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Titel der Diplomarbeit / Title of the Diploma Thesis

„Investigating cross reactivity/cross induction in multireporter plasmids and immunohistochemistry of Integrin α6 in cancerous tissue”

verfasst von / submitted by

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2 Abstract

In order to develop macromolecules for cancer therapy, one has to take a deeper look into the signaling pathways which are associated with the formation of cancer. Amongst others, the three signaling pathways Wnt3a-, Hedgehog- and Notch are of importance, which are also of importance in so called cancer initiating cells. In vitro transfection experiments with pathway reporter plasmids can be used for studying the activity of these pathways within the context of cancer cells.

In this study, luciferase based single reporter plasmids for these signaling pathways were used to investigate plant extracts, which are supposed to be associated with the inhibition of cell division and, therefore, can be taken as a starting point for cancer therapeutics. Further, cross reactivity between the substrates of the luciferase assays need to be examined. Especially when using multiple reporter plasmids for observing the pathway activity, this is an important issue to take into account. In addition to this, investigating the cross induction of the pathways is another issue. For this, a reporter plasmid, 3P-Luc, was characterized which encodes for three different luciferases expressed under the control of promoter elements responsive to these three signaling pathways.

Another major aspect for gaining knowledge about cancer is by means of histological studies. This objective was pursued by performing immunohistochemistry for the surface protein CD49f, which is also known to be upregulated and serving as a potential biomarker for cancer initiating cells.

Taken together, this diploma thesis presents the activity of the Wnt3a-, Hedgehog- and Notch-pathways after treating cells in vitro with two different plant extracts. Furthermore, the cross reactivity between Furimazine and Coelenterazine, substrates for the luciferases NanoLuc and Gaussia Luc, respectively, was investigated. In addition to this, the effect of the inducer plasmids on all three pathways is examined, using the 3P-Luc plasmid and performing all three luciferase assays for this purpose. In the second part of this study, immunohistochemical stainings were carried out on tumor tissues derived from mice, were the murine triple negative breast cancer cell line 4T1 was injected intravenously forming distinct lung lesions in order to specifically visualize expression of CD49f on tumor tissue.

3 Zusammenfassung

Um Makromoleküle für die Krebstherapie zu entwickeln, muss man sich genauer mit den Signalwegen befassen, die mit der Entstehung von Krebs verbunden sind. Von Bedeutung sind unter anderem die drei Signalwege Wnt3a, Hedgehog und Notch, die auch in so genannten tumorinitiierenden Zellen von Bedeutung sind. In vitro Transfektionsexperimente mit Pathway-Reporter-Plasmiden können zur Untersuchung der Aktivität dieser Signalwege im Bezug auf Krebszellen eingesetzt werden.

In dieser Arbeit wurden mit Luciferase-basierten Einzelreporterplasmiden für diese Signalwege jene Pflanzenextrakte untersucht, die mit der Hemmung der Zellteilung in Verbindung gebracht werden und daher als Ausgangspunkt für Krebstherapeutika dienen können. Weiterhin muss die Kreuzreaktivität zwischen den Substraten der Luciferase-Assays untersucht werden. Insbesondere bei der Verwendung mehrerer Reporterplasmide zur Beobachtung der Signalwegaktivität ist dies ein wichtiger Aspekt. Darüber hinaus ist die Untersuchung der Kreuzinduktion der Signalwege ein weiterer Punkt. Dazu wurde ein Reporterplasmid, 3P-Luc, charakterisiert, das für drei verschiedene Luciferasen kodiert, die unter der Kontrolle von Promotorelementen exprimiert werden, welche auf diese drei Signalwege reagieren.

Ein weiterer wichtiger Aspekt für den Erkenntnisgewinn über Krebs ist die Durchführung histologischer Experimente. Dieses Ziel wurde mit Hilfe der Immunhistochemie verfolgt, die das Oberflächenprotein CD49f lokalisieren soll, welches auf Krebszellen nachweislich überexprimiert ist und als potenzieller Biomarker für tumorinitiierende Zellen dient.

