we used 2.5 μg tetracycline/ml medium for 72 hours to reach a Gli2 expression of 15 times on the mRNA level compared to endogeneous Gli2 expression in tetracycline-uninduced cells.

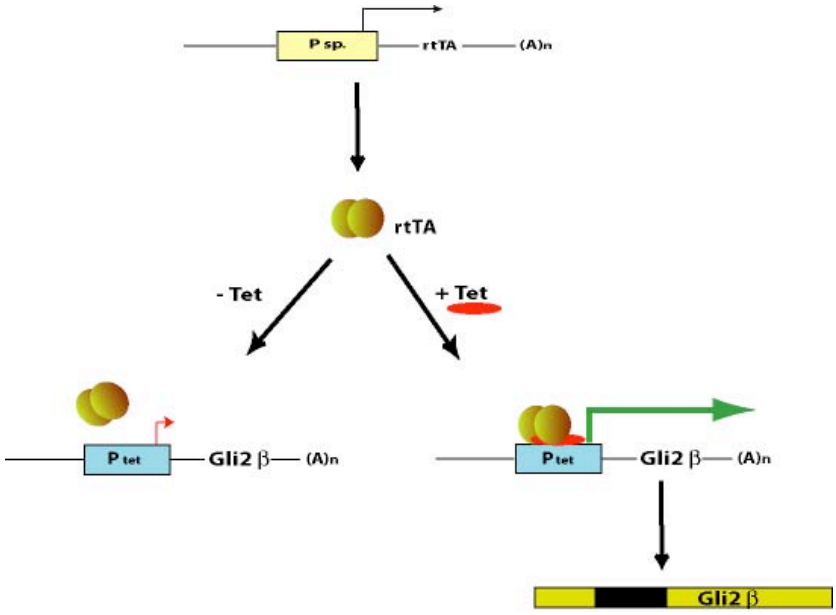


Fig 5

Tetracycline-inducible gene expression. Cells are stably transfected with two plasmids, one expressing the reverse tet controlled trans-activator (rtTA) that can bind to the tet-responsive promoter (P_{tet}) only in the presence of tetracycline (right). Absence of tetracycline prevents the binding of rtTA to P_{tet} and the transgene is not or only very moderately expressed (left).

3.1.4.2 Apoptosis pathways

Cells have to be eliminated from the organism in certain circumstances. Excess tissues during embryogenesis (e.g. the interdigital space during the formation of hands or feet), cells infected with virus or other intracellular parasites, transformed cells are among many other examples. This can mean the elimination of huge cell numbers, as for example the elimination of excess lymphocytes after a terminated immune response. When the infectious agent is eliminated, a whole army of potentially aggressive effector lymphocytes have to be controlled, i.e. killed. If great numbers of cells would die in an uncontrolled manner, the released cellular contents would trigger inflammatory processes and thereby do great harm to the

host. Therefore, the cells must be eliminated in a controlled way which does not induce inflammatory processes.

The term ‘Apoptosis’ (greek: ‘falling leaves’) is describing a programmed and therefore controlled cell death. Cells are induced to commit a ‘suicide process’ in which the cell degrades its own proteins along with alien proteins from potentially infective agents, fragments its DNA and packages its contents in a way that makes it unable to reach the extracellular space. At last, the cell flips its cell membrane inside out, thereby exposing membrane proteins like phosphatidylserine (PS) that are recognized by macrophages or other surrounding cells which then eliminate the apoptotic cell in a controlled manner. For the apoptotic process, the cell must activate specific enzymes, e.g. Caspases, which perform the above described procedures. Apoptosis is therefore an active process of dying which is tightly regulated. There are innumerable proapoptotic molecules driving cell death processes onwards, and equal numbers of antiapoptotic molecules that inhibit or counteract the former. Apoptosis is a process that, after a starting trigger, runs a programmed pathway, with many checkpoints where the balance between pro- and antiapoptotic players decides about life or death. This tight control is necessary as easily seen in the many diseases where apoptosis is overshooting (e.g. autoimmune diseases such as Multiple Sclerosis) or inefficient (e.g. cancers, viral infection). The balance between pro- and anti-apoptotic molecules is often shifted towards protection against apoptosis, for example in cases where cancer cells have loss-of-function mutations in proapoptotic molecules [40-42]. or on the other hand in case of viruses that code for potent antiapoptotic molecules [43]. In both situations, the host cell is protected against the immune system’s actions.

Basically there are two major pathways of apoptosis (see Fig. 7), i.e. the intrinsic, stress induced pathway in which a cell perceives stress signals from within, and the extrinsic, ligand induced pathway, where apoptosis is extracellularly triggered by so called death ligands.

a) The *intrinsic* pathway of apoptosis starts by the stress-induced (e.g. oxidative stress or DNA strand breaks, over p53 induction) disintegration of the mitochondrial membranes that leads to the release of cytochrome c from the mitochondria. Cytochrome c is an essential component of the apoptosome, where Apaf1 and Cytochrome c cleave and thereby activate Procaspase 9. Active Caspase 9 then cleaves and activates the effector Caspases 3, -6 and -7. These Caspases then process the enzymes leading to the features of apoptosis like DNA fragmentation, membrane flipping and others.
b) The *extrinsic* pathway of apoptosis is triggered either by pore formation in the target cell membrane by Perforin or, most importantly by the binding of a death ligand (see below) to its receptor in the extracellular part of the cell. The death signal is transduced to the cytoplasm by receptor conformational changes of the cytoplasmic domain, where, over an adaptor molecule, Procaspase 8 is recruited and activated. Active Caspase 8 then cleaves and thereby activates the effector Caspases 3, -6 and -7 (see below for more detailed information).

3.1.4.2.1 Death-ligand induced apoptosis

Death ligand-death receptor interactions are an important starting point for external apoptosis induction. The death ligands, members of the TNF superfamily of proteins, are secreted by, or membrane bound to, attacking lymphocytes. The most prominent two members of the death ligand family are CD95Ligand (FasL) and Trail (Apo2L) (**T**NF **R**elated **A**poptosis **I**nducing **L**igand). Their receptors are members of the TNF receptor superfamily of type II transmembrane proteins. Six members of this family are of major importance concerning death-ligand induced apoptosis. Two receptors for FasL (one death receptor and one decoy receptor) and four receptors for Trail (two death receptors and two decoy receptors) are well described. Death receptors transduce the death signal to the cytoplasm whereas decoy receptors inhibit the death signal by catching the ligand without a further signal transmission. As decoy receptors antagonize the death signals, the relative number of death receptors / decoy receptors that are bound by death ligands decides about life or death of a cell. Therefore, also the composition of receptors is a checkpoint of apoptosis, buffering the system in order to tightly control death signaling. Figure 6 shows receptors for FasL and Trail.

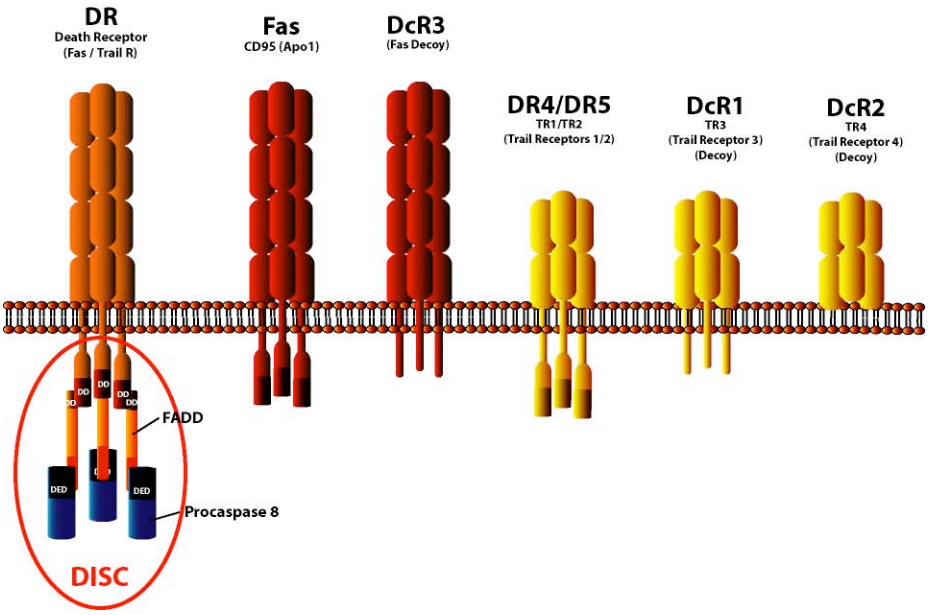


Fig 6

Death receptors of the TNFR superfamily. The left death receptor (orange) is a general death receptor complex comprising a trimerized death receptor, the adapter molecule FADD/TRADD and the bound Procaspase 8 molecules. Red receptors are Fas Ligand (FasL) receptors CD95/Fas (left) and Fas Decoy (right), the latter lacking the Death Domains (DD) which makes FADD/TRADD binding impossible. In yellow the receptors for Trail are shown, Trail receptors 1 and 2 are taken together as the most left yellow receptor, Trail decoy receptors are shown on the left with Decoy Receptor 1 lacking the DDs and Decoy Receptor 2 lacking all domains but the extracellular one.

Upon binding of the ligand, the death receptor is trimerized and its cytoplasmic domain undergoes a conformational change. These two events make the cytoplasmic tail of the death receptor available for the binding of an adaptor molecule, FADD (**F**as **A**ssociated **D**eath **D**omain) or TRADD (**T**NF-**R**eceptor **A**ssociated **D**eath **D**omain), which then recruits Procaspase 8 molecules out of the cytoplasm. Procaspase 8 binds to FADD via its death effector domains (DEDs). The receptor complex, now more recruiting Procaspase 8, is called the DISC (Death inducing signaling complex). According to the best accepted model,

Procaspase 8 molecules undergo autocatalysis when they are brought into close proximity to each other, resulting in active Caspase 8, also called Flice (**F**ADD-**l**ike-**i**nterleukin β-**c**onverting-**e**nzyme). Active Caspase 8 then dissociates from the DISC and cleaves the effector Caspases 3,-6 and -7 which are thereby activated. Active effector Caspases themselves activate the enzymes that are responsible for the apoptotic processes such as DNA fragmentation or membrane flipping (e.g. degradation of DNA repair enzymes (PARP, Poly(ADP-ribose)polymerase), activation of Flipases).

3.1.4.2.2 Caspase 8

Caspases are enzymes with a crucial cysteine residue that cleaves other proteins behind an aspartic acid residue, therefore called **C**ysteine-**Asp**aragine prote**ases**. Caspases are essential for apoptosis pathways, and are classified into initiator Caspases 8 and -9, and effector Caspases 3, -6, and 7 (see above). Initiator Caspases cleave inactive pro-forms of effector Caspases, thereby activating them; effector Caspases in turn cleave other protein substrates within the cell resulting in the apoptotic process. Twelve caspases have so far been identified in humans. Caspase 8 is the initiator of the death ligand-induced apoptosis pathway. Caspase 8 contains two DED (death effector domain), which bind to the DED of the adaptor protein FADD. Upon cleavage, several small subunits of Caspase 8, p43/41, p18 and p10 are formed (numbers indicate their size in kDa). P18 and p10 are the active Caspase 8 molecules that cleave and thereby activate the effector Caspases. This post-transcriptional regulation ensures that Caspases can be very rapidly activated without precedent translation. Caspase 8 is an important apoptosis-promoting molecule, as it does not only initiate receptor-mediated apoptosis, but also links the latter to the internal pathway of apoptosis. It does this by cleaving Bid, a pro-apoptotic member of the Bcl-2 family of proteins. Cleaved and therefore activated Bid translocates to the mitochondrial membrane where it

forms pores together with Bax, another proapoptotic member of the Bcl-2 family. The disintegration of the mitochondrial membrane simulates a stress situation for the cell, as it leads to Cytochrome c release, followed by Caspase 9 activation. By signaling death ligand-mediated apoptosis, and linking it to the internal apoptosis pathway, Caspase 8 is a potent proapoptotic molecule, and blocking its action is therefore a powerful event in apoptosis prevention. **Flip** is a catalytically inactive structural homolog of Caspase 8 and acts in many cell types as a Caspase 8 regulator.

Figure 7 shows an overview over the death-ligand-mediated pathway of apoptosis and its link to the mitochondrial one.

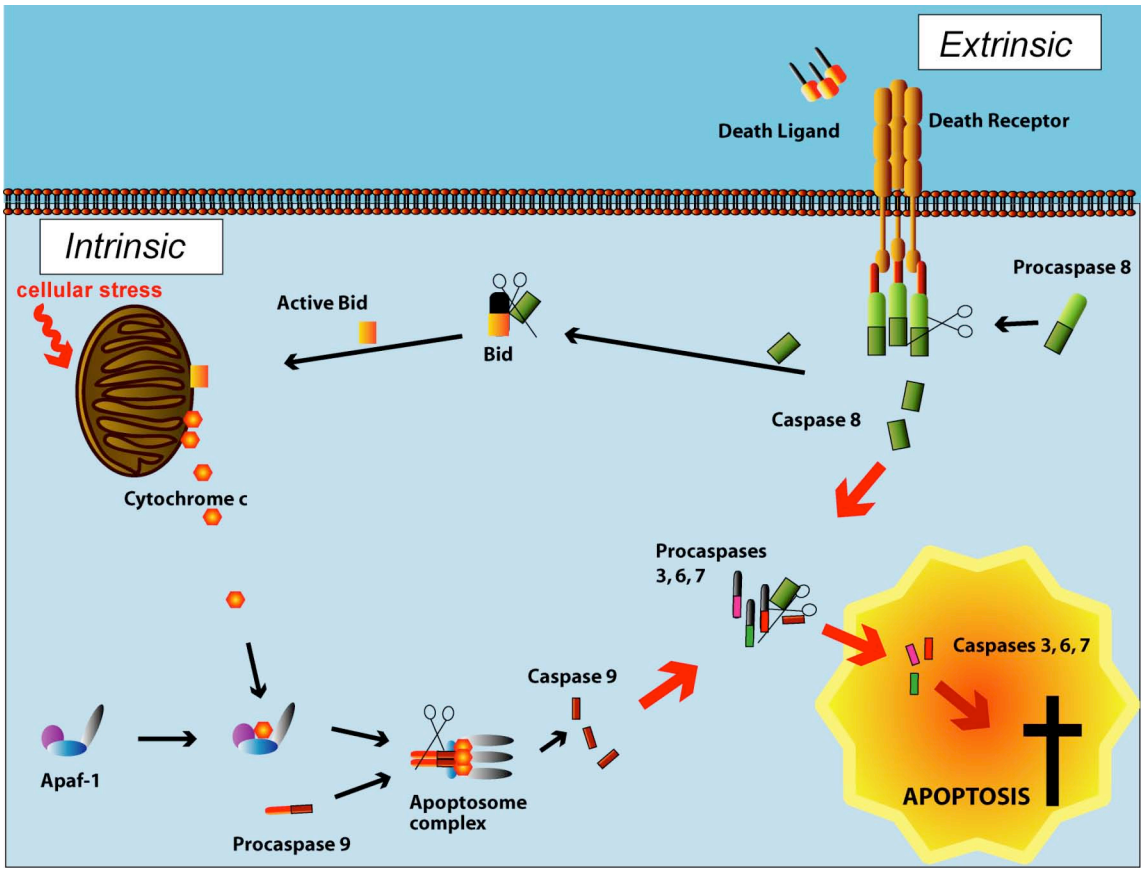


Fig 7

Death ligand-mediated pathway of apoptosis (Extrinsic) and its link to the mitochondrial one (Intrinsic). For details refer to text.

3.1.4.2.3 Flip

Flip (**Fl**ice **i**nhibiting **p**roteins), also called Cflar, Casper or Usurpin, are structural homologs of Caspase 8, either endogeneously expressed by the cell (cFlip, cellular Flip) [44,45] or exogeneously introduced into a host cell by certain viruses (vFlip in Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8 and HVS), as well as the tumorigenic human molluscipoxvirus [46]).

Flip, just like Caspase 8 (see Figure 8), contains two death effector domains (DED) which make it able to bind the death domains (DD) of the adaptor molecules FADD (TRADD), thereby hindering the recruitment of Procaspase 8 molecules to the death inducing signaling complex (DISC). On the one hand, Flip competes against Caspase 8 for FADD binding sites at the DISC, on the other hand, even though Flip may be simultaneously bound to the DISC with Procaspase 8, it does not cleave the latter and thereby silences the affected receptor complex.

cFlip

Two isoforms of cFlip have been described so far on the protein level, however, numerous mRNA splice variants are known. The two protein variants of cFlip are shown in Figure 8 in comparison with Caspase 8. $cFlip_{long}$, just as Caspase 8, has two DEDs which allow binding to FADD/TRADD, but the caspase subunit (Peptidase C14) homolog of $cFlip_{long}$ is inactive. $cFlip_{short}$ has only the two DEDs expressed, and a caspase subunit homolog is entirely lacking. The two isoforms thus bind to the death receptor complex but do not give on the death signal, providing a dead end of the pathway which hinders further recruitment of Procaspase 8 molecules out of the cytoplasm. Therefore, bound cFlip molecules entirely block apoptosis signaling at the level of Caspase 8.

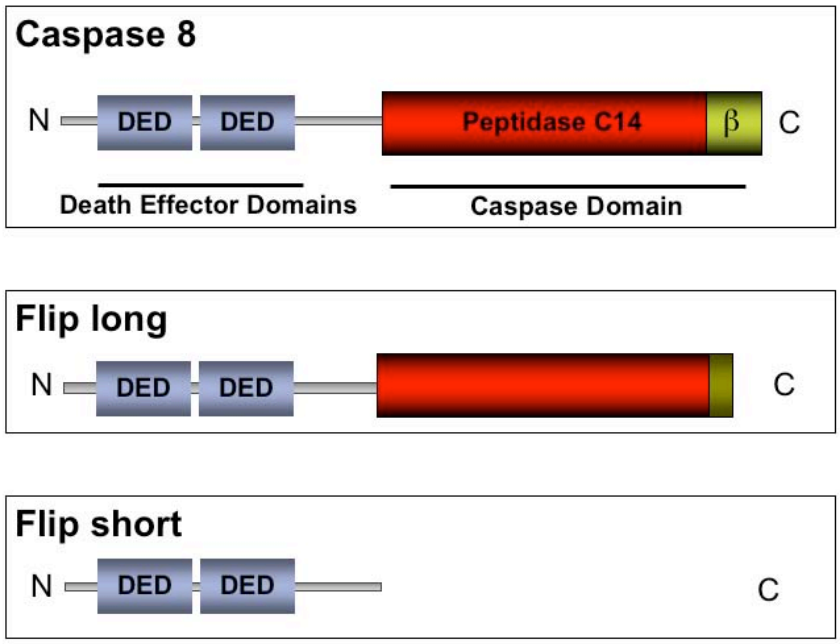


Fig 8

The two splice variants of cFlip on the protein level in comparison with Caspase 8. Flip long comprises two death effector domains (DEDs) and a catalytically inactive caspase domain homolog which makes cFlip long a potent Caspase 8 competitor. cFlip short is made out of the two DEDs only which make it bind to FADD/TRADD, and thereby blocks any Caspase 8 recruitment to the DISC.

cFlip acts as a protector against apoptosis when lymphocytes, which express FasL, coexpress Fas, in order to avoid self-induction of apoptosis. B-cell receptor (BCR) stimulation, for example, has been shown to upregulate cFlip expression [47]. Furthermore, NFκB, which is stimulated by T-cell receptor (TCR) signaling, has been shown to transcriptionally activate cFlip expression [48]. Only when external death signals exceed a certain threshold, the number of activated death receptor complexes exceeds that of inhibitory cFlip molecules, and the apoptosis cascade becomes initiated. This tight regulation represents a buffered balance between life and death of cells, e.g. lymphocytes.

As cFlip is a central player in the regulation of apoptotic cell death, its regulation of expression is of major importance. Several pathways are involved in cFlip regulation, i.e. NFκB and the PI3K/Akt [49-51]. It is highly likely that further pathways may be linked to cFlip expression, especially among those which are involved in apoptosis.

The *cFlip* promoter region is not defined to date, however, the 5'UTR of the *cFlip* gene (chromosome 2, gene locus 2q33) is sequenced (NCBI entry: AB038972) and has become an interesting subject of investigations. *In silico* analysis of the *cFlip* 5'UTR reveals several characteristic features (see Figure 9).

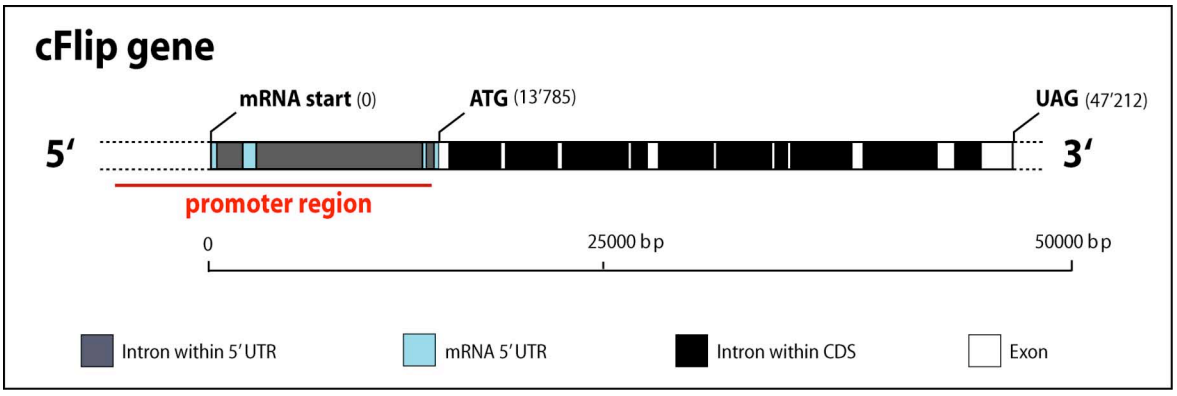


Fig 9

The organisation of the cFlip gene. The putative promoter region is underlined in red, it comprises 3 DNA stretches of differet lengths that are untranscribed into mRNA (grey). The 5'UTR of the mRNA is shown in light blue (4 stretches). The cFlip pre-mRNA has 10 introns of various lengths, making the whole cFlip gene approximately 50 kbases long while the mature mRNA spans a length of 2243 bp only.

The putative *cFlip* promoter region is unusually long (>12 kb), and the pre-mRNA consists of three nucleotide stretches that are excised before mRNA maturation, one of which spans several kilobases of length, therefore the mature mRNA 5'UTR is only 2243 bp in length (NCBI entrance NM_003879).

3.2 Aim of the study

The role of Gli2 as a cell cycle regulator and promoter of proliferation in embryonic development has already been intensively studied. It has been shown that Hedgehog signaling can preserve the replicative potential of epidermic stem cells [52], and as such may also be of major importance for the maintenance of tumor precursor cells in the development of BCC (Hedgehog signaling in BCC reviewed in [53]). However, uncontrolled cell proliferation is just one aspect of cancer, and a developing tumor usually has to cope with immune surveillance and therefore needs instruments to block apoptosis. We argue that a loss of function mutation in *Ptch*, leading to overactivation of Gli2, can promote the development of BCC because it may interfere with the apoptotic machinery of transformed cells by activating anti-apoptotic molecules, and/or by inactivating proapoptotic molecules. The anti-apoptotic molecule Bcl-2 has already been shown to mediate resistance against the intrinsic pathway of apoptosis being of major importance in the survival of tumor cells in situations where the supply of nutrients or oxygen is critical (mitochondrial stress), or when the cells face toxic stress situations due to drug treatment. For the resistance against death signals from attacking lymphocytes, however, Bcl-2 probably plays a minor role. Thus, the search for further apoptosis related genes that lie downstream of Gli2 is self-evident. The aim of the present study was to shed light on the apoptosis resistance of BCC and thereby contribute to the elucidation of the link between elevated Gli2 levels and tumor development in BCC.

The pursuit of this aim was done in three steps:

1. In the first phase, Gli2 target genes were identified that are related to apoptosis using gene expression arrays. As a prime candidate, cFlip was identified and a detailed analysis of the dependence of cFlip from the transcription factor Gli2 was carried out. We focused our interest on cFlip, as its upregulation upon Gli2 overactivation was very prominent, and cFlip is regarded as a very potent inhibitor of the extrinsic apoptosis pathway.
2. In the second phase, we manipulated the expression of the Gli2 target cFlip in a model cell line to assess its relevance in apoptosis prevention. As there might be several factors contributing to the Gli2-mediated protection from apoptosis, it was necessary to estimate the role of cFlip in that protection. This was done by comparing the protective effect of Gli2 in situations where cFlip expression was high or low. (cFlip expression manipulation by RNAinterference).
3. In the third phase, the *in vitro* data were verified *in vivo*. We confirmed and validated the data obtained in the model cell line in basal cell carcinoma.

4 Materials and Methods

4.1 Statistical analyses

In all experiments which addressed gene expression comparisons, (i.e. gene expression in uninduced compared to tetracycline-induced cells or control BCC tissue compared to siRNA treated BCC tissue), we used the *unpaired one-sided Student t-test* to calculate whether results are significant.

4.2 Basic DNA and RNA applications

4.2.1 Purification of genomic DNA

Genomic DNA was extracted from HaCat cells using the DNeasy Tissue Kit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer's protocol.

4.2.2 Polymerase chain reaction (PCR)

All reactions were carried out in a Protocol^{TM} thermal cycler (AMS Biotechnology, Bioggio-Lugano, Switzerland).

template DNA	:	100 ng	
fw primer	:	0.5 μM	
rv primer	:	0.5 μM	
10xPCR buffer	:	1 x	(Invitrogen, Luzern, Switzerland)
MgCl	:	1.5 mM	(Invitrogen)
dNTP's	:	0.2 mM	(Invitrogen)
T'aq Polymerase	:	1 U	(Invitrogen)

Program:

1. initial denaturation:	95 °C, 2 min
2. denaturation:	95 °C, 45 sec
3. primer annealing:	x °C, 45 sec (melting temperatures listed in table 9.1.6)
4. elongation:	72 °C, 2.5 min
5. final elongation:	72 °C, 10 min
6. chilling:	4 °C, infinite

steps 2. to 4. were cycled 35 times before moving to step 5.

Exception: The Bcl-2 promoter fragment (blunt end) was generated using Pfu Polymerase (Stratagene, Amsterdam, The Netherlands) and the corresponding buffer using cycling conditions as described above for Taq polymerase.

4.2.3 Total RNA extraction

Total RNA was purified from HaCat cells using the RNeasy Kit (Qiagen) according to the manufacturer's protocol.

4.2.4 cDNA Synthesis

2 μg of total RNA was mixed with 0.25 μl oligo $(dT)_{18}$ (500 μg/ml), denatured at 70 °C for 10 minutes and chilled on ice.

4 μl 5x 1st strand buffer, 2 μl 100 mM DTT, 1 μl 10mM dNTP's and 1 μl (200 U) Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV-RT) was added, and the mix adjusted with ddH_2O to a final volume of 20 μl. The mix was incubated at 37 °C for 1 hour, followed by 10 minutes incubation at 70 °C to inactivate the enzyme reaction. All chemicals used for cDNA synthesis are from Invitrogen.

4.2.5 Real Time Polymerase chain reaction (RT-PCR)

All reactions were carried out in a Mx4000 cycler (Stratagene)

template cDNA	:	1	μl	
fw primer	:	0.3	μM	
rv primer	:	0.3	μM	
TaqMan Universal Mastermix	:	1	x	(Qiagen)
TaqMan Probe	:	200	nM	

The final volume was adjusted with dH_2O to 20 μl, and the mix transferred into 96 well Optical reaction plates (Applied Biosystems, California, USA).

4.2.6 DNA/RNA precipitation

DNA/RNA solutions were mixed with 0.1 volumes of NaAc and 2.5 volumes of 100 % Ethanol in a 1.5 ml Eppendorf reaction tube and precipitated on ice for 1 hour, followed by full speed centrifugation in a microcentrifuge (13500 x g) at RT. The supernatant was discarded and the pellet washed with 300 µl 70 % Ethanol. The pellet was air dried and resuspended in an appropriate volume of TE.

4.2.7 DNA cloning

PCR products, carrying restriction sites integrated in the primer sequences, and vector plasmids were restriction digested at corresponding restriction sites in order to generate sticky ends, run on an agarose gel for purification, excised in order to get rid of the cut edges of the PCR products and ligated using the Quick Ligation Kit (New England Biolabs, Boston, USA) according to the manufacturer's protocol. PCR primers are listed in table 9.1.6.

Exception: The Bcl-2 promoter fragment was blunt-end ligated into the pGL3 luciferase expression vector (Promega, Catalys, Wallisellen, Switzerland) as described below.

All inserts were checked after bacterial transformation for right orientation and sequence by PCR (see screening for recombinants) and sequencing.

4.2.8 Vector plasmids

plasmids used are listed in table 9.1.5.

4.2.9 Restriction digests

0.5 – 1 µg DNA (plasmid or PCR product) was restriction digested using restriction enzymes as listed in table 9.1.9. Reaction conditions were set following the recommendations of the enzyme provider company.

4.2.9.1 Blunt-end generation of Bcl-2 promoter PCR product and pGL3 vector

The PCR product of the Bcl-2 promoter fragment was blunt-end ligated into pGL3. Therefore we digested the vector with ApaI (New England Biolabs), (blunt-end cutter, see table 9.1.9). The PCR product of the Bcl-2 promoter fragment was generated with Pfu Polymerase (Stratagene), as this polymerase has a 3' to 5' exonuclease activity, leading to a blut-end PCR product that could be directly cloned into the ApaI-digested vector.

4.3 Bacterial transformation

4.3.1 Plasmid transformation into bacteria

Competent E.coli HB 101 (provided from F. Bachmann, Inst. of Med. Microbiology, Basel) were thawed on ice and 30 ng of plasmid was mixed to 30 μl of bacteria. The mix was kept on ice for 10 minutes before heat-shocked at 42 °C for 50 seconds. After the heat shock, the mix was chilled on ice for 5 minutes, then resuspended in 300 μl of antibiotic-free LB medium and incubated at 37 °C on a shaker for 1 hour to allow for antibiotic resistance to be expressed. 150 μl of the mix was then plated on Agar plates containing the plasmid-related antibiotic, and plates incubated at 37 °C. Colonies were picked and analysed after 12 hours of incubation.

4.3.2 Screening for recombinants

Bacterial colonies were picked with a sterile toothpick and traces were transferred into PCR tubes. A PCR mix containing a primer binding the plasmid and a primer

binding the insert was added to the tube, and a PCR was run for each clone to be analysed. The PCR products were run on an agarose gel. Those colonies that had uptaken a plasmid containing the correct insert were identified by a band of correct size on the agarose gel.
Primer sequences for colony PCR are shown in table 9.1.6.

4.3.3 Plasmid propagation

Bacterial recombinants were picked from the agar plate and transferred into sterile culture tubes (Greiner, Mettmenstetten, Switzerland) with 4 ml of LB medium containing the plasmid-related selection antibiotic, and the mix was incubated for 12 hours at 37 °C on a shaker (200 rpm). Plasmids were purified from this culture using the NucleoSpin® plasmid purification kit (Macherey-Nagel, Oensingen, Switzerland) according to the manufacturer's protocol. Inserts were then checked by sequencing (ABI prism™ 310 Genetic Analyzer, Applied Biosystems) using a primer binding the insert shortly 5' upstream of the insert (table 9.1.6) according to the manufacturers protocol.

4.4 Cell culture

HaCat cells were grown in Dulbecco's modified eagle's medium (DMEM) (Sigma, Buchs, Switzerland) with 10 % fetal calf serum (FCS) and 1 % glutamine in 75 ml culture flasks (TPP, Trasadingen, Switzerland) at 37 °C in a 5 % CO_2 incubator. Before reaching confluence, or prior to experiments, cells were detached with trypsin, washed with growth medium, spun down for 5 minutes at 1000 rmp in a centrifuge (Hettich, Tuttlingen, Gemany). Double stable HaCat lines expressing N-terminally His-tagged human Gli2 (NHis-Gli2) under the control of the tetracycline

repressor were obtained from Dr. F. Aberger (University of Salzburg, Austria). Blasticidine (8 μg/ml), (Calbiochem, Läufelfingen, Switzerland) and Zeocine (25 μg/ml), (Invitrogen) were added in order to keep stability of transfectants. NHis-Gli2 expression was induced by the addition of 2.5 mg/L tetracycline (Sigma, Buchs, Switzerland) to the medium.

4.5 Affymetrix gene chip mRNA expression screening

Tetracycline-induced and -uninduced NHis-Gli2 cells were compared for genes differentially expressed on the mRNA level, using the Affymetrix gene chips Human **U133A_2.0** (Affymetrix, St. Clara CA, USA) using the Life Sciences Training Facility in the Biozentrum/Pharmazentrum, University of Basel. Equipment and software for RNA quality control (Agilent Bioanalyzer), expression data validation (real-time PCR) and array data management (MIMAS) as well as analysis (Agilent GeneSpring, Ingenuity PathwayAnalysis) were available and instructed at Pharmazentrum 5th floor Room 5021 Floor plan.

4.6 Cell transfections

500 μl of cell suspension (2 x 10^5 cells/mL) were plated in 24 well plates (Greiner) 24 hours prior to transfection. Transfections with either plasmid or siRNA were carried out using Lipofectamine 2000 (Invitrogen) as a transfection reagent according to the manufacturer's protocol. Briefly, siRNA (to a final concentration of 33 nM) or plasmids (final concentration 1200 ng/ml) were preincubated in Lipofectamine 2000 (final concentration 0.5 %) and Optimem® + Glutamax (Gibco, Luzern, Switzerland) for 1h at room temperature (RT), then mixed with growth medium before adding to the cells. Cells were then incubated at 37 °C in a 5 %

CO_2 incubator for 24 hours. Subsequently, the transfection mix was removed, fresh growth medium was added and the cells were further incubated at 37 °C until ready for subsequent experimental steps. SiRNA gene silencing or plasmid expression was screened 48 hours post-transfection.

In case of double plasmid transfection using EGFP expressing plasmid (BDBiosciences, Erembodegem, Belgium) as a transfection efficiency normalizer, the plasmids were mixed at a 3:1 (plasmid to EGFP expression plasmid) ratio (total final concentration 1200 ng/ml) in Optimem® prior to addition of Lipofectamine 2000.

4.7 siRNA gene silencing

SiRNAs used for transfection were targeting GFP (irrelevant siRNA as negative control), Gli2 or cFlip. The RNA target sequences are shown in table 9.1.4. The gene silencing effect of the siRNAs was analysed 48 hours after siRNA transfection by RT-PCR (see below), setting RNA expression of $siRNA_{GFP}$ transfected cells as 100 % expression.

4.7.1 siRNA sequence determination

Three siRNA sequences targeting cFlip were designed following the recommendations of the literature [54]. RNAi activity of each siRNA was assessed by RT-PCR and the most effective siRNA was chosen for RNAi experiments (see table 9.1.4).

4.8 RNA expression measurement

Approximately 6 x 10^5 cells were trypsinized and washed twice with 1 x PBS before total RNA was isolated using an RNeasy mini kit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer's protocol. cDNA was synthesized with Moloney Murine Leukemia Virus reverse transcriptase (M-MLV RT) (Invitrogen) according to the manufacturer's instructions. Quantitative mRNA measurements were carried out by RT-PCR on a Mx4000 cycler (Stratagene, Amsterdam, The Netherlands) using qPCR Rox&Go mastermix (Q-Bio gene, Basel, Switzerland), with GAPDH as a normalizer representing a housekeeping gene. Primer sequences used for RT-PCR are shown in table 9.1.6

4.9 Protein expression measurement

4.9.1 Flow cytometry

Relative protein expression of genes under investigation was carried out by fluorescence activated cell sorting (FACS) on a FACSCalibur flow cytometer (Beckton Dickinson BD Biosciences, Allschwil, Switzerland).

4.9.1.1 Surface staining of extracellular membrane proteins

Briefly, cells were trypsinized and washed twice with 1 x PBS, then incubated for 45 minutes at RT with the first antibody according to the manufactor's recommendations. Cells were then washed twice with 1 x PBS and incubated for 1 hour at RT in the dark with the fluorochrome-conjugated antibody according to the manufactor's recommendations.

Before flow cytometry, cells were washed 3 times with 1 x PBS.

4.9.1.2 Intracellular and nuclear staining

Briefly, cells were trypsinized and transferred into FACS tubes (BD Falcon, Beckton Dickinson), washed once with 1 x PBS, then fixed with 0.2 % PFA for 20 minutes at RT, washed again once with 1 x PBS and then permeabilized with ice-cold 90 % Methanol for 30 minutes on ice. Cells were then washed twice with FACS buffer containing saponine (see Buffers), and incubated with the first antibody according to the manufactor's recommendations for 45 minutes at RT, washed again twice with FACS buffer and incubated with the fluorochrome-conjugated antibody according to the manufactor's recommendations for 1 hour at RT in the dark. Right before flow cytometry, cells were washed twice with FACS buffer and once with 1 x PBS.

Antibodies used are listed in table 9.1.10.

4.10 Apoptosis induction in cultured cells

HaCat cells (NHis-Gli2 cells or HaCat wildtype) were plated 1 x 10^5 cells per well in a 24 well plate, and Gli2 expression was induced with tetracycline on the following day. In case of siRNA treated cells, cells were transfected with siRNA targeting GFP (irrelevant siRNA as a negative control), Gli2 or cFlip after 24 hours of tetracycline treatment. Cells were then induced to apoptosis after 60 hours of tetracycline treatment (48 hours of siRNA treatment) using soluble recombinant Trail (100 ng/ml) (Alexis, Lausen, Switzerland) that had been preincubated for 10 min on ice with a cross-linking enhancer for ligands (2 μg/ml), (Alexis). Apoptosis was evaluated after 36 hours of Trail treatment.

4.11 Apoptosis evaluation

4.11.1 DNA fragmentation

In order to detect the fraction of apoptotic cells, cells were collected and transferrred to FACS tubes along with the supernatant. Cells were centrifuged and resuspended in a hypotonic buffer containing propidiumiodide (PI), and incubated for 30 minutes at RT, then analysed in a flow cytometer (Beckton Dickinson).

4.11.2 Apopercentage© assay

In order to detect apoptotic cells in adherent culture in 24 well plates, cells were washed twice with 1 x PBS and incubated with Apopercentage© dye for 1 hour according to the manufacturer's instructions. Apoptotic cells were stained red and detected under a light microscope (Nikon Eclipse TE200, Nikon, Egg, Switzerland). The cell cultures were photographed with a digital camera and the relative amount of apoptotic events was defined as red pixel number per diameter in the Adobe Photoshop® software.

4.11.3 Caspase 8 activity evaluation

Caspase 8 activity was assessed in apoptosis-induced cells using the Caspase-Glo® Assay (Promega) according to the manufacturer's instructions. Luminescence was measured in a Safire^{2} ^{TM} luminometer (Tecan, Männedorf, Switzerland).

4.12 Nuclear and cytoplasmic protein extraction

HaCat cells were trypsinized and washed twice with 1 x PBS, then incubated in 3 x pellet volume of EMSA buffer on ice, dounced 30 times using an Eppendorf pipette, and vortexed vigorously. The suspension was transferred in a 1.5 ml Eppendorf tube and spun down at 1000 x g in a 4 °C centrifuge. The supernatant, containing the cytoplasmic protein fraction, was transferred into a fresh tube. The pellet was washed with 3x pellet volume of EMSA buffer and spun down again. The resulting pellet was resuspended in 3 x pellet volume of EMSA buffer, and KCl slowly added to a final concentration of 300 mM. The mix was incubated on ice for 30 min, and centrifuged at 13500 x g in a 4 °C centrifuge for 15 min. The supernatant, containing the nuclear fraction was transferred into a fresh tube.
To both protein fractions, glycerol was added to a final concentration of 5 %, the samples snap frozen in liquid nitrogen and stored at -80 °C.