Zusammenfassend stellt diese Diplomarbeit die Aktivität der Wnt3a-, Hedgehog- und Notch-Signalwege nach der in vitro Behandlung von Zellen mit zwei verschiedenen Pflanzenextrakten dar. Darüber hinaus wurde die Kreuzreaktivität zwischen Furimazin und Coelenterazin, Substraten für die Luciferasen NanoLuc bzw. Gaussia Luc, untersucht. Außerdem wird die Wirkung der Induktorplasmide auf alle drei Signalwege untersucht, wobei das 3P-Luc-Plasmid verwendet wird und zu diesem Zweck alle drei Luciferase-Assays durchgeführt werden. Im zweiten Teil dieser Arbeit wurden immunhistochemische Färbungen an Tumorgeweben von Mäusen durchgeführt, bei denen die murine Triple-negative Brustkrebs-Zelllinie 4T1 intravenös injiziert wurde, um die Expression von CD49f auf Tumorgewebe spezifisch zu visualisieren.

4 Aim of the thesis

The aims of this work were as follows:

1. Verify the effect of a plant extract on Wnt3a activity and study effects on Hedgehog- and Notch- pathways
2. Investigate a potential cross reactivity between the substrates Furimazine (for NanoLuc) and Coelenterazine (for Gaussia Luc) when the assays were performed in one experiment
3. Determine potential cross induction between Wnt3a-, Hedgehog-, and Notch-pathways when activating individual pathways with inducer plasmids
4. Analyze the expression of CD49f in transplanted murine triple negative breast cancer in lung lesions by means of immunohistochemistry

ad 1: One of the first aims was to investigate the effect of plant extracts of *Peucedanum ostruthium* and *"plant A"* (identity not disclosed) on the activity of the Wnt3a-, Hedgehog- and Notch- pathway. The goal was to verify results and to gain more knowledge about the plant extract, which had shown in previous work an effect on the Wnt3a-pathway, and to see whether a similar effect occurs with the Hedgehog- and Notch- pathways. The plant extracts were dissolved in DMSO and diluted with HEPES buffered saline (HBS) to treat reporter plasmid transfected cells at concentrations of 25, 50, 100 and 200µg/ml.

ad 2: For investigating the cross reactivity between the substrates Furimazine and Coelenterazine when the NanoLuc and Gaussia Luc assays were performed in one experiment, HEK293T cells were seeded into two different plates (NanoLuc assay plate and Gaussia Luc assay plate) and transfected with the reporter genes TOP-NLuc or CBF-GLuc. The NanoLuc assay plate was read with Coelenterazine (from the lysate) and with Furimazine (also from the lysate). Vice-versa the Gaussia Luc assay plate was read with Furimazine from the lysate and with Coelenterazine from the supernatant.

ad 3: For detecting the cross induction between the signaling pathways, HEK293T cells were transfected with the 3P-Luc plasmid and co-transfected it with the inducer plasmids, pAd (Wnt3a inducer), phGli1 (Hedgehog inducer) and phICN1 (Notch inducer). After the transfection and co-transfection with one of the inducers, all three luciferase assays, NanoLuc, Firefly Luc and Gaussia Luc, were performed in one experiment.

ad 4: As the surface protein CD49f can serve as a biomarker for cancer stem cells, it was an important task to visualize its expression on tumorous tissue. Therefore, HE staining and immunohistochemistry was applied by using a Rabbit Monoclonal antibody to Integrin α6 as a primary antibody and the Goat-Anti-Rabbit Biotinylated Secondary Antibody. With the help of Avidin, Biotin and DAB, a brown color was developed to specifically visualize the CD49f on the surface of the lung cancer tissue of mice.

The following figure illustrates the different topics, which are presented in this thesis.

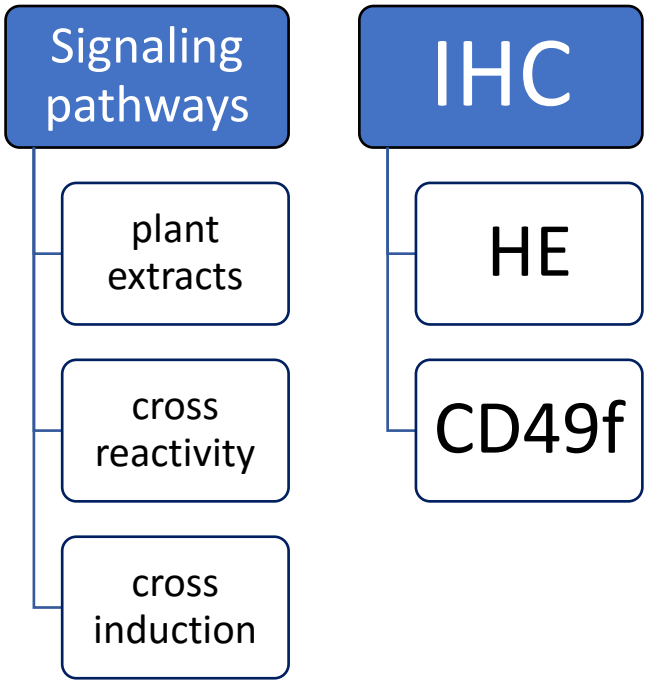


Figure 1: Schematic representation of different topics covered in this thesis.

5 Introduction

5.1 Cancer signaling pathways

Genetic mutations can lead to over proliferation of cells and to the escape of control mechanisms that usually prevent cells from turning into cancer cells. Cell growth and mitosis, apoptosis and cell motility are originally controlled by signaling pathways, whose genes can mutate and further lead to cancer progression. This is manifested in changes in the tumor microenvironment, angiogenesis, and inflammation. In addition to this, tumor suppressors can no longer inhibit the cell division. Critical negative regulators of signaling are eliminated and signals for cell growth are constantly given. (Sever and Brugge 2015) In this thesis, Wnt3a-, Hedgehog- and Notch- pathways play a central role as targets for experiments where their activation and inhibition are investigated when treated with different components.

5.1.1 Wnt3a-pathway

The Wnt pathway is an important pathway in metazoan beings. “Wnt” is a fusion of two words: *wingless* (= the name of the Drosophila segment polarity gene) and *integrated* or *int-1 (*= the name of the vertebrate homolog). A broad spectrum of cellular processes is controlled by the Wnt signaling pathway, for example cell fate determination, polarity, motility, organogenesis, and primary axis formation and the latest research has shown that this pathway is also involved in the renewal of embryonic stem cells, and on the other hand, is also very important for embryonic cell differentiation. This is seen as a controversial ability, which makes the Wnt3a-pathway interesting to explore. (Komiya and Habas 2008) (Merrill 2012) Embryogenesis is a phase, where signaling pathways play a decisive role. This is why they are tightly regulated and why the expression of the Wnt proteins and Wnt antagonists are tightly regulated temporally and spatially during this crucial development phase. Knowing this, a deregulation of the Wnt signaling pathway can lead to catastrophic consequences for the embryonic development. To put this in other words, it is now well known that a defective Wnt signaling pathway can lead to numerous human diseases. Cancers of the skin, colon and breast, human birth defect disorders including neural tube closure birth, spina bifida, and skeletal defects are diseases, that can be caused by a dysregulation of the Wnt signaling pathway. (Komiya and Habas 2008)

Wnt signaling pathways can be subdivided into three categories: the canonical Wnt pathway, the noncanonical planar cell polarity pathway, and the noncanonical Wnt/calcium pathway. Through binding of Wnt ligand to a Frizzled family receptor, the Wnt pathway is activated and the biological signal (depending on the pathway category) is given to the Dishevelled protein inside the cell. The canonical Wnt pathway is responsible for gene transcription, whereas the noncanonical planar cell polarity pathway regulates the cytoskeleton and further, cell shape. The noncanonical Wnt/calcium pathway controls calcium homeostasis inside the cell. Wnt signaling pathways use two types of cell communication: nearby cell-cell communication (paracrine) or same-cell communication (autocrine). (Nusse and Varmus 1992) (Nusse 2005) (Haiwei, et al. 2010)