4.13 Western blot

Protein samples were run on an SDS PAGE minigel (Bio-Rad, Reinach, Switzerland), (30 µg for nuclear extracts, 20 µg for cytoplasmic extracts), and the gel blotted onto a nitrocellulose membrane (Whatman, Middlesex, UK) in a semi-dry blotter (Bio-Rad). The membrane was blocked in blocking solution (see buffers) for 30 minutes at RT. The first antibody was applied in blocking solution overnight at 4 °C. The secondary antibody (HRP conjugated) was applied at RT for 1 hour. Washing steps were carried out in PBS containing 0.1 % Tween 20 (Fluka, Buchs, Switzerland). The Immobilon™ Western Chemoluminescent HRP substrate (Millipore, MA, USA) was used for detection on a CL-XPosure™ Film (Pierce, Perbio, Lausanne, Switzerland). Membranes were stripped (see below) and reprobed with anti-βActin antibody as a control for equal loading.

4.13.1 Membrane stripping

Nitrocellulose membranes were stripped in stripping solution (see buffers) for 30 minutes at 50 °C and then air-dried at RT for >3 hours.

4.14 cFlip promoter studies

4.14.1 Reporter constructs

Potential Gli2 binding sites in the putative cFlip promoter region were identified through in silico analysis acording to the Gli2 binding consensus sequence GACCACCCA found in the promoters of the published Gli2 targets Gli1, FoxE1 and Bcl-2. Four sequences lying 5' upstream of the cFlip coding sequence comprising at least two potential Gli2 binding sites were PCR amplified from genomic DNA of HaCat cells and cloned 5' upstream of a luciferase gene into a pGL3 basic vector (Promega). Briefly, the PCR primers for amplification were designed to contain restriction sites for BglII (A'GATCT, New England Biolabs), the PCR products were restriction digested with BglII directly after PCR, and the resulting inserts were gel purified in order to get rid of the cut overhangs. The vector was opened up using BglII restriction enzyme and the purified inserts were integrated using the Quick T4 Ligation Kit (New England Biolabs) according to the manufacturer's instructions. Primer sequences for PCR are shown in table 9.1.6.

As a positive control for functional Gli2 activity, the Bcl-2 promoter fragment was also PCR amplified and the PCR product cloned into pGL3 basic. The Bcl-2 promoter fragment was blunt-end ligated into pGL3 as described above. The vector was used to transform E.coli HB 101 for propagation (see below).

4.14.2 Promoter activity evaluation

HaCat NHis-Gli2 cells were plated 1 x 10^5 cells per well in 24 well plates, and Gli2 expression was induced by tetracycline as described above. For each construct to be transfected, a tetracycline-uninduced, basic Gli2 expressing culture was also maintained. After 24 hours of tetracycline treatment, reporter constructs were cotransfected into induced as well as into uninduced (basic Gli2 expressing) HaCat NHis-Gli2 along with the EGFP expression vector EGFP-N1 (Clontech, California, USA) to screen transfection efficiency as described above. Cells were kept in culture for another 48 hours, and luciferase activity was analysed using the Luciferase Assay System (Promega) according to the manufacturer's protocol. Briefly, cells were washed with 1 x PBS and lysed with a cell lysis solution included in the kit. The lysate was transferred into 96 well plates (Greiner), and EGFP expression was evaluated for each well using the Safire$^{2\ TM}$ luminometer (Tecan). Subsequently, the luciferase substrate was added to each well and luminescence was measured with the Safire$^{2\ TM}$ luminometer. Luminescence was normalized to EGFP expression and overall luciferase activity was calculated with Microsoft Excel software.

4.14.3 Electrophoretic mobility shift assay (EMSA)

4.14.3.1 Probe preparation

Oligonucleotides for gel shift assays (see table 9.1.2), as identified in a luciferase reporter assay, were ordered in single strands at Microsynth, Balgach, Switzerland.

Sense strands were radiolabelled with P^{32} using polynucleotide kinase (New England Biolabs), the radiolabeled strands were annealed to the antisense strands and the double strand probes were gel purified in a 4 % polyacrylamide gel by

excision. The probes were measured for radioactivity in a scintillator (Packard Instrument Company, Geneva, Switzerland), and diluted with d(H_2O) to an activity of 5000 cpm/μl. The probes were stored at -20 °C in a lead container.

4.14.3.2 Gel shift assays

Nuclear extracts (10 μg) from HaCat NHis-Gli2 were mixed with poly(dIdC) (2 μg), BSA (2 μg) and 2 μl 10 x EMSA buffer and the reaction volume adjusted with distilled water to 20 μl. In the case of supershift analysis, 1 μg anti-Gli2-antibody (H-300), (Santa Cruz Biotechnology, Heidelberg, Germany) was added to the mix. For detection of free oligonucleotides, nuclear extracts were replaced by an equal volume of d(H_20). The reaction mix was preincubated at RT for 30 minutes, then 1 μl of radiolabeled probe was added to the reaction, and the mix again incubated for 30 minutes at RT. After incubation, the reactions were chilled on ice and run on a native 4 % polyacrylamide gel for 1 hour at 100 V. Gels were dried in a vacuum drier (Univapo, Uniequip, Munich, Germany) for 2 hours, and a phosphoimager plate exposed on the dried gels overnight. The plates were scanned and analysed with a phosphoimager (Molecular Imager®FX, Bio-Rad).

4.14.3.3 Competition experiments

For competition, increasing excess concentrations of an unlabelled Gli2-binding oligonucleotide were preincubated with the probes and run on the gel using the same conditions as above. Excess concentrations of unlabeled oligonucleotides are 10:1 (unlabeled oligo concentration : labeled oligo concentration), 100:1, 1000:1, and 4000:1 in the case of bs_3 (see results table 1).

4.14.3.4 Pulldown of Gli2 protein with target DNA

5' biotinylated Gli2-binding oligonucleotides were synthesized at Sigma-Genosys, Steinheim, Germany. Sequences of the positive control-, negative control-oligonucleotides and the binding site bs_3 from the putative cFlip promoter are listed in table 9.1.2.

Streptavidine-coated beads (Amersham/GE-Healthcare, Otelfingen, Switzerland) were prepared according to the manufacturer's protocol, and 60 µl of working suspension were transferred to a 1.5 ml Eppendorf reaction tube. 30 pmol biotinylated oligo (in 300 µl of 1 x PBS) were added to the tube, mixed by flipping the tube and incubate at RT for 30 minutes on a stirring wheel. The beads were then spun down at 1200 x g for 1 minute and the supernatant discarded. The pellet was washed twice in 1xPBS, then 1x in EMSA buffer (see buffers and media) for equilibration. The pellet was then resuspended in 500 µl EMSA buffer, and 7.5 µg poly dIdC (competitor) was added to the tube and mixed by careful pipetting. The mix was incubated at RT for 25 minutes before complete protease inhibitor cocktail (Roche) and subsequently 120 µg protein extract were added and carefully mixed by pipetting. The mix was incubated at 4 °C overnight. The beads were thereafter washed 3-5 times with EMSA buffer. After the last wash, the beads were resuspended in 50 µl PBS with 0.5 % SDS, incubated at 65 °C for 12 min and chilled on ice. The eluted beads were then spun down at 2000 x g, and the supernatant containing the pulled-down protein transfered into a fresh tube.

4.15 Basal cell carcinoma (BCC) tissue

BCC specimens from surgical excisions were obtained from Dr. P. Häusermann and Dr. S. Büchner (Dermatology, University Hospital of Basel, Switzerland) immediately after surgery, and transferred into DMEM containing 10 % FCS, 1 % glutamine and 1 mg/ml gentamycine (Gibco) before they were cut into small pieces of approximately 1 mm^3.

4.15.1 Transfection of BCC tissue

The small pieces of BCC tissue were transferred into wells of a 96 well plate (Greiner) comprised with a Lipofectamine 2000 - siRNA mix prepared as described above for siRNA transfection of cells, except for the fact that a concentration of 470 nM siRNA was used for tissue transfection. The BCC tissue pieces were incubated with the transfection mix for 24 hours at 37 °C in a 5 % CO_2 incubator, then transferred into fresh DMEM containing 10 % FCS, 1 % glutamine and 1mg/ml gentamycine and incubated for another 48 hours at 37 °C. SiRNAs used for transfection were targeting EGFP (irrelevant siRNA as negative control), Gli2 or cFlip. The RNA target sequences are shown in table 9.1.4. After 72 hours total incubation time, tissue pieces were embedded in Tissue-Tek® (Sakura, California, USA) and snap-frozen in liquid nitrogen.

4.15.2 Apoptosis induction in BCC tissue pieces

BCC tissue pieces were incubated in DMEM containing 10 % FCS, 1 % glutamine and 20 ng/ml soluble recombinant human Trail along with a cross-linking enhancer (anti Flag antibody) for 36 hours at 37 °C in 5 % CO_2.

4.15.3 Preparation of cryosections

Frozen BCC tissue pieces were cut with a Microm HM 560 Cryo-Star Cryostat (Microm, Volketswil, Switzerland) and transferrred onto glass slides (Sigma-Aldrich, Buchs, Switzerland), air dried for 12 hours at RT and then used for immunohistochemistry or stored at 4 °C.

4.15.4 Immunohistochemistry on BCC cryosections

Air dried cryosections on glass slides were fixed with acetone for 10 minutes at 4 °C. Cryosections were then rehydrated in 1 x PBS for 5 minutes and the first antibody, diluted in 0.5 % BSA-PBS, was applied on the slides. For antibody specificities see table 9.1.10. The slides were incubated in a wet chamber at RT overnight (12 hours).

The slides were then washed 3 times in 1 x PBS for 5 minutes each and incubated with the secondary antibody diluted in 0.5 % BSA-PBS for 3.5 hours at 4 °C in a wet chamber. After the second incubation, slides were again washed 3 times with 1 x PBS, then incubated with the ABC-Peroxidase complex (Dako Cytomation, Baar, Switzerland) for 1 hour in a wet chamber at RT, followed by 3 times washing with 1 x PBS. Subsequently the ready to use AEC+ High Sensitivity Substrate Chromogen (Dako Cytomation) was applied on the cryosections for color reaction. The color reaction was stopped by washing the slides in tap water for 3 minutes, then the cryosections were mounted with 1 drop of Crystal Mount (Biomeda, California, USA).

Analysis of color intensity, directly correlating to protein expression, was carried out with a light microscope (Nikon Eclipse TE200, Nikon) and the Openlab® software (Improvision Ltd, Coventry, UK) runnning on a MacIntosh Computer according to the software instructions. Briefly, cryosections were photographed and tumor tissue (defined by cryosection areas stained with the BerEP4 antibody

which distinguishes BCC tissue from surrounding stroma) was masked and color intensities of masked pixels was measured. The results were transferred in a Microsoft Excel sheet, and the color intensity values of tumor tissues stained with an isotype control antibody was subtracted.

4.15.5 Apoptosis induction in Basal cell carcinoma (BCC) tissue

BCC tissue pieces were prepared and siRNA treated as described elsewhere [55]. SiRNA treated and –untreated tissue pieces were induced to apoptosis after 36 hours of siRNA treatment using soluble recombinant Trail (200 ng/ml culture medium) (Alexis, Lausen, Switzerland) that had been preincubated for 10 min on ice with a cross-linking enhancer for ligands (4 μg/ml) (Alexis), and tissue pieces were kept in Trail-containing culture medium for another 30 hours.

4.15.6 Apoptosis evaluation in Basal cell carcinoma (BCC) tissue

Cryosections of tissue pieces were prepared as described elsewhere [55]. BCC tumor was located on slides using the Ber-EP4 antibody (Dako), and apoptosis was assessed morphologically by microscopy.

4.16 Antibodies

Gli2-Immunohistochemistry (IHC): primary antibody: anti-human Gli2 (N-20 goat polyclonal) (Sta. Cruz, CA, USA), secondary antibody: rabbit anti goat Ig-biotinylated (Dako Cytomation, Zug, Switzerland), followed by ABComplex/Horseradish peroxidase (HRP)-conjugated (Dako), detected by AEC substrate (Dako). Gli2-Western blot: primary antibody: anti-human Gli2 (H-300, rabbit IgG) (Sta. Cruz), secondary antibody: HRP-conjugated goat anti rabbit Ig (Dako). Electric mobility shift assays (EMSA): anti-human Gli2 (H-300, rabbit IgG) (Sta. Cruz). cFlip immunohistochemistry: primary antibody anti-human cFlip antibody (rabbit polyclonal) was produced as described elsewhere [56], secondary antibody: goat anti rabbit Ig-biotinylated (Dako), followed by peroxidase (HRP)-conjugated ABComplex/Horseradish (Dako) and AEC substrate (Dako). cFlip Western blot: primary antibody anti-human cFlip antibody (rabbit polyclonal) was produced as described elsewhere [56], secondary antibody HRP-conjugated goat anti rabbit Ig (Dako). cFlip FACS: primary antibody anti-human cFlip antibody (rabbit polyclonal) was produced as described elsewhere [56], secondary antibody PE-conjugated goat anti rabbit Ig (Dako).

Bcl-2 IHC and Western blot: primary antibody (mouse IgG) (Dako), secondary antibody HRP-conjugated goat anti-mouse Ig (Dako).

Ber-EP4 IHC: primary antibody (mouse IgG) for BCC tissue identification in IHC (Dako), secondary antibody HRP-conjugated goat anti-mouse Ig (Dako).

Beta-Actin negative control for EMSA or loading control for Western blot: primary antibody: anti human β-actin (mouse IgG) (Abcam, Cambridge, UK), secondary antibody HRP-conjugated goat anti mouse Ig (Dako).

Trail-Receptors 1-4, IHC: primary antibody (mouse IgG) (Alexis), secondary antibody goat anti mouse Ig-biotinylated (Dako), followed by peroxidase (HRP)-conjugated ABComplex/Horseradish (Dako) and AEC substrate (Dako), FACS:

primary antibody (mouse IgG) (Alexis), secondary antibody FITC-conjugated goat anti mouse Ig (Dako).

Isotype controls for IHC are mouse IgG1 (Dako) or serum from rabbit before cFlip immunization ([56].

4.17 Buffers and Media

4.17.1 Basic solutions

4.17.1.1 1xPBS, pH 7.2

NaCl	137 mM
KCl	2.7 mM
KH_2PO_4	1.5 mM
Na_2HPO_4	12 mM

4.17.1.2 Paraformaldehyde stock (PFA)

1.5 M paraformaldehyde in 20 mM NaOH

4.17.2 Solutions for FACS

4.17.2.1 FACS buffer

PBS	1 x
saponine	3 M
BSA	5 M
n-goat serum	5 %
sterile filter	0.45 μm

4.17.2.2 Fixing solution for FACS

0.2 % PFA in 1xPBS

4.17.2.3 Hypotonic FACS buffer containing propidium iodide (PI)

PBS	0.5 x
Triton-X-100	0.1 %
PI	50 μg/ml

4.17.3 Solutions for Western blot

4.17.3.1 1xRunning buffer for SDS-PAGE, pH 8.3

Tris base	50 mM
Glycine	19.9 mM
SDS	0.1 %

4.17.3.2 Transfer buffer for semi-dry blotting

Tris-base	48 mM
Glycine	39 mM
Methanol	20 %
SDS (10 %)	0.0375 %

4.17.3.3 Z' solution for blocking, pH 7.4

Tris	100 mM
$MgCl_2$	100 mM
Tween 20	0.5 %
Triton X-100	0.5 %
BSA	0.5 %
FCS	5 %

4.17.3.4 Stripping solution pH 6.8

Tris-HCl	50 mM
SDS	2 %
β-Mercaptoethanol	0.7 %

4.17.4 Solutions for EMSA

4.17.4.1 EMSA buffer

HEPES pH7.9	20 mM
NaCl	75 mM
DTT	1 mM
$MgCl_2$	2 mM

4.17.4.2 Tris-Glycine running buffer

Tris	25 mM
Glycine	230 mM

4.17.5 Media

4.17.5.1 Cell culture growth medium

DMEM, 10 % FCS, 1 % Glutamine

4.17.5.2 Cell culture growth medium for HaCat NHis-Gli2

DMEM, 10 % FCS, 1 % Glutamine, Blasticidine (8 μg/ml), Zeocine (25 μg/ml)

(Gli2 expression induction with 2.5 μg/ml tetracycline)

4.17.5.3 Transfection medium

Optimem® + Glutamax medium (final 12 % of total transfection volume) incubated with Lipofectamine 2000 (final concentration 0.5 %) and siRNA or plasmid vectors were mixed with DMEM.

4.17.5.4 LB-Medium for E.coli

Tryptone	1	%
Yeast extract	0.5	%
NaCl	1	%

add H_2O to final volume, autoclave

chill until the medium has reached <50 °C, then add antibiotic (Kanamycine: 30 μg/ml; Ampicilline: 100 μg/ml).

4.17.6 LB agar plates

15 g Bacto-agar were added to 1 l of LB medium and autoclaved. Liquid agar was cooled down to 50 °C, and antibiotic was added to the appropriate final concentration (Kanamycine: 30 μg/ml; Ampicilline: 100 μg/ml).

4.17.7 Solutions for DNA/RNA applications

4.17.7.1 TE buffer (TRIS-EDTA Buffer, pH 8.0)

Tris-HCl	10 mM
EDTA	1 mM

in ddH2O

sterile filter 0.45 μm and autoclave

4.17.7.2 DNA/RNA precipitation buffer

Sodium acetate pH 5.2	3M	0.1 volumes of DNA/RNA solution
Ethanol	100 %	2.5 volumes of DNA/RNA solution

4.17.8 Solutions for agarose gel electrophoresis

4.17.8.1 5x TBE buffer, pH 8.0

Tris-HCl	5 M
Boric acid	4 M
EDTA	10 mM

4.17.8.2 Gel loading buffer, pH 8.0

Glycerine	30 %
Tris HCl	10 mM
EDTA	10 mM
Bromphenolblue/Xylene cyanol	tip of a spatula

4.17.8.3 Gel staining solution

TBE buffer	1 x
Ethidium bromide	1 mM

5 Results

5.1 Inducibility of Gli2 expression on HaCat NHis-Gli2 by tetracycline

Gli2 expression on tetracycline-treated and untreated HaCat NHis-Gli2 cells or the control cell line HaCat control was assessed by RT-PCR at mRNA level and by Western blot at protein level (Fig. 10). We reached an upregulation of Gli2 mRNA expression by 14 times on average after 72 hours of tetracycline treatment on HaCat NHis-Gli2 whereas its expression was unaffected by tetracycline in the control cell line HaCat control (Fig. 10A). Protein expression induction was assessed in a time-course experiment, showing a substantial rise in Gli2 protein 6 hours post-induction by tetracycline in NHis-Gli2 cells (Fig. 10B). HaCat control cells did not alter Gli2 protein expression upon tetracycline treatment.

In a time-course experiment the kinetics of mRNA expression upon tetracycline treatment showed a first phase of Gli2 upregulation (10 x) after 12 hours, followed by further rises in Gli2 mRNA after 48 and 72 hours reaching a Gli2 elevation of over 16 fold (Fig. 10C).

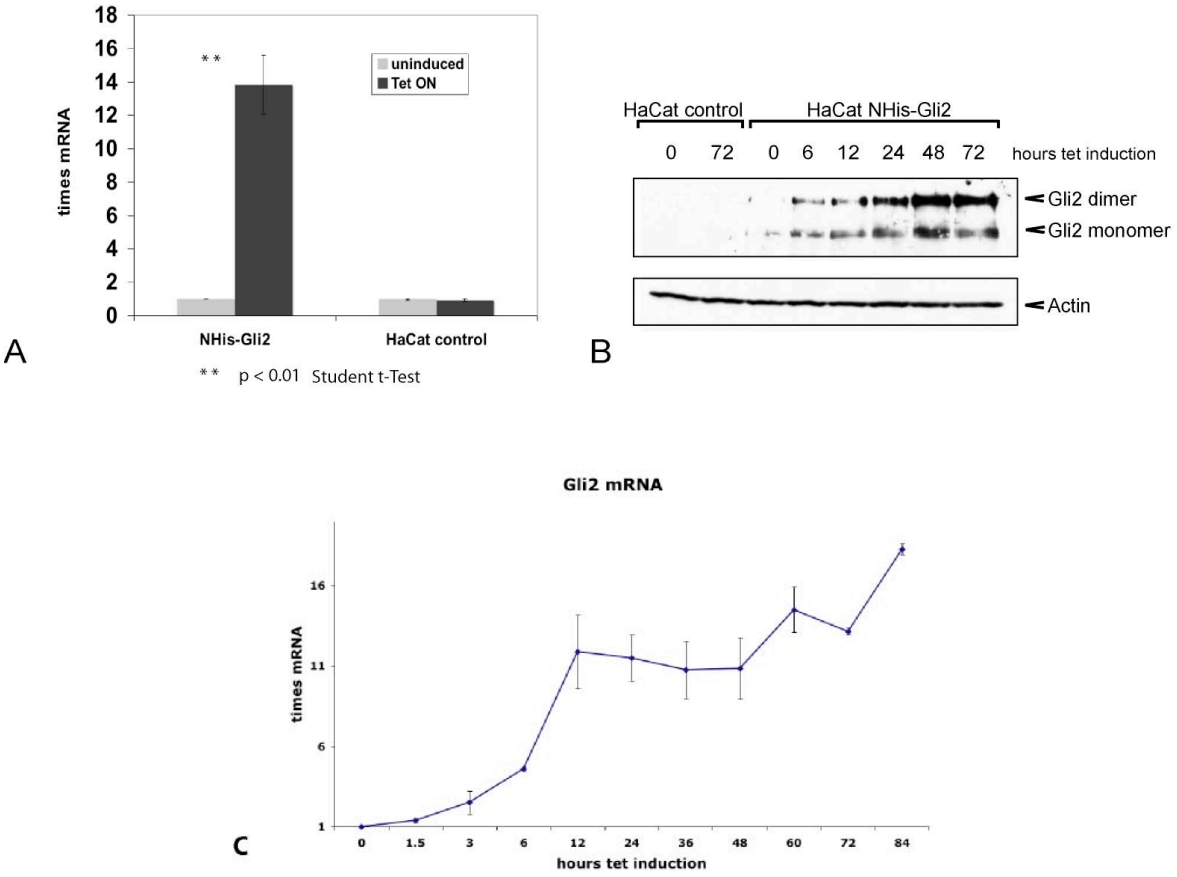


Fig 10

Induction of Gli2 in the transgenic cell line NHis-Gli2 HaCat.

Gli2 mRNA upregulation was assessed after 72 hours of tetracycline induction (A), whereas a timecourse experiment shows Gli2 protein expression significantly upregulated already after 6 hours of tetracycline induction. A single band at 130 kDa is seen in untreated NHis-Gli2 cells only, however, higher expression of Gli2 protein gives rise to a second band at 260 kDa representing dimeric Gli2 (B). A mRNA expression timecourse experiment shows a first rise in Gli2 mRNA after 6 hours of tetracycline treatment and further rise after 48 and 72 hours (C). The expression of Gli2 mRNA was standardized by the corresponding GAPDH mRNA levels, and normalized to that in Tet-Off control cells, which was set to 1. Results represent the mean of three experiments including standard deviations (A,C), or are representative of three different experiments (B).

5.2 Overexpressed Gli2 in HaCat NHis-Gli2 is functional

Bcl-2 is a published direct transcriptional target of the transcription factor Gli2 [38]. Therefore, as a control for functionality of the overexpressed Gli2 protein, we assessed the mRNA and protein expression of Bcl-2 in HaCat NHis-Gli2. A substantial upregulation of Bcl-2 mRNA (65 x) and a clear Bcl-2 protein overexpression could be detected upon 72 hours of tetracycline treatment (Fig. 11)

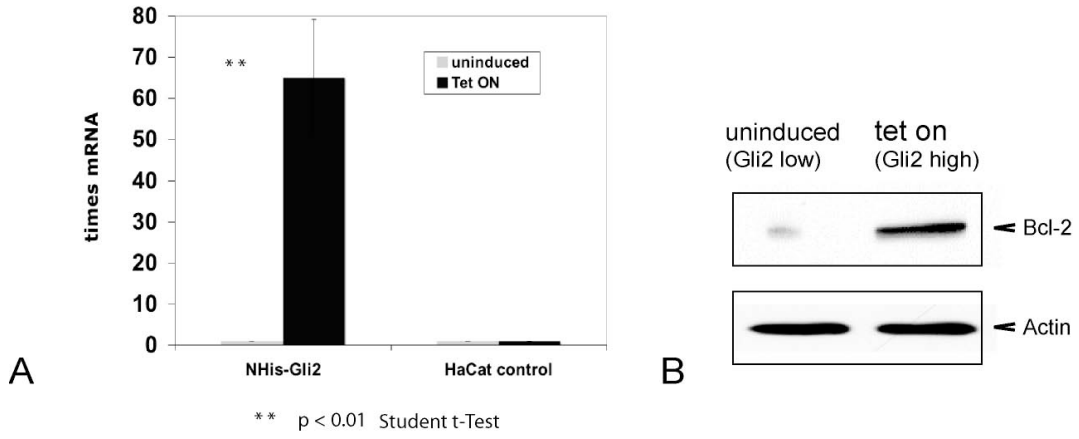


Fig 11

Transcriptional regulation of the Bcl-2 gene by Gli2.

72 hours of tet induction lead to overexpression of Bcl-2 at mRNA (A) and Protein (B) levels in NHis-Gli2 cells. The expression of Bcl-2 mRNA (A) was standardized by the corresponding GAPDH mRNA levels, and normalized to that in Tet-Off control cells, which was set to 1. Results represent the mean of three experiments including standard deviations (A), or are representative of three different experiments (B).

Furthermore, we cloned the promoter region of the Bcl-2 gene, comprising the three published Gli2 binding sites [38] (table 2, Discussion), upstream of a luciferase gene in the luciferase expression vector pGL3 basic. This vector was then transfected into HaCat NHis-Gli2, and Gli2-dependent luciferase expression

was assessed. We detected a 4.8-fold elevation of luminescence upon 72 hours of tetracycline treatment, showing that the transgenic Gli2 was able to bind the Bcl-2 promoter in HaCat NHis-Gli2 and thereby raising the expression of luciferase protein (Fig. 12).

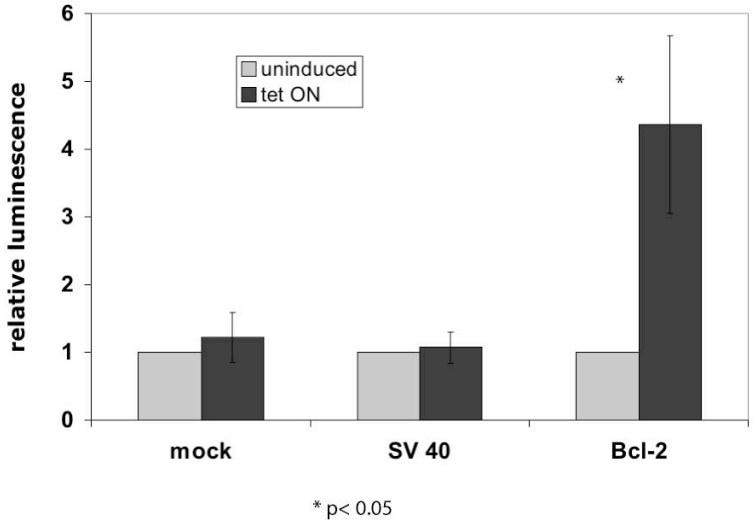


Fig 12

Luciferase reporter of Bcl-2 promoter activity upon Gli2.

The Bcl-2 promoter reacts on elevated Gli2 (tetracycline induction of Gli2 expression), as seen in the right bars. Dark grey bars represent the relative luciferase activity in tet on cells whereas light grey bars represent the default luciferase activity of tet off cells (set to 1). Neither the mock transfected cells (luciferase gene without a promoter) nor the cells transfected with the luciferase gene carrying an SV40 promoter (positive control for luciferase expression) reacted on elevated Gli2 levels (left and middle bars). Results represent the mean of five experiments including standard deviations.

5.3 Affymetrix Gene Chip Analysis

In order to identify apoptosis-related Gli2 target genes, we screened differential expression of mRNA between tetracycline induced and non-induced NHis-Gli2 HaCat cells using the Human U133A_2 gene chip. Table 9.1.1 shows those

apoptosis-related genes which were found to have altered mRNA levels in response to tetracycline treatment. As expected, Gli2 mRNA is upregulated along with its already published direct target genes Bcl-2 and FoxE1. These 2 genes serve as an internal control, their upregulation shows the functionality of Gli2 as a transcriptional activator. Among the other affected apoptosis related genes, the upregulation of Caspase 10 (a Caspase 8 homologue with partially overlapping functions, [57]), cFlip (a Caspase 8 inhibitor) [44,45] , and TOSO (a regulator of Fas-induced apoptosis, [58]) are most prominent. As Fas expression has been shown to be absent on basal cell carcinoma cells and is virtually absent also on our model cell line NHis-Gli2 HaCat (see below), we argued that TOSO is of minor importance in our model. Therefore, we concentrated our further investigations on cFlip, which acts as an inhibitor of not only Fas, but also of Trail receptors 1 and 2 (see below) at Caspase 8 level. As Caspase 8 expression is unaffected by elevated Gli2 levels, the rise of Caspase 8 inhibitors may be of major importance in terms of protection against death-ligand mediated apoptosis in HaCat keratinocytes.

5.4 cFlip expression is elevated upon Gli2 upregulation

Based on above findings on Affymetrix gene chip analysis, we assessed the expression levels of cFlip mRNA and protein in dependence of Gli2 levels in HaCat NHis-Gli2 cells by RT-PCR and FACS.

Only in HaCat NHis-Gli2, but not in the control cell line, cFlip levels rose 4-fold on mRNA levels, and a time-course experiment clearly indicated a rise in cFlip protein as early as 6 hours post-induction by tetracycline (Fig. 13). FACS analysis confirmed the shift in cFlip protein expression upon tetracycline treatment, although the antibody used did not allow the quantification of the cFlip overexpression.

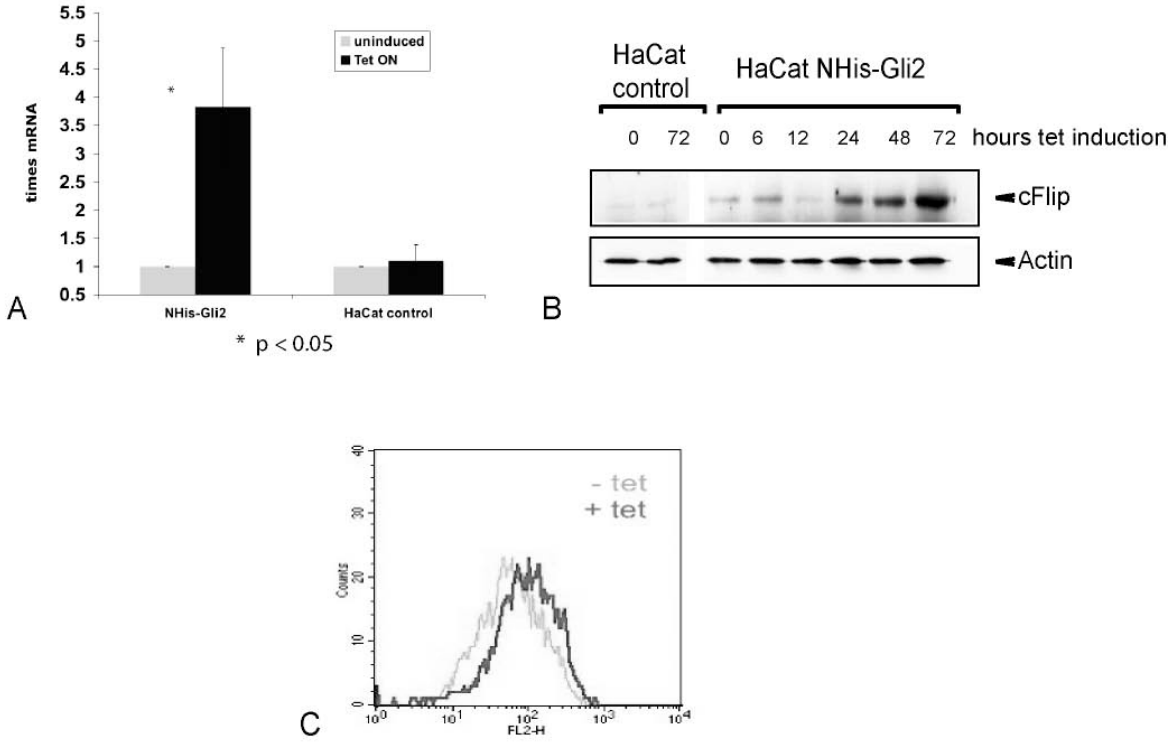


Fig 13

Transcriptional regulation of the cFlip gene by Gli2.

cFlip mRNA upregulation was assessed after 72 hours of tetracycline induction (A). In a timecourse experiment cFlip protein expression was significantly upregulated already after 6 hours of tetracycline induction as seen in a Western blot at 25 kDa, representing Flip short (B). In a FACS experiment, a clear shift in cFlip protein expression is seen after 72 hours of tetracycline treatment (C). The expression of cFlip mRNA was standardized by the corresponding GAPDH mRNA levels, and normalized to that in Tet-Off control cells, which was set to 1. Results represent the mean of three experiments including standard deviations (A) or are representative of three different experiments (B,C).

These findings show that cFlip expression lies downstream of the expression of the transcription factor Gli2. On mRNA level, we screened the expression of both isoforms of cFlip, $Flip_{long}$ and $Flip_{short}$, and we found that the two molecules were evenly upregulated (Fig. 14).

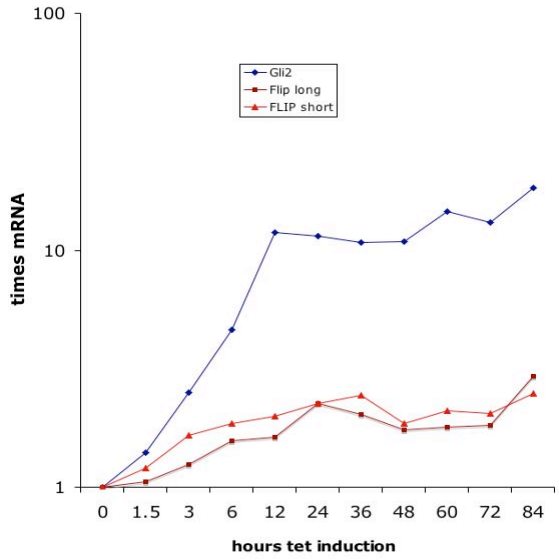


Fig 14

Timecourse of Gli2 and cFlip mRNA upon tetracycline treatment.

Both cFlip isoforms Flip long and Flip short react equally on elevated Gli2 expression after tetracycline-induced Gli2 upregulation.

5.4.1 Kinetics of cFlip upregulation are similar to those of direct targets Gli1 and Bcl-2

Fig. 15 shows the time-course of mRNA upregulation of cFlip in comparison to that of the published direct targets Gli1 and Bcl-2. The reaction of cFlip on elevated Gli2 levels is not as potent as that of the known direct targets, however, the time of onset of upregulation as early as 1.5 hours after tetracycline induction, is equal for all four mRNAs. This finding hints to a potential direct activation of the cFlip promoter by Gli2, as some delay of mRNA rise would be expected if Gli2 was targeting cFlip indirectly via a third molecule, whose protein synthesis would take more time.

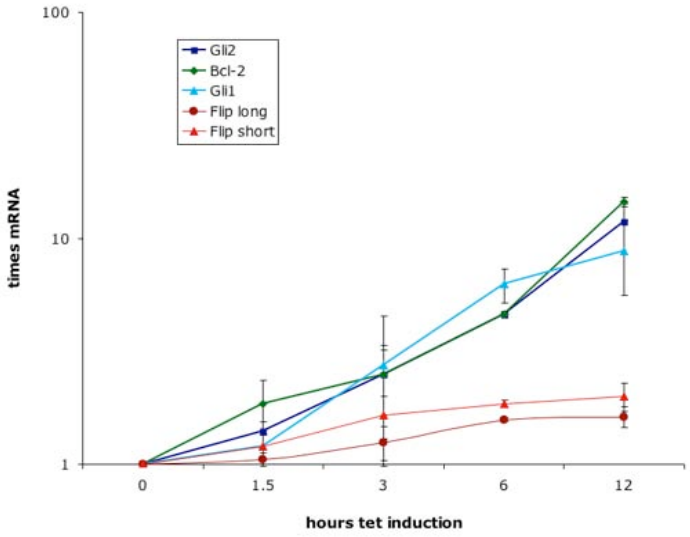


Fig 15

Timecourse of cFlip mRNA in comparison to the direct Gli2 targets Gli1 and Bcl-2 upon tetracycline-induced Gli2 upregulation.

The onset of mRNA upregulation of both cFlip isoforms is taking place as early as that of the direct transcriptional targets Gli1 and Bcl-2. Although the maximal cFlip upregulation (12 h) is not equal to that of Gli1 or Bcl-2, a significant rise in its mRNA expression is already seen after 1.5 hours of tetracycline treatment. Results represent the mean of three experiments including standard deviations.

5.4.2 *In silico* analysis of the 5' flanking region of the cFlip gene

We analysed the upstream region of the cFlip gene to identify potential Gli2 binding motifs in order to narrow the cis-element on which Gli2 may bind as a transactivator. We were able to identify 13 potential Gli2 binding sites in 4 clusters (I-IV) (see table 1 and figure 16). A cluster was defined as a DNA stretch that carries at least two potential Gli2 binding sites with each binding site having not more than two mismatches from the published Gli binding consensus sequence 5' GACCACCCA 3'. The three known direct Gli2 targets Gli1, Bcl-2 and FoxE1 have this consensus sequence. Appendix section 9.2 shows the 5' upstream region of the cFlip gene with the four clusters I-IV marked in yellow.

Table 1 gives a summary of the four defined clusters with the corresponding potential Gli2 binding sites.

Name	Candidate binding site	direction	Deviation from consensus
Cluster I (-941 to + 290)*	tgggaggcc	3' → 5'	2 mismatches
	gaccagcct	5' → 3'	2 mismatches
	tgggaggcc	3' → 5'	2 mismatches
	gatcgccca	5' → 3'	2 mismatches
	tgggtgcgc	3' → 5'	2 mismatches
Cluster II (+2058 to +2864)*	gaccacgca	5' → 3'	1 mismatch
	taccaccca	5' → 3'	1 mismatch
	tgggtggcg	3' → 5'	2 mismatches
	tgggacgtc	3' → 5'	2 mismatches
Cluster III (+8199 to + 8612)*	cacctccca	5' → 3'	2 mismatches
	aggctggtc	3' → 5'	2 mismatches
Cluster IV (+13059 to +13775)*	tgggtggag	3' → 5'	2 mismatches
	gaccaccca	5' → 3'	0 mismatches
Bcl-2 promoter	gaccaccaa	5' → 3'	1 mismatch
	gcacaccca	5' → 3'	2 mismatches
	cgccaccca	5' → 3'	2 mismatches

Table 1

Candidate Gli2 binding motifs (second lane) in the putative cFlip promoter region as defined by in silico analysis. Sixteen candidate binding sites have been found, gathering in four clusters (I-IV). The binding sites in the Bcl-2 promoter are taken as a reference (already published to bind Gli2). The third lane indicates the orientation of the binding motif, with

Gli2 potentially binding in cis (green) or trans (red). Deviations from the published Gli binding consensus sequence is indicated in the fourth lane. (position from mRNA start codon*)**

5.4.3 The putative cFlip promoter region is reacting on elevated Gli2 levels

The four clusters I-IV of the 5' flanking region of the coding sequence of cFlip, each comprising at least 2 potential Gli2 binding sites as identified by *in silico* analysis, were PCR amplified, checked by sequencing and the products ligated upstream of the luciferase gene in the luciferase expression vector pGL3 basic. The vector was transfected into our model cell line. After tetracycline treatment, cells showed a significant rise in luciferase activity only with clusters II and IV as a luciferase promoter, indicating a direct binding of Gli2 to either of the fragments (Fig. 16). Cluster IV contains one 100 % homolog of the Gli binding consensus sequence ($bs_{Bcl\text{-}2}$) and is thus expected to show promoter activity. The relatively low luciferase activation (1.9 x) might be explained by the fact that only a single copy of the binding sequence is present. Cluster II comprises of 4 potential Gli2 binding sites ($bs_{1\text{-}4}$), two of which have 1 mismatch, the other two have two mismatches from the consensus sequence. This cluster shows a more pronounced Gli2-responsive promoter activity (4.4 x).