Of all three pathways, the canonical Wnt pathway is the most explored signaling pathway. It regulates the gene transcription by regulating the cellular amount of β-catenin. (MacDonald, Tamai and He 2010)

To explain the pathway in more detail, it is best described when categorized in a two-state model: OFF (without Wnt ligand) and ON (with Wnt ligand). Without Wnt-protein (OFF state), cytoplasmic β-catenin is targeted for degradation by two scaffolding proteins, Axin and Adenomatous polyposis coli (APC). This leads to the amino-terminal phosphorylation of β-catenin by the kinases CKIα and GSK3. β-TrCP recognizes phosphorylated β-catenin, which is then ubiquitinated and degraded by the proteasome, thus keeping a low amount of free β-catenin in the cell. With Wnt protein (ON state), Wnt ligands bind to two types of receptors: seven-pass transmembrane Frizzled (FZD) receptor and single-pass low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6). The resulting complex (Wnt - FZD - LRP5/6) activates intracellular domains of both receptors. This leads to the recruitment of Dishevelled (DVL) and Axin, which inhibits the phosphorylation of β-catenin, thus its degradation. Increasing cellular levels of β-catenin facilitates the binding of β-catenin with the TCF/LEF transcription factors and thus activates the Wnt-regulated gene expression. This signaling mechanism regulates cell proliferation and differentiation and is a key mechanism for regulating stem cells. This also means that mutations in the Wnt pathway can be the reason for many illnesses including cancer. (MacDonald and He 2012)

In tumor cells, this signaling pathway can be activated in the OFF-state without the binding of the Wnt protein to the receptor. A loss-of-function mutation of a protein of the destruction

complex leads to this dysregulation. An example for this is colon carcinoma caused by the hereditary disease FAP (Familial Adenomatous Polyposis), where the tumor suppressor APC is involved. With a mutated APC, the complex can no longer be formed and β-catenin can no longer be degraded. Hence, β-catenin-dependent genes are no longer stimulus-dependent and can be transcribed on their own. This is why these genes play a central role in the cell cycle regulation and the activation of cell division and why such a mutation can lead to the development of cancer. (Antwerpes 2019) The following figure shows the signaling pathway in the ON, OFF and mutated state:

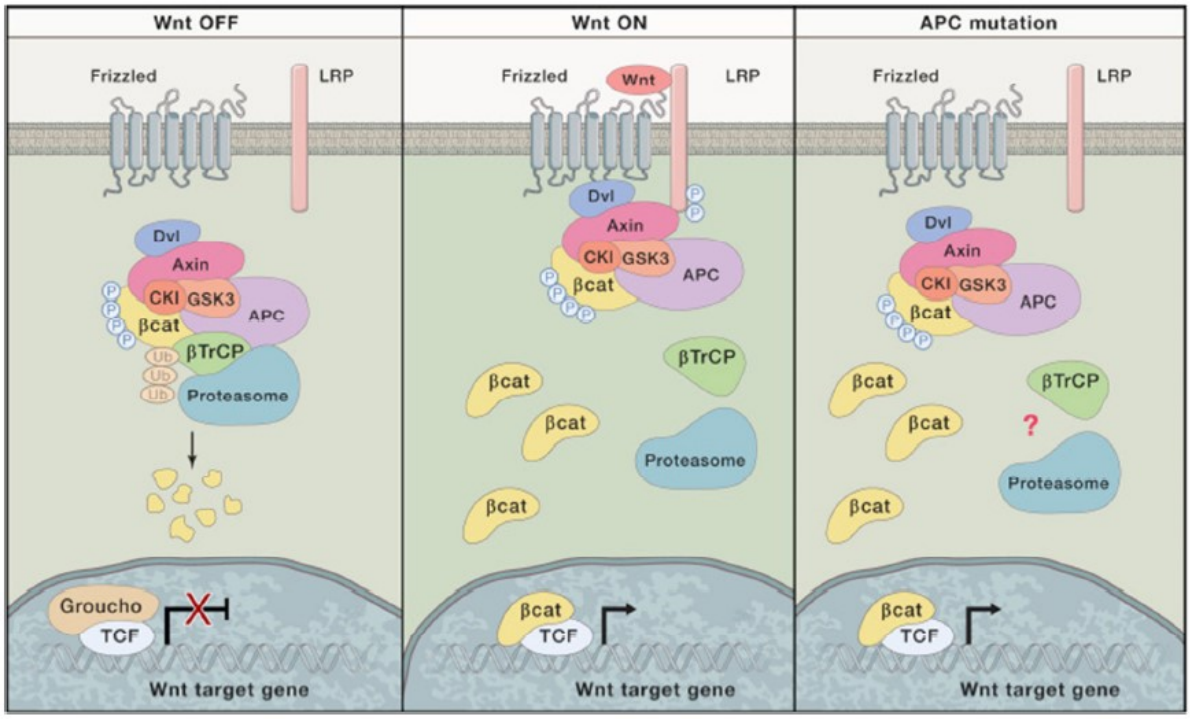


Figure 2: Wnt-pathway in different activation states. (Figure reproduced from (Nusse and Clevers 2017)

5.1.2 Hedgehog-pathway

Also remarkably involved during the phase of embryogenesis, the Hedgehog- (Hh) pathway is a major pathway in mammalians. According to the species, three different proteins have been characterized and differentially produced in Hedgehog-pathway: Sonic, Indian, and Desert Hedgehog. Sonic Hedgehog (Shh) is the main protein in humans and is expressed at the organogenesis, an early phase of embryogenesis. As it is a key regulatory pathway in this developing stage, limb formation and development of the central nervous system are under the control of Shh. It also plays a crucial role for the lung, where it is also responsible for bronchial budding. To investigate the effect of a dysfunction, it has been explored that mice

models with the inhibition of Shh pathway cause serious lung deformities with hypoplasia and tracheal abnormities and non-viable phenotypes. It could be proven that numerous lung illnesses are related to Shh dysregulation, including lung fibrosis. (Giroux-Leprieur, et al. 2018)

Shh pathway can be activated by two modes: the canonical and the non-canonical mode. Activation of the Hedgehog-pathway trough canonical mode is induced by binding of Shh protein on its receptor, Patched (Ptch). Ptch has an inhibitory effect on Smoothened (Smo), a second membranous protein, which is suppressed after the binding of Shh on Ptch (Ptch1 or Ptch 2). By activating Smo, glioma-associated oncogene (Gli) proteins are activated, then translocate into the nucleus to facilitate target gene transcription. Normally, Gli is kept in the cytosol by the suppressor of fused homolog (SUFU). When Shh binds to Ptch, Smo is activated and SUFU releases Gli to go into the nucleus and causes the transcription of genes. (Giroux-Leprieur, et al. 2018)