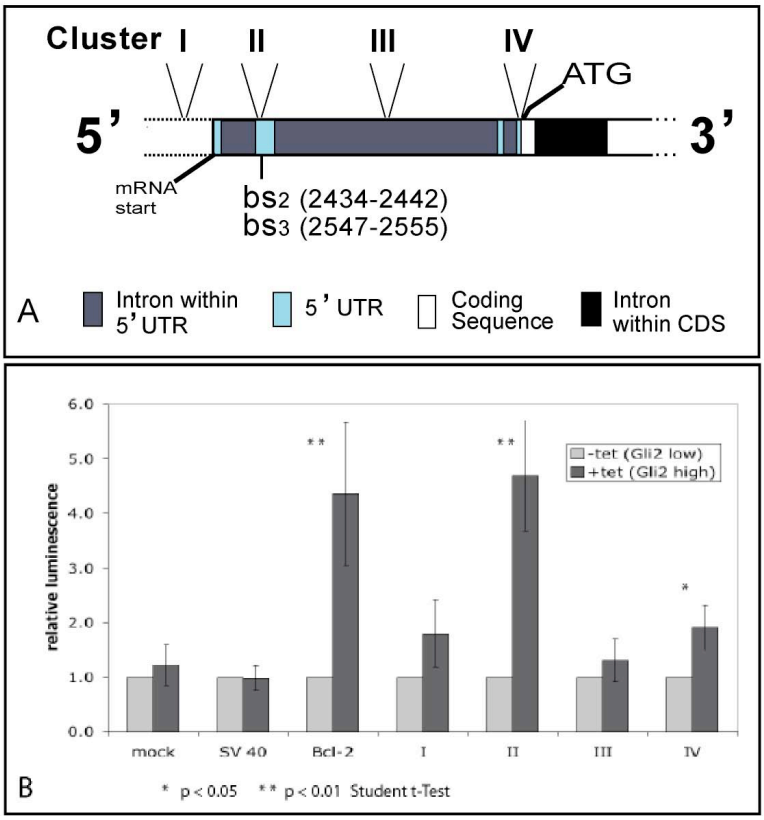


Fig 16

The putative cFlip promoter region is reacting on Gli2 upregulation

In silico analysis of the 5' upstream region of the cFlip gene revealed four clusters of Gli2 binding motif candidates. Two binding sites within cluster II (bs_2 and bs_3) as well as one binding site within cluster IV (bs_{Bcl-2}) are indicated as they could be shown to directly bind Gli2 in gel shift assays (see below) (A). Clusters II and IV show promoter activity when cloned upstream of a luciferase gene in a reporter vector (B). Luciferase activity rises significantly when transfected NHis-Gli2 cells are induced to upregulate Gli2 by tetracycline (dark bars). Luciferase activity of uninduced cells expressing basic Gli2 levels are set as default (light grey bars). Results represent the mean of three experiments including standard deviations.

5.4.4 Electrophoretic mobility shift assay (EMSA)

We continued the investigation for direct Gli2 target sequences within the putative cFlip promoter region by focusing on the sequences found in cluster II. The direct match in cluster IV with the Gli2 binding consensus sequence GACCACCCA

served as a positive control. The negative control, mutated sequence GACCAGGCG was taken in a reverse orientation [59]. All oligos for the assay were designed with the central 9 nucleotide binding sequence flanked by those 17 nucleotides on both sides which are also present in the original genomic DNA stretch. Oligos are listed in table 9.1.2.

As shown in Fig. 18, two out of the four candidate binding site sequences whithin cluster II, bs_2 and bs_3, were shifted when incubated with nuclear protein extracts from HaCat NHis Gli2, showing interaction of the Gli2 protein with the radiolabeled cFlip promoter sequence.

Most interestingly, as seen in Fig 17, and more pronounced in Fig 18, a second bandshift was appearing at a lower size (shifted oligo 2). We assume that the two shifts represent monomeric and dimeric Gli2 bound to the radiolabeled $bs_{Bcl\text{-}2}$ and bs_3, as Gli2 was seen in the Western blot (Fig. 10) in two bands, which both are binding to $bs_{Bcl\text{-}2}$ in a pulldown experiment (see below). Therefore, we refer to the second specific band as shifted oligo 2, which is found right above a presumably unspecific band (Fig. 18). The latter band is regarded as unspecific because it is also appearing in the control EMSA where the negative control oligo bs_{mut} was applied (Fig. 17).

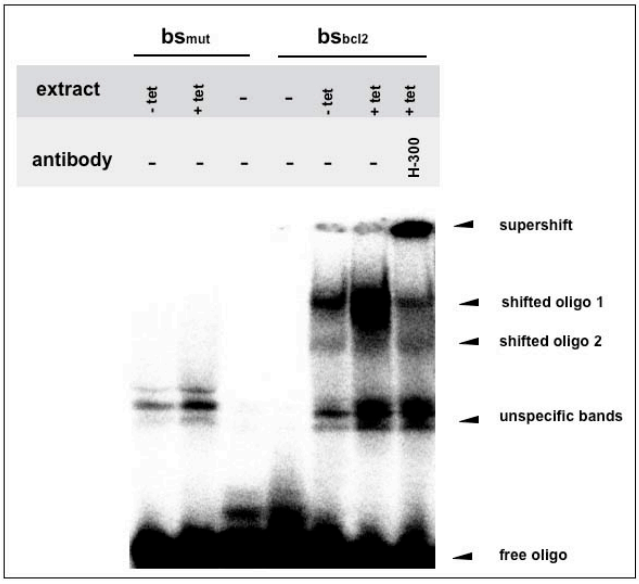


Fig 17

Control EMSA checking functionality of the experimental setup.

A radiolabeled negative control oligonucleotide (bs_{mut}) on the one hand, and the radiolabeled positive control Gli2 binding oligonucleotide (bs_{Bcl-2}) on the other hand were run on PAGE along with, or without nuclear extracts from NHis-Gli2 HaCat cells that were either tetracycline- induced (+tet) or uninduced (-tet). While bs_{mut} did not shift at all, bs_{Bcl-2} was clearly shifted along with nuclear extracts (shifted oligo1), and the shifted signal was much stronger when nuclear extracts were taken from tetracycline induced cells, indicating a higher Gli2 expression in the latter. A second band (shifted oligo 2) seen below, is also believed to be specifically shifted by Gli2, although its signal is much weaker than the first one. The two shifted oligos might represent shifts by dimeric and monomeric Gli2 respectively, according to transgenic Gli2 seen in Western blots. When nuclear extracts were preincubated with an anti-Gli2 antibody (H-300), a supershifted signal was clearly appearing, confirming that Gli2 bound the oligonucleotide bs_{Bcl-2}.

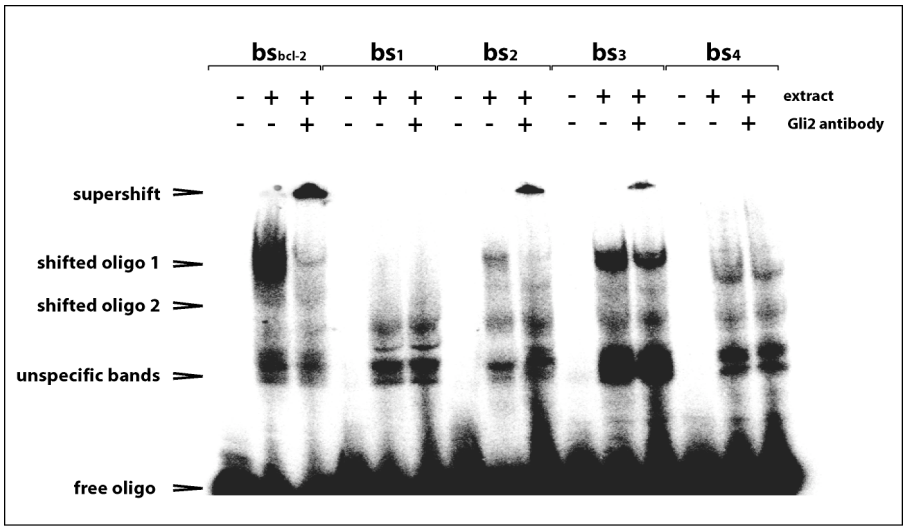


Fig 18

Overview over the Gli2 binding capacities of the four binding site candidates from cluster II.

The same setup as in Fig 17 is taken for all four binding site candidates (bs_1-bs_4). Only bs_2 and bs_3 are shifted along with nuclear extracts, and both are specifically binding Gli2 as can be seen in the supershifted signal with the Gli2 antibody. Bs_4 shows a shifted signal which is unrelated to Gli2, as its size does not match the one of the positive control, and no supershift with the Gli2 antibody is observed.

Candidate sequences bs_1 and bs_4 did not shift along with nuclear extracts. A supershift of the signals can be observed in probe sequences bs_{Bcl-2}, bs_2 and bs_3, when the nuclear protein extracts were preincubated with a Gli2 antibody, confirming specific interactions of the probes with Gli2. As a negative control, an anti-βActin antibody was used, showing no supershift in both probes bs_{Bcl-2} and bs_3 (Fig. 19).

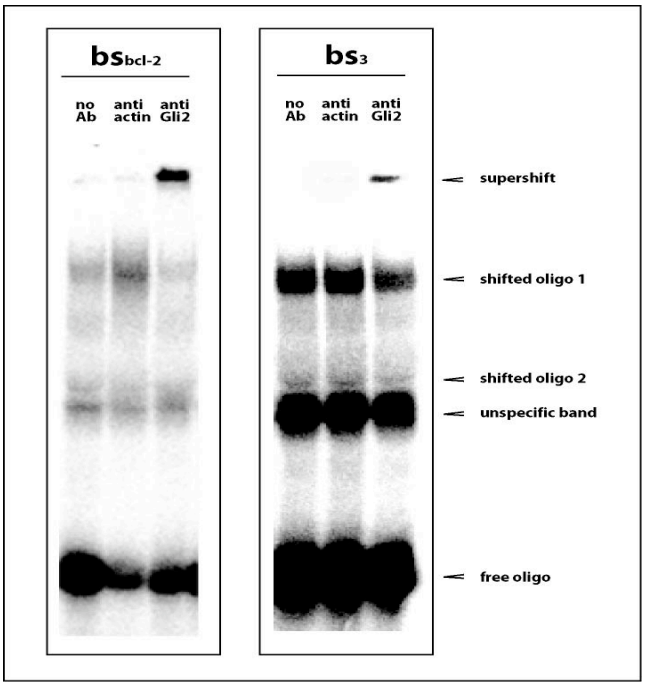


Fig 19

Control of supershift specificity in bs_{Bcl-2} and bs_3 when supershifted with anti-Gli2 antibody.

An anti-βactin santibody was incubated with nuclear extracts as a negative control, and did not supershift the oligonucleotide, confirming specificity of Gli2 binding in Figures 17 and 18.

In order to further confirm specificity of binding site interaction with Gli2, we performed competition assays of bs_2 and bs_3 with unlabeled bs_{Bcl-2}. When premixed with an excess of unlabeled specific bs_{Bcl-2} oligo as a competitor (100:1 in bs_2 and 400:1 in bs_3), we observed a fading of the shifted signal, confirming that bs_2 and bs_3 are specifically binding to Gli2 (Fig. 20)

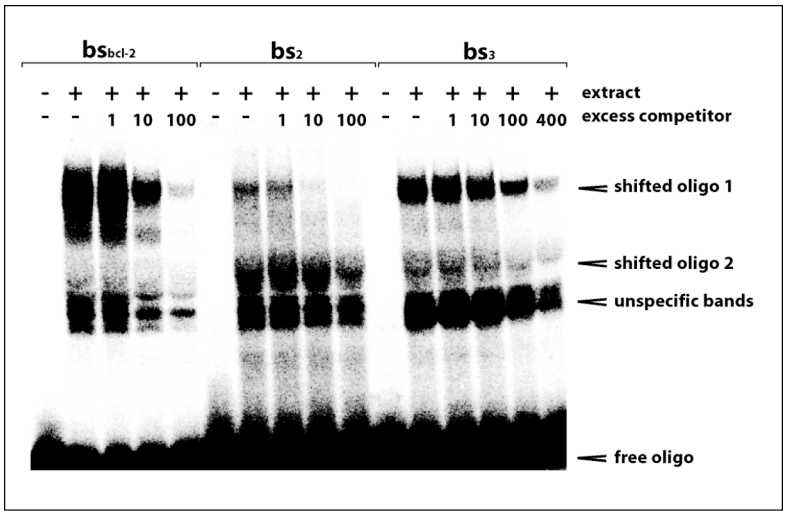


Fig 20

Competition assays to confirm Gli2 binding to bs_2 and bs_3.

Signals of both shifted oligonucleotides (shifted oligo 1 and shifted oligo 2) fade when radiolabeled bs_2 and bs_3 oligonucleotides were preincubated with unlabeled bs_{Bcl-2} as a competitor for Gli2 binding. Bs_3 however seems to have a higher Gli2 affinity as it needed a higher competitor excess until it faded.

As the bs_3 oligo shift was not easily competed with unlabeled oligos, we used a higher concentration of specific competitor (1000:1), and could observe a clear fading of the shifted radiolabeled oligos. There was no competition when the negative control mutant oligo bs_{mut} was used at the same concentration for competition, indicating that bs_3 is specifically shifted by binding of Gli2 protein (see Fig. 21).

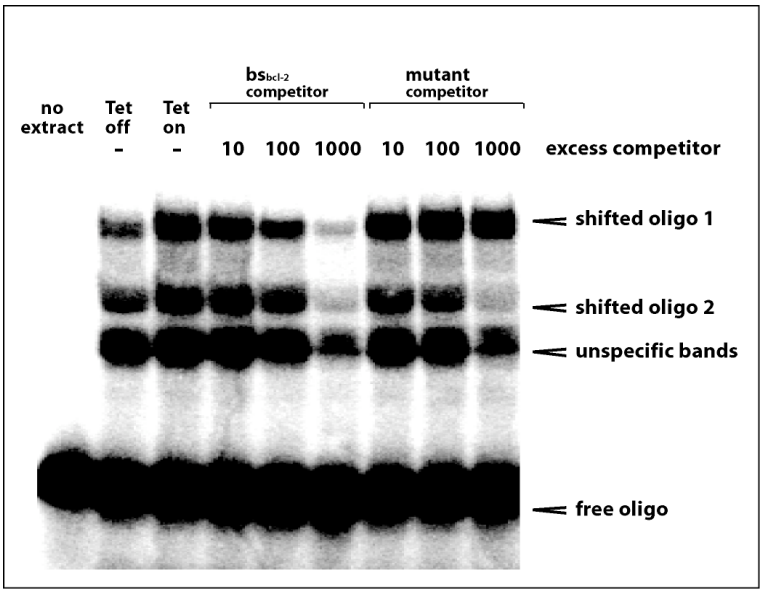


Fig 21

bs_3 specificity analysis.

As bs_3 was clearly outcompeted with an excess of 1000 times unlabeled bs_{Bcl-2}, it was checked whether the competition effect was unspecific. bs_{mut} does not bind Gli2 and thus served as a negative control for competition. Indeed, bs_3 could be competed by 1000 times excess of unlabeled bs_3 but not by the same amount of unlabeled bs_{mut} (no fading of the signal of shifted oligo 1). However, the fading of the shifted oligo 2 seems to be an unspecific effect as it is also seen in the case of excess unlabeled bs_{mut} (last lane).

In addition, a much stronger signal in the shift of both cFlip Gli2 binding sites bs_2 and bs_3, as well as of the control binding consensus sequence from the Bcl-2 promoter, bs_{Bcl-2}, could be observed, when we compared nuclear protein extracts of tetracycline-induced cells with nuclear extracts from tetracycline-uninduced cells. This indicates that recombinant Gli2 is functional in terms of binding capacity, and substantially upregulated in tetracycline-treated cells (Fig. 22).

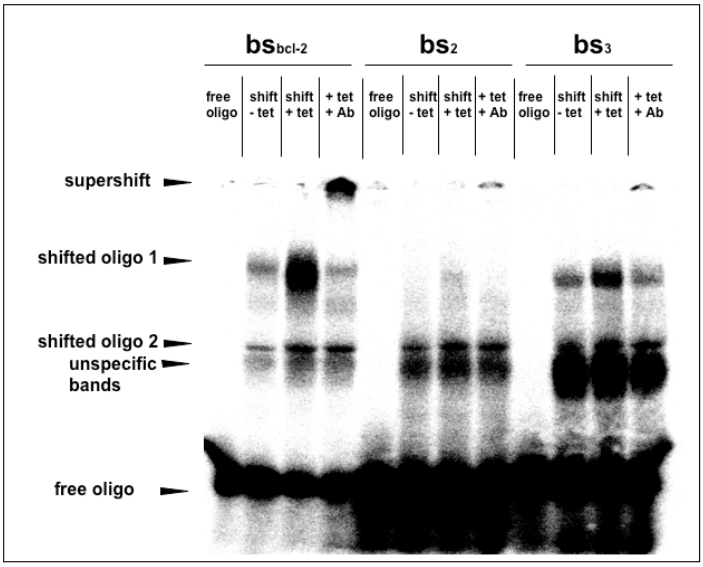


Fig 22

Overview over the Gli2 binding activities of bs_2 and bs_3.

5.4.5 Gli2 monomer and dimer from NHis-Gli2 cells can be pulled down with the Gli2 binding motif from the Bcl-2 and from the cFlip promoter

Using biotinylated Gli2-binding motifs from the Bcl-2 or the cFlip promoter bound to streptavidine-coated beads, we were able to specifically pull down monomeric and dimeric Gli2 from nuclear extracts of NHis-Gli2 cells. Thus, the shifted bands seen in the EMSAs indeed correspond to Gli2 monomer and dimer: The biotinylated Gli2 binding consensus oligo from Bcl-2 bs_{Bcl-2} (GACCACCCA) and bs_3 from cFlip (TGGGTGGCG) were both pulling down two proteins equal in size with the two proteins corresponding to monomeric and dimeric Gli2 as shown in Fig. 23, whereas the negative control oligo or the beads alone did not.

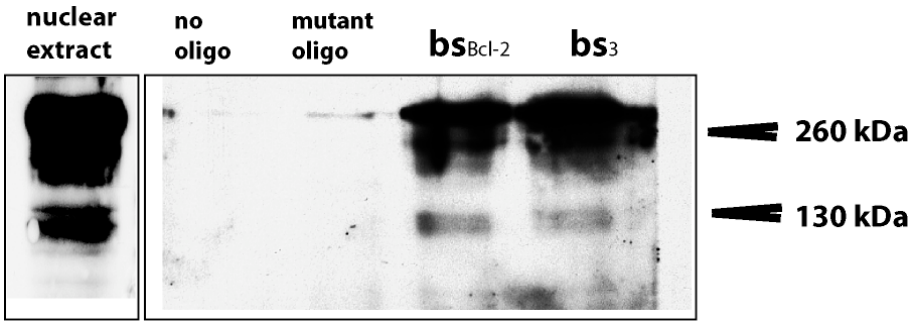


Fig 23

Pulldown of monomeric and dimeric Gli2 using biotinylated binding sites bs_{Bcl-2} and bs_3 bound to streptavidine-coated beads.

While streptavidine beads alone (second lane) and beads bound to bs_{mut} (third lane) did not pull down any protein that could be detected with an anti- Gli2 antibody, beads bound to bs_{Bcl-2} (positive control) (fourth lane) or to bs_3 (fifth lane) pulled down two proteins equal in size to monomeric and dimeric Gli2 (first lane) as detected by an anti-Gli2 antibody.

5.5 Death receptor expression on HaCat NHis-Gli2

The expressions of Fas (CD95) and Trail receptors (Death receptors TR1 or DR4, TR2 or DR5, and Decoy receptors TR3 or DcR1 and TR4 or DcR2) were assessed by RT-PCR on mRNA level (data not shown) or by FACS on protein level.

According to FACS data, Fas protein expression was absent on HaCat NHis-Gli2 (data not shown), therefore we assessed its mRNA expression level by RT-PCR. The high C_T value (C_T 37) of the positive signal implies that Fas expression is virtually absent in these cells (data not shown).

Trail decoy receptors 3 and 4 could not be detected on protein level, only death receptors 1 and 2 (TR1 and TR2) were found to be expressed on both, the HaCat NHis-Gli2 and HaCat control (Fig. 24).

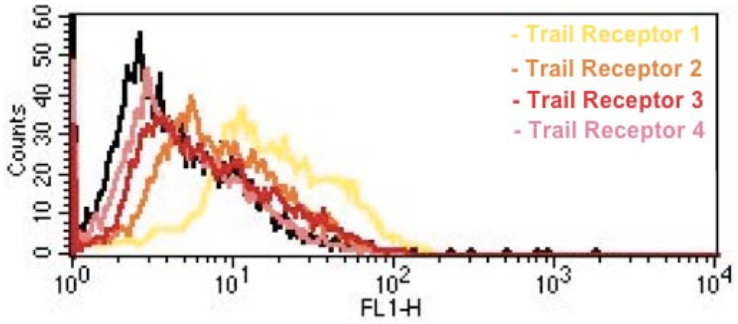


Fig 24

FACS analysis of Trail receptor (TR) expression on HaCat NHis-Gli2.

Only TR expression on NHis-Gli2 cells is shown, representative also for HaCat control as TR expression patterns match perfectly between the two cell lines. TR1 and TR2 are both expressed while TR3 and TR4 are not exceeding background signals of the antibody isotype control (black line).

TR1 and TR2 are the mediators of Trail-induced apoptosis. As on the one hand, these two receptors are sufficiently expressed and on the other hand, the anti-apoptotic decoy receptors TR3 and TR4 are absent, we assume that the model cell line HaCat NHis Gli2 is susceptible to apoptosis induction by Trail. Therefore we focused further steps of our investigation on the apoptosis pathway via TR1 and TR2, triggered by Trail.

5.5.1 Functionality of TR1 and TR2

We were able to induce apoptosis in HaCat NHis-Gli2 cells using soluble recombinant human Trail (100 ng/ml) along with an enhancer. The recombinant Trail protein is flag-tagged, and its function depends on trimerization via an anti-flag antibody. Nearly 50 % of the cells showed substantial DNA fragmentation after 24 hours of treatment with soluble Trail as shown in Fig. 25.

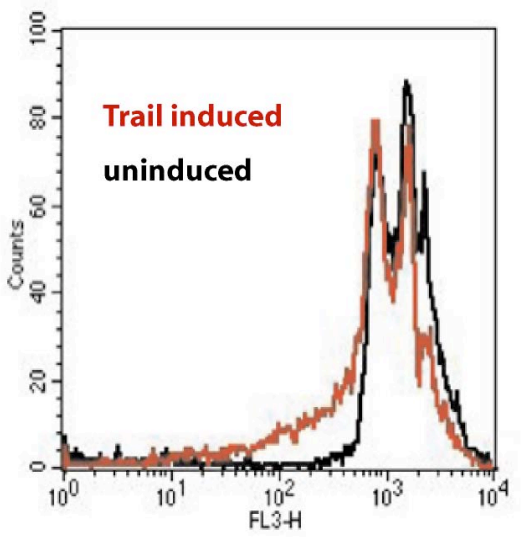


Fig 25

Apoptosis induction in NHis-Gli2 HaCat by soluble recombinant Trail measured by FACS.

Propidium iodide (PI) staining of the cells shows a substantial increase of the subdiploid cell fraction (red line) after Trail treatment, indicating significant numbers of apoptosis events. Subdiploid cells are shown right to the left peak (representing diploid cells). The right peak represents supradiploid cells (dividing cells).

Trail-induced Caspase 8 activation was assessed by Western blot (see below), showing that apoptosis is indeed induced via Trail. Therefore, we assume that TR1 and TR2 expressed on NHis-Gli2 and HaCat control, are functional.

5.6 Gli2 overexpression protects HaCat NHis-Gli2 cells from Trail-induced apoptosis

We induced apoptosis in HaCat NHis-Gli2 using soluble human Trail. About 30 % of cells died after 36 hours of Trail treatment, a percentage which is highly reduced when the cells are induced to upregulate Gli2 by tetracycline. There was no reduction in apoptosis, however, when the cell line HaCat control was treated with

tetracycline. Thus, Gli2 evoked a protective effect in these cells, acting against Trail-mediated apoptosis (Fig. 26).

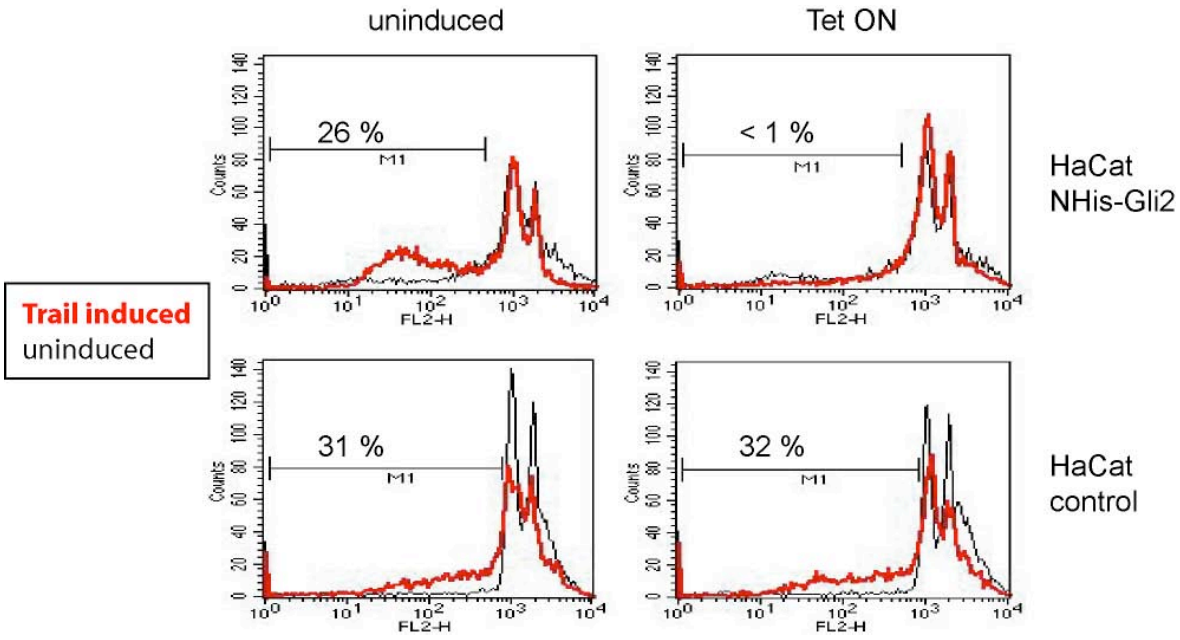


Fig 26

High expression of Gli2 and cFlip protects NHis-Gli2 cells from Trail-mediated apoptosis.

Apoptotic cell fraction was measured as subdiploid DNA in FACS. NHis-Gli2 cells were protected from Trail-induced apoptosis when Gli2 was overexpressed by tetracycline induction (upper panels). Tetracycline did not affect the apoptotic potential of HaCat control cells (lower panels). Red lines represent Trail induced cells and black lines untreated cells. Numbers indicate percent subdiploid cells.

We further analysed Trail-induced apoptosis in tetracycline induced and -uninduced NHis-Gli2 HaCat cells using the Apopercentage™ assay kit. A red dye is specifically binding to apoptotic cells as they flip membrane components inside out, which can be perceived as a reliable marker for apoptotic events. The dye is taken up by apoptotic cells in the course of membrane flipping, whereas healthy cells do not take up the dye at all and necrotic cells do not retain the dye during a washing step. By light microscopy, apoptotic cells are easily detected and can be quantified on a digital image with Photoshop® by red pixel quantification. Fig. 27

shows apoptotic cells (red) in uninduced (low Gli2) or tet-ON (high Gli2) NHIS-Gli2 HaCat cells, treated with or without Trail confirming the results obtained by FACS analysis (Fig. 26).

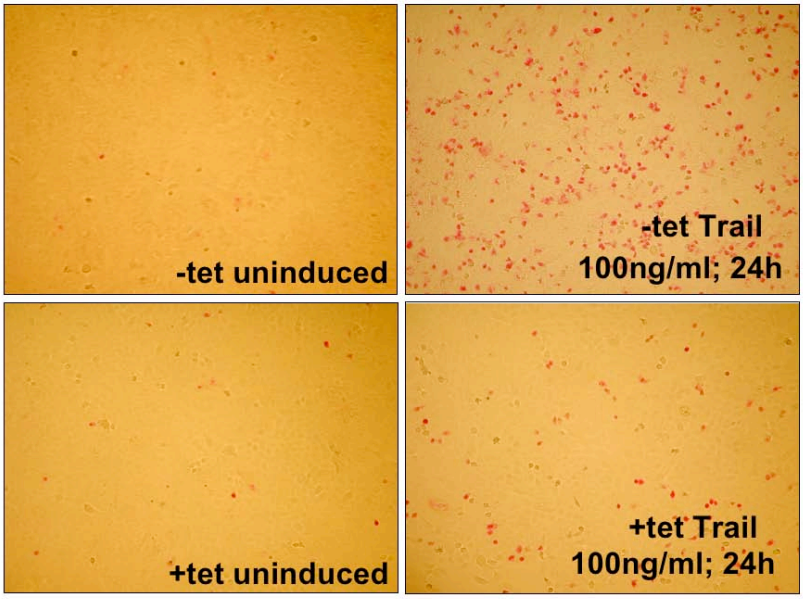


Fig 27

High expression of Gli2 protects NHis-Gli2 cells from Trail-mediated apoptosis.

Apoptotic cells were measured as membrane-flipped cells in culture plates. NHis-Gli2 cells were protected from Trail-induced apoptosis when Gli2 was overexpressed by tetracycline induction (lower panels). Red spots represent membrane-flipped and thus apoptotic cells.

5.6.1 Importance of cFlip in Gli2-mediated protection against apoptosis

As the anti-apoptotic cFlip is substantially upregulated upon elevated Gli2 in our model cell line, we attempted to assess its importance in the protection against Trail-mediated apoptosis. To study this, HaCat NHis-Gli2 were treated with tetracycline to upregulate Gli2, while at the same time cFlip expression was downregulated by RNA interference.

SiRNA against cFlip were designed (see table 9.1.4) which downregulated cFlip mRNA by about 50 % (Fig. 29) as well as cFlip protein in the tet-off situation (Fig 28). When cells were treated with tetracycline, with a 14 times overexpression of Gli2, cFlip was downregulated by only 30 % on the mRNA level (Fig. 29), but still markedly on the protein level (Fig. 28).

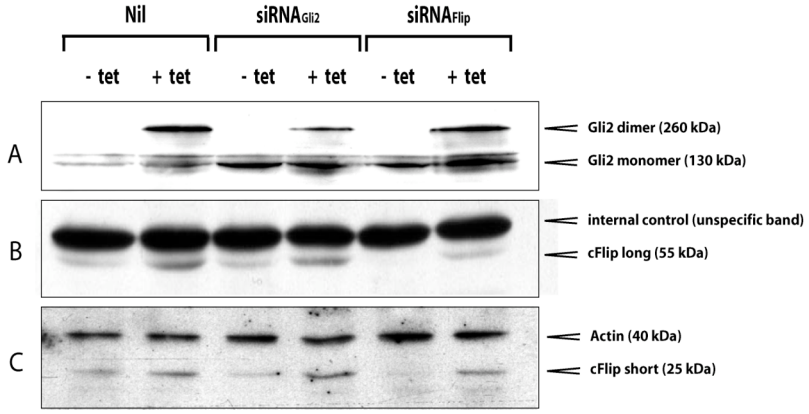


Fig 28

Gli2 (A) and cFlip (B and C) protein expression in NHis-Gli2 cells upon siRNA treatment.

Gli2 was specifically downregulated with $siRNA_{Gli2}$ (A) and a decrease in cFlip proteins of both isoforms in cytoplasmic extracts of the same cells could observed (B and C). A marked reduction of cFlip protein of both isoforms could be achieved by treatment with $siRNA_{Flip}$ (B and C). The specificity of RNAi is shown by the fact that Gli2 expression was unaffected by $siRNA_{Flip}$ (A).

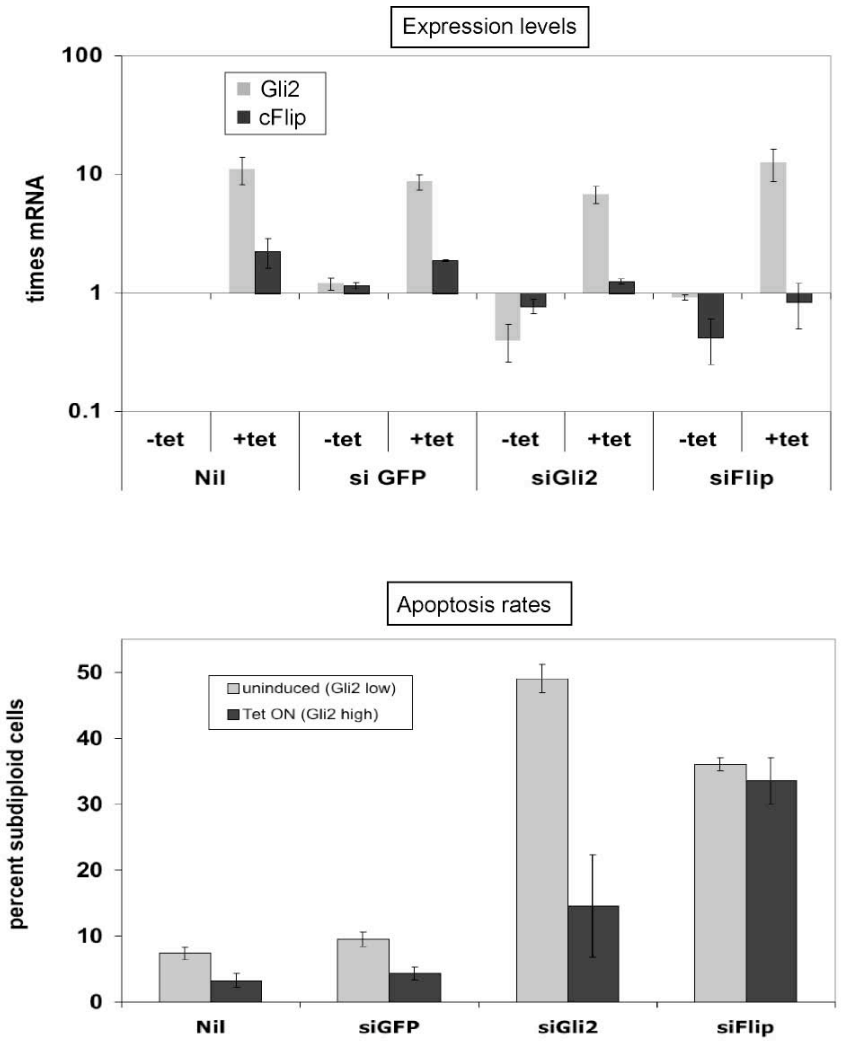


Fig 29

Apoptosis levels in NHis-Gli2 cells are dependent on Gli2 and cFlip expression. mRNA levels of Gli2 (upper panel blue bars) are shown next to corresponding cFlip levels (purple bars) for each siRNA treatment. Apoptosis was measured by subdiploid DNA, and is inversely correlated to cFlip expression levels, with lowest apoptosis rates in tetracycline-induced, siRNA untreated cells and highest apoptosis rates in tetracycline-uninduced, $siRNA_{Gli2}$ -treated cells (lower panel red bars). Results represent the mean of three experiments including standard deviations.

Despite high Gli2 levels in the cells, there was a substantial increase in apoptosis after Trail induction when cFlip was kept low by siRNA treatment (Fig 29), as

shown by the higher proportion of fragmented DNA. We therefore assume an important role for cFlip in the protection against Trail induced apoptosis mediated by Gli2. This was confirmed in another apoptosis assay where apoptotic cells were directly stained in culture by the Apopercentage® assay as shown in Fig. 30. Red pixel values indicated on the figure represent the proportion of apoptotic cells which was substantially higher in the case where cFlip expression was kept low by siRNA, despite Gli2 expression was high. As control, siRNA against GFP was applied yielding similar results as with untreated cells as shown above.

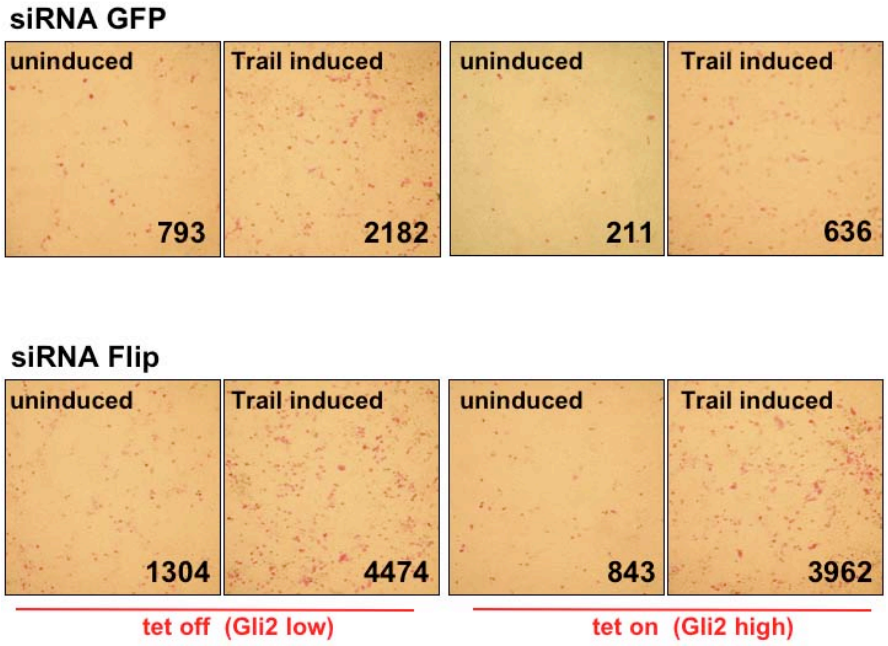


Fig 30

High expression of Gli2 and cFlip protects NHis-Gli2 cells from Trail-mediated apoptosis. Apoptotic cells are measured as membrane-flipped cells in culture plates. NHis-Gli2 cells are protected from Trail-induced apoptosis when Gli2 is overexpressed by tetracycline induction, (right boxes upper panels) similar to cells transfected with the irrelevant siRNA GFP (upper panels). cFlip gene-silencing with specific siRNA rescued the apoptotic potential of NHis-Gli2 cells even though they were tetracycline-induced and expressed high Gli2 levels. The result shown is representative of three different experiments, data with HaCat control cells or HaCat NHis-Gli2 cells without siRNA transfection are not shown.

5.6.2 Apoptosis prevention in Gli2 overexpressing cells is acting at the level of Caspase 8

Interaction of Trail with its receptors cleaved Procaspase 8 to active Caspase 8 (p18 subunit) in tetracycline-uninduced NHis-Gli2 HaCat cells (Fig. 31). Upregulation of Gli2 by tetracycline prevented Caspase 8 activation, indicating that Gli2 overexpression protects NHis-Gli2 cells from death-ligand induced apoptosis at the level of Caspase 8 activation via the upregulation of the Caspase 8 inhibitor cFlip.

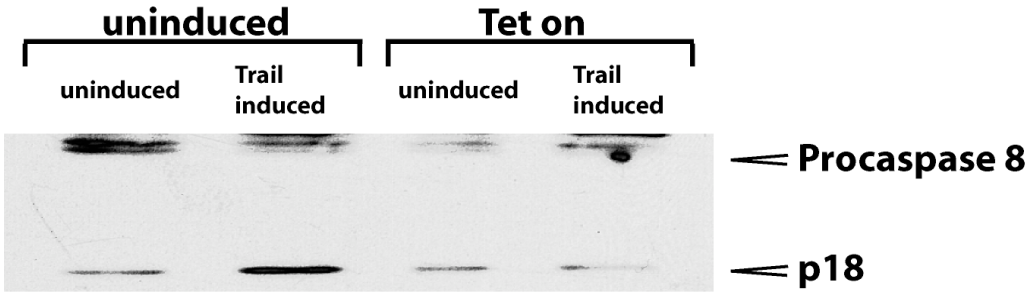


Fig 31

Gli2 mediated protection against Trail-induced apoptosis is taking place at the level of Caspase 8 activation.

The active subunit p18 of Caspase 8 is generated when NHis-Gli2 cells are treated with soluble recombinant Trail, as seen in a Western blot using anti-Caspase 8 antibody (second lane). When cells are expressing high Gli2 levels (tet on), Caspase 8 activation is significantly reduced as is seen in the right two lanes.

The involvement of cFlip was confirmed with the Caspase-Glo® 8 Assay that links Caspase 8 activity to luciferase activity. This assay indirectly reports Caspase 8 activity through luminescence levels. When cFlip levels were downregulated with cFlip-specific siRNA, luminescence was clearly increased upon Trail treatment even in the tet on situation where Gli2 was high (Fig. 32). Similar results could be obtained when Gli2 was targeted by siRNA.

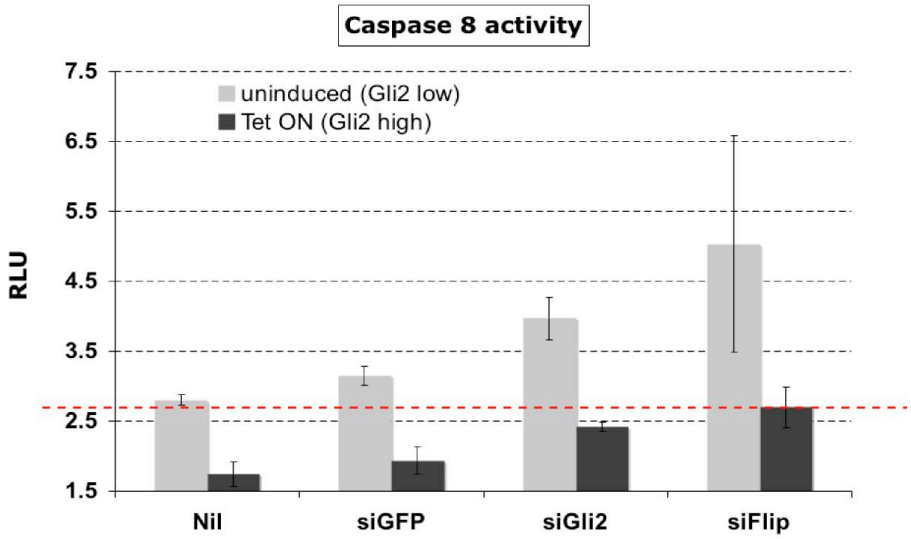


Fig 32

Caspase 8 activation in NHis-Gli2 cells is dependent on cFlip expression.

Apoptosis was induced by Trail treatment and measured by Caspase 8 activity and is inversely correlated to cFlip levels (seen in Fig 29). Highest Caspase 8 activity was measured in tet off cells (low Gli2) where cFlip was downregulated by specific siRNA$_{Flip}$ (right grey bar). Lowest Caspase 8 activation was seen in siRNA untreated tet on cells (high Gli2, high cFlip, left black bar). Caspase 8 activation of siRNA untreated tet off cells was almost entirely rescued in tet on cells that were siRNA$_{Flip}$ treated (high Gli2 but low cFlip, left grey bar compared to right black bar, marked by the dotted red line). Results represent the mean of three experiments including standard deviations.

Altogether, all these results show that cFlip is central in apoptosis prevention by Gli2 overexpression.

5.7 Gli2 and cFlip in Basal cell carcinoma (BCC) specimens

In order to assess the potential influence of Gli2 on cFlip expression in BCC, we analysed biopsies of BCC patients. Cryosections of BCC tissue were first stained for the presence of an epidermal antigen specific for BCC tissue. Furthermore, the sections were stained for Gli2 expression in order to exclude low Gli2 expressing

tumors and restricting further investigations to high Gli2 expressing (derived from overactivated Hedgehog signaling) tumors. Four BCCs that satisfied this prerequisite were taken for further investigations. In order to modulate Gli2 expression, small BCC pieces were treated with siRNA against Gli2, and the impact of Gli2 downregulation on cFlip expression was assessed. As a positive control for Gli2-dependent protein expression, we also analysed Bcl-2 expression on untreated and on $siRNA_{Gli2}$ treated tumor tissues, as Bcl-2 has been shown to be directly regulated by Gli2. The following situations were analysed:

- Untreated tumor tissue
- $siRNA_{GFP}$ treated tissue (unrelated siRNA as negative control)
- $siRNA_{Gli2}$ treated tissue

All tumor samples were stained for the expression of

- BerEP4 (epithelial antigen identifying BCC tumor tissue)
- Gli2
- cFlip
- Bcl-2 (positive control for Gli2-dependent protein expression)

As shown in Fig. 33, we succeeded to efficiently downregulate Gli2 in all four tumor specimens tested (30 % residual Gli2 expression). In response to the Gli2 downregulation, we also found Bcl-2 expression being significantly lowered in all samples (Bcl-2 expression downregulated to 50 %), which serves as a positive control. As predicted, cFlip expression was also significantly downregulated when Gli2 was lowered (downregulated to 40 % residual expression relative to untreated tissue). From these results we can conclude that cFlip is also *in vivo* in BCC a downstream target of Gli2, when Gli2 is highly expressed and derived from overactivated Hedgehog signaling.

In addition, we targeted cFlip expression in the BCC samples with siRNA against cFlip. In these tissue samples, we succeeded to lower cFlip expression by 57 % compared to untreated tissue (43 % residual expression), (data not shown).

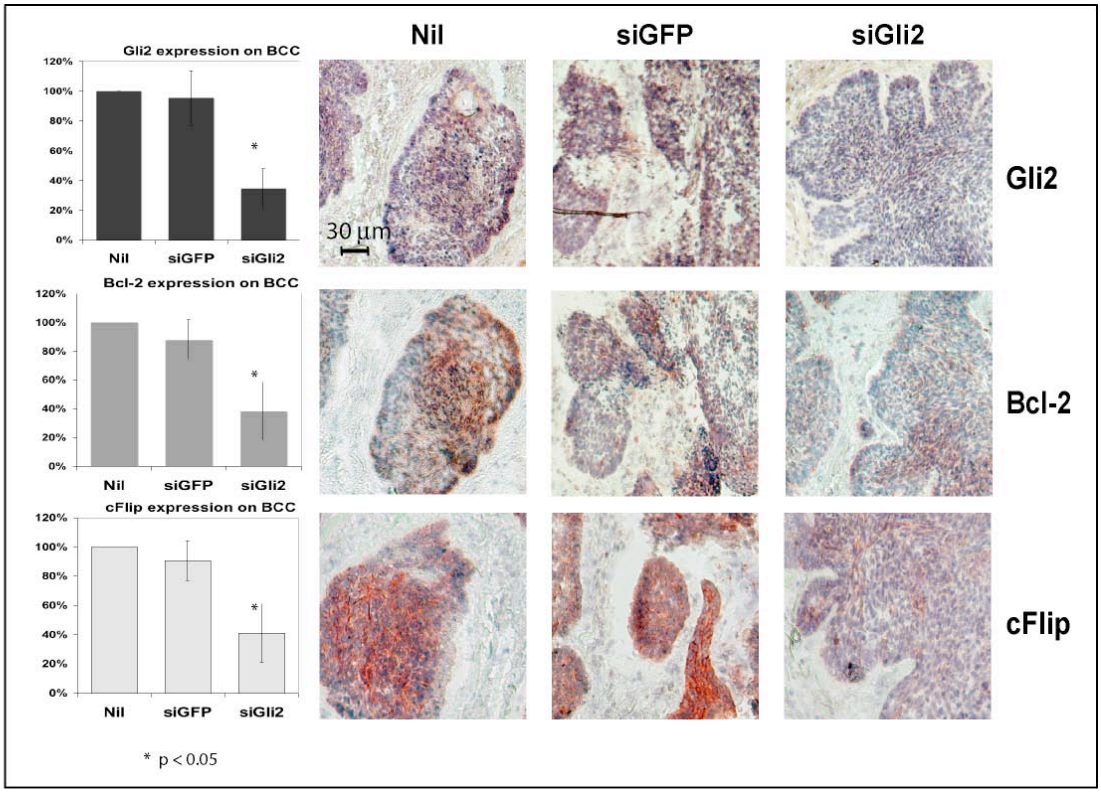


Fig 33

Expression of Gli2 and its target proteins in BCC tissue. Basic expression levels are shown in the left bars and in the left pictures (Nil). Control siRNA treated tissue showed no significant alteration in protein expression (middle bars and middle pictures (siGFP)). Treatment of BCC tissue with specific siRNA targeting Gli2 lead to a significant reduction in the expression of Gli2 itself and of its target proteins Bcl-2 and cFlip (right bars and right pictures (siGli2)). Results represent the mean of relative expression levels in four independent BCCs including standard deviations.

5.8 Trail receptors are expressed on BCC

We stained BCC tissue as described above also for Trail receptors 1 to 4. While TR 2 and 4 were clearly expressed, we could not observe any substantial TR1 or TR3 expression on the BCC tested (see Figure 34). The decoy receptor TR 4 seems to be strongly expressed, but the high color intensity may be explained by potential difference in affinity to the secondary antibody. However, as TR2 is expressed, despite the presence of TR4, we can expect a certain Trail susceptibility of BCCs at least in the situation where high amounts of Trail are used to saturate TR4 and induce apoptosis through TR2.

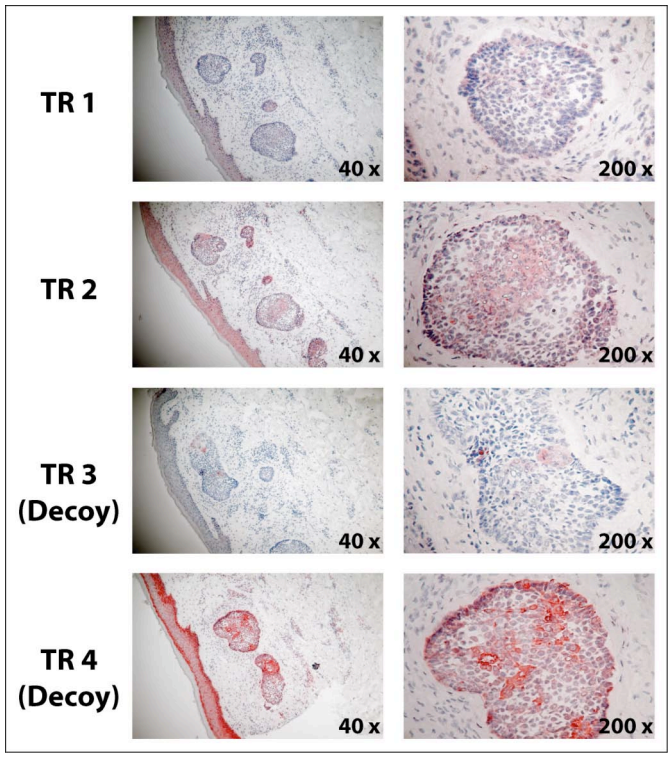


Fig 34

Expression of Trail receptors in one BCC tissue. Left panels show an overview over the tissue including epidermis and dermis (magnification 40 x), right panels show a higher magnification (200 x) of the BCC nodules. Color intensities between different stainings are not quantitative data as antibody affinities to each protein may vary. The results are representative for two BCC specimens.

5.9 Apoptosis is induced in BCC tissue specimens by soluble recombinant human Trail, and is increased when Gli2 expression is silenced by RNA interference

Induction of apoptosis in BCC tissue specimens was successful as can be seen in Hematoxilin and BerEP4 stainings of cryosections of BCC cultured for 36 hours with 200 ng/ml soluble recombinant human Trail along with a cross-linking enhancer (anti Flag antibody) (Fig. 35). Untreated BCC tissue fractions did not show signs of apoptosis and disintegration of the tumor nodules. BCC tissue pieces that showed high Gli2 expression (Nil, siGFP) were rather resistant to very high concentrations of Trail applied (200 ng/ml), while those pieces that had lowered Gli2 levels due to siRNA treatment against Gli2 (siGli2) were showing apoptosis and tissue disruption. Thus, as in the cell line, high Gli2 has a protective effect against Trail-mediated apoptosis in BCC.

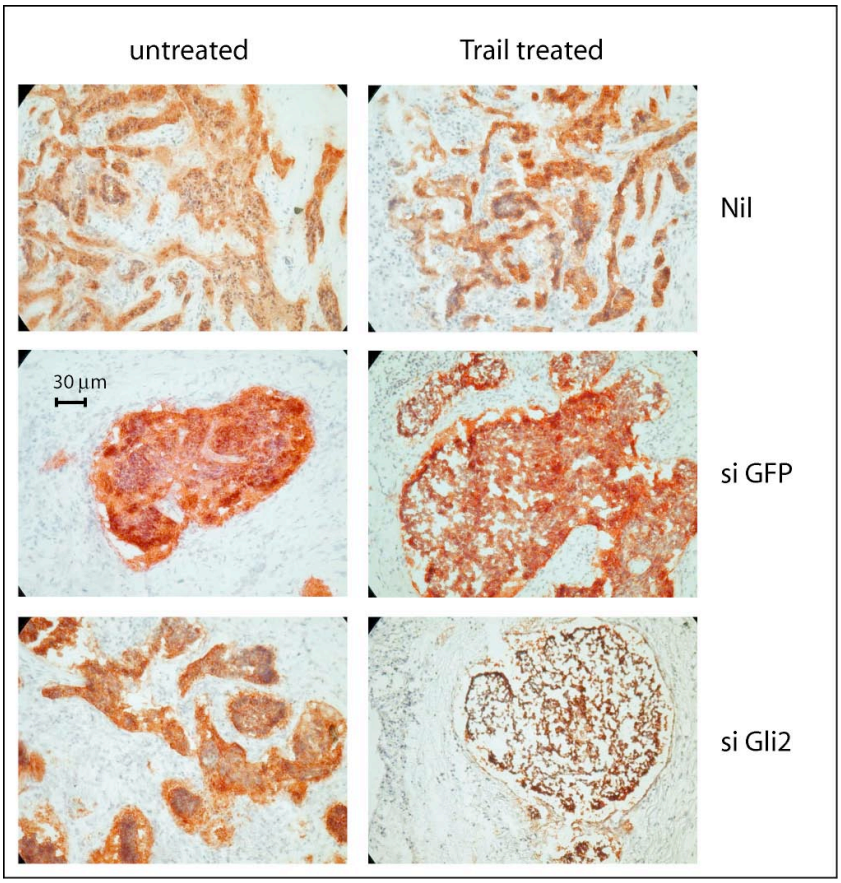


Fig 35

Apoptosis in Trail-treated (right panels) and –untreated (left panels) BCC tissue pieces. Trail-untreated tissue pieces mainly show intact BCC tumor nodules. Those pieces which were treated with Trail and Gli2-specific siRNA showed features of BCC tissue destruction and apoptosis (lower right panel) in contrast to siRNA-untreated and control siRNA-treated pieces (upper right and middle right panels).

6 Discussion

When cells turn malignant and transform into tumor cells, they face several environmental pressures, such as the surveillance and attacks of the immune system. These hostile environmental conditions exert a selective pressure on newly transformed cells promoting the development of immune evasion mechanisms. These mechanisms include preventive measures to circumvent the attacks of cytotoxic lymphocytes. Besides the possibility of a developing tumor to secrete apoptosis-inducing ligands which kill infiltrating lymphocytes (counter attack theory, reviewed in [60,61]), tumor cells can either shut down the expression of death receptors [56,62] or upregulate molecules that interfere with apoptosis pathways downstream of the death receptor signaling [63-65]. Both mechansims are observed in various tumors. BCC cells lack the expression of Fas (CD95) [55], have only moderate expression levels of Trail receptors (Figure 34) and are therefore poorly sensitive for immune effctor cells that attack with FasL (CD95L) or Trail. However, on an earlier developmental stage, where tumors are not yet established but cells are already transformed, which is seen for example in actinic keratosis as a precancerous state of squamous cell carcinoma (SCC), death receptors are still expressed [56]. Thus, these cells need mechansims to block apoptosis induction downstream of death receptors. Therefore, the high expression of anti-apoptotic molecules such as Bcl-2 or cFlip in developing tumors is not surprising.

BCCs are often associated with mutations in the PTCH gene and as a consequence a deregulated hedgehog signaling, leading to elevated levels of the transcription factors Gli1, Gli2 and Gli3. Overexpression of Gli1 and Gli2 as the primary mediators of hedgehog signaling has no effect on the expression of the surface death receptor proteins Fas, TNFR or the Trail receptors 1-4, neither does it alter the expressions of death ligands such as FasL (CD95L) or Trail (RT-PCR

and FACS data not shown). However, Gli2 overexpression induces the expression of the anti-apoptotic molecule Bcl-2, conferring some resistance against the intrinsic pathway of apoptosis by interfering with Bax and Bak and thereby inhibiting Cytochrome c release from the mitochondria. High expression of Bcl-2 may protect developing tumors from stress-induced cell death in situations where limited oxygen or nutrients, or drug pressure would trigger the intrinsic pathway of apoptosis.

In this work, we could show that Gli2 overexpression mediated protection against death-ligand induced apoptosis via the upregulation of the Caspase 8 inhibitor cFlip. We showed in the human keratinocytic cell line NHis-Gli2 HaCat as well as *ex vivo* in BCC biopsies that cFlip is interacting with Gli2. Performing gel-shift assays, we confirmed that Gli2 is acting as a transactivator of cFlip by binding to a putative promoter region or cis-element of the cFlip gene. The 5' UTR of cFlip is very long and carries several untranslated sequence stretches of various lengths, spanning a distance of >15 kilobases in total. This highly complex structure of the 5' UTR region makes promoter studies extremely difficult, therefore, we focused on potential Gli binding sites on the 5' flanking region of the cFlip gene. We *in silico* analysed the entire 5' UTR of cFlip and identified 4 potential Gli candidate binding regions, all comprising at least two possible binding sites that differed from the Gli consensus sequence by two mismatches at the utmost. We screened the promoter activity for these four clusters (Figure 16) and were able to show activity in two of them by luciferase assays. One of the two included the Gli consensus sequence 5' GACCACCCA 3' that has been shown by Regl et al [38] for Bcl-2 and by Agren et al. [66] for Ptch1 to bind the transactivators Gli1 and Gli2. The other cluster included two sequences differing by two mismatches each from the consensus sequence 5' GACCACCCA 3'. Both were able to bind Gli2 as shown in gel shift assays (Figure 22).

The exact Gli binding motif 5' GACCACCCA 3' was originally identified by Kinzler and Vogelstein [67]. However, in the meantime, three Gli proteins have been described with some overlapping functions and targets (see Introduction), and the sequence of their target binding sites varied slightly, with two mismatches from the consensus sequence. As we could demonstrate an interaction of cFlip with Gli2, we focused our interest on transcriptional targets of Gli2. Table 2 lists the Gli2 transcriptional targets known to date, and their deviations from the consensus sequence.

Gene name of Gli2 target	Sequence	Deviation from consensus	Reference
Gli1	5′ CGGGTGGTC 3′	1	[39]
Bcl-2	5′ GACCACCAA 3′ 5′ GCACACCCA 3′ 5′ CGCCACCCA 3′	1 2 2	[38]
FoxE1	5′ GATCTCCCA 3′ 5′ GGACACCCA 3′ 5′ GCCCACCCG 3′ 5′ GACCGCCCG 3′	2 2 2 2	[68]
Ptch1	5′ TGGGTGGTC 3′	0	[66]
cFlip	5′ TGGGTGGCG 3′ 5′ TACCACCCA 3′ 5′ GACCACCCA 3′	2 1 0	this study

Table 2
Gli2 target genes and their Gli2 binding motifs

It is difficult to define a rule for the Gli2 binding sequence in Gli2 transcriptional target genes, however, the motifs defined so far show that there are no more than

2 mismatches allowed from the Gli2 binding motif first defined as 5′ GACCACCCA 3′. All nucleotides except a double C on positions 7 and 8 (or a double G on positions 3 and 4 if inversed) may apparently be replaced by either a purine or a pyrimidine, but the relative positions of replacing nucleotides may follow a rule still unelucidated.

Binding sites bs_2 and bs_3 in the putative promoter cluster II specifically interacted with Gli2 protein, as the supershift (shown in Figure 18) showed specificity of interaction of the shifted DNA oligo with Gli2. No supershift was observed when an irrelevant antibody was applied as a negative control (anti β-actin, Fig 19). However, we could not show interaction of any of the binding sequences with Gli1, as there was no supershift when we used an anti-Gli1 antibody (data not shown). This finding may be an artifact, as Gli2 protein is strongly overexpressed relative to Gli1, and the Gli1 supershift may simply not be visible. Thus, we can not exclude the possibility that Gli1 was also binding the cFlip binding sites bs_2 and bs_3. In our gel shift assays, we observed two shifted bands, which were both fading in the competition experiment (Figure 20), (designated shifted oligos 1 and 2). As we also observed two bands in the Gli2 Western blots (Figure 10), we argue that two forms of Gli2 can bind to cFlip, and they were running at two different velocities in the acrylamid gel. We assume that these bands represent monomeric and homodimeric Gli2, due to the fact, that the second band had exactly the double size of the first band (260 kDa compared to 130 kDa for monomeric Gli2) and that both bands got stronger in a time-dependent manner under tetracycline induction. In support of our assumption, according to Nguyen et al. overexpressed five-zinc finger proteins are able to aggregate to dimers or oligomers of higher order by interaction of the first two zinc fingers, leaving the last three zinc fingers functional for DNA binding [13]. In addition, we could show in pull-down experiments (Figure

23) that bs_{Bcl-2} and bs_3 can be used to bind and thus pull down both proteins seen in the Western blots (Figure 10). These proteins are equal in size and relative amounts to the putative monomeric and dimeric Gli2. Altogether, the pulldown experiments give strong evidence that the two bands observed in Western blots indeed represent monomeric and dimeric Gli2. The chance that an unknown protein of 260 kDa, pulled down with the Gli binding sites bs_2 and bs_3 would crossreact with our Gli2 antibody H-300 is unlikely.

Taken together, all results obtained clearly show that Gli2 can act as a transcriptional activator of the cFlip gene by binding to cluster II (see Figure 16) in the 5' region of the cFlip gene. However, we are so far unable to identify a detailed cFlip promoter region, as cluster II lies very distant from the transcriptional start site (+2400 bp approximately) of cFlip, and is more likely to act as a regulatory cis-element than as a proximal promoter. Cluster II seems to play an important role for the cFlip gene regulation, but it most probably acts as a regulatory cis element, binding Gli2 which in turn transactivates cFlip as a secondary element of the transcriptional complex.

The core promoter of cFlip is still unpublished and difficult to define, however, several putative CAAT boxes (NF1 binding) and a TATA box (binding of TATA binding protein and formation of the transcription initiation complex) can be found, although not in reasonable proximity to the transcriptional start site (-1536, -404 and -1440 bp, respectively). *In silico* core promoter predictions using the *Center For Biological Sequence Analysis* (http://www.cbs.dtu.dk/services/Promoter/) predict a core promoter with a high probability (score 1.069; 'highly likely') at position -321. Interestingly, in close proximity to this position (at -330), there is a GC-box located. GC boxes are binding sites for sp1 transcription factors, which mediate the binding of RNA polymerase II at the right position into the

transcriptional complex. GC-boxes are typical for gene promoters that lack a TATA box. Accordingly, there is a putative CAAT box but no TATA box in proximity to the predicted core promoter at position -321 in the 5' region of the transcriptional start site of the cFlip gene.
The predicted core promoter region of cFlip is located within cluster I of the four putative Gli2 binding regions clusters I to IV, however, no significant response to Gli2 overexpression could be observed in reporter experiments using cluster I as a promoter in the luciferase reporter vector pGL3 (see Figure 16). However, unrelated to Gli2, there was substantial overall luciferase activity observed with cluster I (data not shown), supporting the possibility that the core promoter of the cFlip gene may be located at position -321, 5' of the transcriptional start site.

Gli2 transactivates the expression of Bcl-2, as has been shown previously [38]. Bcl-2 is an antagonist of the intrinsic, stress-induced pathway of apoptosis [69], but is of minor importance in the extrinsic, death-ligand induced pathway. Therefore, we argued that the protection against Trail mediated apoptosis seen in Gli2 overexpressing cells must have a reason beyond Bcl-2 expression. cFlip, an inhibitor of a very early step of extrinsic apoptosis induction, may play an important role in the protection. When we experimentally upregulated Gli2 in our model cell line, while keeping cFlip expression low by RNAi, we observed that the apoptosis resistance of the cells was overcome (Fig 29). Therefore, cFlip is an important player in the prevention of apoptosis mediated by Gli2. Affymetrix gene chip analysis revealed additional apoptosis-related Gli2 target molecules (table 9.1.1), however, none of them turned out to play such an important role as cFlip. CARD 10 (caspase recruitment domain family, member 10) is a molecule that interacts with Bcl-10 and might be involved in the Apaf-1/Caspase-9 pathway [70]. It is therefore not influencing the extrinsic pathway of apoptosis. TOSO, a further Gli2 target revealed in the Affymetrix analysis, is implicated in the inhibition of the Fas/FasL pathway of apoptosis, but does not affect Trail mediated apoptosis [58].

Caspase 2 and Caspase 10 are both upregulated upon Gli2 overexpression (see table 9.1.1). Caspase 10 is a Caspase 8 homolog and may have some overlapping functions [57] whereas Caspase 2 supports Trail -mediated cleavage of Bid [71], thus, being a positive mediator of Trail -induced apoptosis. However, with Gli2-dependent upregulation of Caspases -10 and -2, we would rather expect an apoptosis supporting action of Gli2 via Trail. Therefore, cFlip is the only antiapoptotic molecule that is activated as a Gli2 target which is a negative regulator of Trail mediated apoptosis.
Among the molecules that are downregulated upon Gli2 overexpression, we found Bax and Apaf-1 to be slightly repressed. Both molecules are mainly involved in the intrinsic pathway of apoptosis (see Figure 7), and are thus only secondarily affecting Trail-mediated apoptosis. However, the Gli2-mediated downregulation of proapoptotic molecules is an interesting finding and could contribute to the inhibition of the intrinsic apoptosis pathway by Bcl-2.

It has now become possible to apply the RNAi technology for the specific downregulation of mRNA and protein in tumor biopsies and tissues [72]. Using this technology, we succeeded to efficiently downregulate Gli2 protein in BCC tumor tissue *ex vivo*. This allowed us to measure the expression of Gli2 target genes in dependence from Gli2 levels in this tumor. Bcl-2 as a known direct target of Gli2 was efficiently downregulated following decreased Gli2 levels and thus served as an internal control of functionality of the assays. We were able to show that cFlip is not only coexpressed with Gli2 in BCC, but is also regulated downstream of Gli2 in this tumor. Upon silencing of Gli2, cFlip levels were significantly lowered (see Fig. 33). The fact that the downregulation of Bcl-2 protein was not as efficient as that of cFlip could be due to the relatively long half life of Bcl-2 protein compared to that of cFlip (10 h-25 h, compared to < 3 h respectively, depending on the cell types) [73-77]. On the other hand, Gli proteins are short lived [78,79], which is typical for zinc finger transcription factors. The high turnover rate of zinc finger transcription

factors ensures a tight control of target genes. Therefore, applying siRNA on cells or tissue should result in a relatively fast decline of Gli2 protein expression, leading to rapid downregulation of short-lived downstream proteins such as cFlip, while Bcl-2 protein downregulation is retarded although its mRNA is degraded as rapidly as that of cFlip.

Trail treatment of BCC ex *vivo* only induced minor apoptotic cell death and tissue disruption (Fig. 35). This might be due to the fact that BCC express high Gli2 and therefore also high cFlip, the latter protecting the tumor cells from apoptosis. Downregulation of Gli2 with specific siRNAs before Trail treatment led to marked cell death and disruption of the BCC nodules. That this is due to the effect of the siRNA transfection which may induce strong cellular stress and unspecific apoptosis is unlikely, as unrelated GFP-specific siRNAs did not lead to tissue damage. Thus, consistent with the results obtained in NHis-Gli2 HaCat cells, downregulation of Gli2 in BCC tissues leads to the reduction of the apoptosis resistance through the concomitant downregulation of the anti-apoptotic cFlip. Our results provide the proof of concept that Gli2 specific siRNA could be a valuable therapeutical option for the future BCC treatment.

Conclusion

Many tumors including Basal Cell Carcinoma (BCC) develop mechanisms to avoid the attack of the immune system. One such mechanism is the resistance against apoptosis. The formation of sporadic BCC is often the result of overactivated Hedgehog (Hh) signaling due to mutations in *Patched* or *Smoothened*. This leads to overexpression of Hh mediators, the Gli transcription factors. Gli1 and Gli2 have been shown to regulate the expression of the anti-apoptotic molecule Bcl-2, thereby interfering with the intrinsic pathway of apoptosis. We found that Gli2

counteracts the extrinsic death receptor-mediated apoptotic pathway by regulating the expression of the Caspase 8 inhibitor cFlip which is known to inhibit apoptosis. We have been able to demonstrate that Gli2 directly binds to the cFlip promoter and thereby increases the apoptosis resistance of the cell. In addition, we demonstrate the link between Gli2 and cFlip not only in a model cell line but also ex vivo in BCC tissues. Our finding that Gli2 drives cFlip expression provides evidence that a deregulated Hedgehog signaling, as found in many cancer types, may contribute to the immune evasion of the tumor. Gli2 gene silencing may therefore become a new treatment strategy for tumors originating from deregulated hedgehog signaling.

7 Literature

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9 Appendices

9.1 Tables

9.1.1 Apoptosis-related Gli2-targets revealed in Affymetrix gene expression profiling

9.1.1.1 Gli2-induced

Gene Name	Affymetrix Accession No	Fold mRNA increase	Function
Gli2	208057_s_at	0.207	Zn finger transcription factor, GLI-Kruppel family member
Bcl-2	203685_at	0.155	Antiapoptotic, inhibits CytC release from mitochondria
FoxE1	206912_at	0.132	forkhead box E1, thyroid transcription factor 2
cFlip	211317_s_at	0.43	Antiapoptotic CASP8 and FADD-like apoptosis regulator
CARD 10	210025_s_at	0.345	caspase recruitment domain family, member 10
Caspase 2	209812_x_at	0.239	apoptosis-related cysteine protease
Caspase 10	211888_x_at	0.223	apoptosis-related cysteine protease
TOSO	221602_s_at	0.341	regulator of Fas-induced apoptosis

9.1.1.2 Gli2-repressed

Gene Name	Affymetrix Accession No	Fold mRNA decrease	Function
BAX	211833_s_at	0.124	Bcl-2-associated X protein
Apaf-1	204859_s_at	0.123	apoptotic peptidase activating factor

9.1.2 Oligonucleotides for EMSA

Name	Sequence	Position from Start ATG
Pos control	5' gatctaagagctcccgaagaccacccacaatgatggttgtatgt 3'	-
Neg control	5' gatctaagagctcccgaagaccaggcgcaatgatggttgtatgt 3'	-
Bs_1	5' tgagctatgcataagccgtttgaccacgcatggagaattttacc 3'	-11341 to -11297
Bs_2	5' acgcatggagaattttaccacccagagacacgcgagtggccctg 3'	-11316 to -11272
Bs_3	5' tggtaacatttcagccggtgggtggcggggattaggcgtgaagc 3'	-11206 to -11162
Bs_4	5' ggtgtcgccctgggctcctgggacgtcggggcactgtcccccga 3'	-10922 to -10878

9.1.3 Oligonucleotides for Gli2 protein pulldown

Name	Sequence
Pos control 5' Biotin	5' ttgatctaagagctcccgaagaccacccacaatgatggttgtatgt 3'
Neg control 5' Biotin	5' ttgatctaagagctcccgaagaccaggcgcaatgatggttgtatgt 3'
Bs_3 5' Biotin	5' tttggtaacatttcagccggtgggtggcggggattaggcgtgaagc 3'

9.1.4 SiRNAs

Target gene	mRNA target sequence	Pos. on mRNA
GFP	5' gcaagcugacccugaaguucau 3'	800 - 821 on vector EGFP-N1
Gli2	5' cugagguggucaucuaugaga 3'	1477 - 1498
Flip	5' ucugaugugucccucauuaauu 3'	799 - 820
Flip long	5' ugaagaauguggaauucaagg 3'	1521 - 1542
Flip short	5' auagaucccuuucuauagaac 3'	26 - 47

9.1.5 Vector plasmids

Name	Purpose	provider
pGEM®-T	Basic cloning vector	Promega, Catalys, Wallisellen, Switzerland
pGL-3 basic	Luciferase expression reporter without promoter	Promega
pGL-3 control	Luciferase expression reporter positive control (SV40 promoter)	Promega
pEGFP-N1	EGFP expression vector	BD Biosciences, Erembodegem, Belgium

9.1.6 Primers

9.1.6.1 PCR primers

Name	Sequence	Annealing temp. for PCR
pBcl-2 fw	5' ccacggactaggtgttcagg 3'	57 °C
pBcl-2 rv	5' ctcctcctcctggtcctgc 3'	57 °C
pFlip A fw	5' tatgctggtctctgactgggagct 3'	59 °C
pFlip A rv	5' gcgaaggctgaggtggcagcggca 3'	59 °C
pFlip B fw	5' tacattagatctctaccgcagctcctagtaggtg 3'	55 °C
pFlip B rv	5' tacattagatcttcctctggaggctcaaggagac 3'	55 °C
pFlip C fw	5' tacattagatctcagtggcgcaatcttagc 3'	55 °C
pFlip C rv	5' tacattagatctgaggcaaggagttcaagac 3'	55 °C
pFlip D fw	5' tacattagatcttttctgccctcaggtgtg 3'	55 °C
pFlip D rv	5' tacattagatctgagggctgacttcatttc 3'	55 °C

9.1.6.2 Sequencing primers

Name	Sequence	vector	Pos. on vector
SeqEGFP-MCS	5' ccgtcagatccgctagcgcta 3'	pEGFP-N1	580 - 600
pGL3 Seq	5' caggtgccagaacatttctctatc 3'	pGL3 basic	4791 - 4814

9.1.6.3 Primers for RT-PCR

Name	Sequence	Pos. on mRNA	Product length
GAPDH fw	5' gaaggtgaaggtcggagtc 3'	81 – 99	225 bp
GAPDH rv	5' gaagatggtgatgggatttc 3'	306 – 287	
Flip long fw	5' ttggccaatttgcctgtatg 3'	1250 – 1279	78 bp
Flip long rv	5' ctcggctcaccaggacaca 3'	1328 – 1310	
Flip short fw	5' gcagcaatccaaaagagtctca 3'	855 – 876	88 bp
Flip short rv	5' ccaagaattttcagatcaggacaat 3'	943 – 919	
Gli2 fw	5' acggctgacattcggctaac 3'	4501 – 4520	82 bp
Gli2 rv	5' cccaaatgctccctaccatct 3'	4583 – 4563	
Gli1 fw	5' ggctgcaccaaacgctataca 3'	1180 – 1200	76 bp
Gli1 rv	5' acatgggcgtcaggacca 3'	1256 – 1238	
Bcl-2 fw	Qiagen Product N° 241117		
Bcl-2 rv			

9.1.7

Probes for RT-PCR

Name	Sequence	Pos. on mRNA	dye
GAPDH	5' caagcttcccgttctcagcc 3'	258 – 277	FAM
Flip long	5' ccgagcaccgagactacgacagcttt 3'	1281– 1306	FAM
Flip short	5' ccttcaaataacttcaggatgataacaccctatgcc 3'	882 – 916	FAM
Gli2	5' ttggccaaaacctttcaaaggatatgca 3'	4532– 4559	ROX
Gli1	5' atcctagctcgctgcgaaaacatgtcaag 3'	1202– 1230	ROX
Bcl-2	Qiagen Product N° 241117	Qiagen Product N° 241117	FAM

9.1.8 Antibiotics

Name	Application	Working concentration	provider
Kanamycine	selection medium for EGFP-N1	30 μg/ml	Gibco
Ampicilline	selection medium for pGL3	100 μg/ml	Sigma
Blasticidine	selection medium for NHis-Gli2 stability	8 μg/ml	Calbiochem
Zeocine	selection medium for NHis-Gli2 stability	25 μg/ml	Invitrogen
Tetracycline	Induction of Gli2 expression in NHis-Gli2	2.5 mg/L	Sigma
Gentamycine	BCC tissue culture	1 mg/ml	Gibco

9.1.9 Restriction enzymes

Name	Cutting site	purpose	provider
ApaI	CCC˅GGG GGG˅CCC	Blunt-end cloning of Bcl-2 promoter fragment into pGL3	NEB
NdeI	CA˅TATG GTAT˅AC	Cloning of Flip promoter fragments into pGEM-T and pEGFP-N1	NEB
SacII	CCGC˅GG GG˅CGCC	Cloning of Flip promoter fragments into pGEM-T and pEGFP-N1	NEB
BglII	A˅GATCT TCTAG˅A	Cloning of Flip promoter fragments into pGL3	NEB

9.1.10 Antibodies

Application	Target protein	specificity	conjugation	origin	conc.
FACS/ Western blot	Gli2	Goat anti human	-	St.Cruz (H-300)	1 μg/ml
FACS/IHC/ Western blot	cFlip	Rabbit anti human	-	housemade	N.A. 1:50
Western blot	cFlip	Rat anti human	-	Alexis (Dave-2)	1 μg/ml
FACS	Trail receptor 1	Mouse anti human	-	Alexis	10 μg/ml
FACS	Trail receptor 2	Mouse anti human	-	Alexis	10 μg/ml
FACS	Trail receptor 3	Mouse anti human	-	Alexis	10 μg/ml
FACS	Trail receptor 4	Mouse anti human	-	Alexis	10 μg/ml
IHC	Bcl-2	Mouse anti human	-	Dako Cytomation	1 μg/ml
IHC	Ber-EP4 epithelial Ag	Mouse anti human	-	Dako Cytomation (N1554)	1:1 ready to use
IHC	Gli2	rabbit anti human	-	St. Cruz (N-20)	1 μg/ml
FACS/IHC	Isotype control	Mouse IgG1	-	Dako Cytomation	1 μg/ml

FACS/IHC	Pre-immmue serum	Rabbit Ig	-	housemade	N.A. 1:50
FACS	Mouse IgG	Goat anti mouse Ig	FITC	Sigma	1.5 μg/ml
FACS	Rabbit Ig	Goat anti rabbit Ig	FITC	Sigma	5 μg/ml
IHC	Mouse Ig	Goat anti mouse Ig	Biotin	Sigma	3.5 μg/ml
IHC	Goat Ig	Rabbit anti goat Ig	Biotin	Dako Cytomation	2 μg/ml
IHC	Rabbit Ig	Goat anti rabbit Ig	Biotin	Dako Cytomation	3.3 μg/ml
IHC	Mouse Ig	Goat anti mouse Ig	HRP	Dako Cytomation	10 μg/ml
Western blot	Rabbit Ig	Goat anti rabbit	HRP	Dako Cytomation	0.15 μg/ml
Western blot	Rat IgG	Rabbit anti rat	Biotin	Dako Cytomation	0.2 μg/ml
Western blot	β-Actin	Mouse anti human Ig	HRP	Abcam (AC-15)	0.16 μg/ml

9.2 cFlip gene, full sequence

CFLAR gene locus: Chromosome 2 ="2q33
NCBI entry: AB 038972
total on genome: 51830 bp (gene start @ 2122)
total length of gene: 74214 bp

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intron: 2437..>2605
gap: 2606...4094
intron: 4095...4536
exon: 4537...4892
intron 4893..>5242
gap 5243..10222
intron <10223..15492
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intron 15559..15766
exon 15767..16184
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exon 49252..50090
exon 50091..51830

**Coding sequence: join(15904..16184,19085..19190,22028..22163,26464..26546,
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Curriculum vitae

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Erklärung

Ich erkläre, dass ich die Dissertation *'The apoptosis resistance of a keratinocytic cell line and of basal cell carcinoma is mediated by the transcription factor Gli2 via cFlip upregulation'* nur mit der darin angegebenen Hilfe verfasst und bei keiner anderen Universität und keiner anderen Fakultät der Universität Basel eingereicht habe.

Basel, im März 2007

Erwin Kump