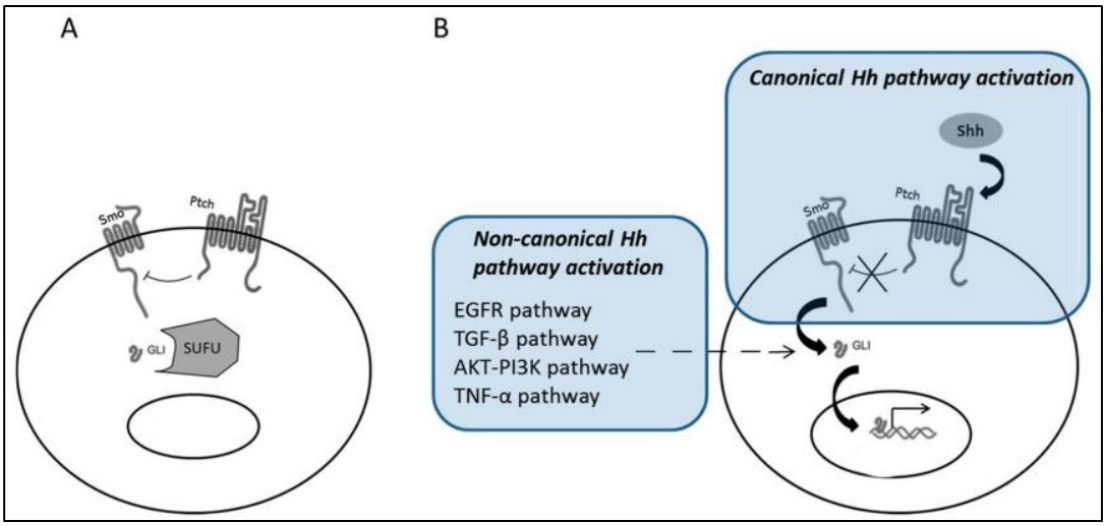


Figure 3: (A) Inactivated and (B) activated Hedgehog pathway through Shh. (Figure reproduced from Giroux-Leprieur, et al. 2018)

(A) No Shh, Gli is held outside from nucleus by SUFU and inactivated. (B) 1. Canonical Hh pathway activation: Shh binds to Ptch, Smo is no longer suppressed, Gli is translocated to the nucleus and activates gene transcription. 2. Non-canonical Hh pathway activation: Shh-independent. EGFR-, TGF-β, AKT-PI3K-, TNF-α- pathways are capable of directly translocating Gli into the nucleus to activate gene transcription. This form of Hh-activation is associated with Smo inhibitor resistant basal cell carcinomas. (Giroux-Leprieur, et al. 2018)

Moreover, Hedgehog-pathway is associated with the development of breast cancer. Patients with triple-negative breast cancer (TNBC, breast cancer tumor which lack hormone receptors and HER2 receptors) still have a poor prognosis in contrast to normal breast cancer patients. Research has shown that the non-canonical Hedgehog-signaling appears to be a fundamental mechanism in developing TNBC. However, it could not be proven that the non-canonical

signaling is a direct reason for TNBC development, whereas the canonical signaling of the Hedgehog-pathway is more explored. Breast cancer is associated with massive Shh upregulation, which causes progression and changes in the tumor microenvironment. (Riobo-Del Galdo, Montero and Wertheimer 2019)

5.1.3 Notch-pathway

The Notch signaling pathway is a key for numerous processes, such as cell division, apoptosis, maintaining stem cells and cell fate decision. As a cytoplasmatic receptor with four homologous proteins named Notch1, Notch2, Notch3, and Notch4, it is able to bind two ligand families: Delta-like (Dll1 -3 and -4) and Jagged (Jagged -1 and -2). The Notch ligands and their receptors have the structure of single-pass transmembrane proteins. (Bazzoni and Bentivegna 2019)

In Figure 4, the structure of the Notch receptor and the ligands is shown: The Delta and Jagged ligands are comparable: Both have no intracellular domain, while their extracellular part consists of EGF-like repeats and a distal Cysteine-rich region, named Delta/Serrate/Lag-2 (DSL) domain, which is the responsible region for interacting with Notch receptors. The extracellular domain of Notch receptors consists of EGF-like repeats and has a Cysteine-rich region called Lin -12, which noncovalently attaches the extracellular Notch to the membrane-joined intracellular Notch. This domain (=Notch intracellular; NICD) consists of four parts: an RBPJK associate molecule (RAM) region, ankyrin repeats (which are responsible for the communication between NICD and CBF 1, a transcriptional activator), a transactivation domain (TAD), and a PEST domain, which is involved in Notch degradation. (Bazzoni and Bentivegna 2019)

Figure 5 shows the steps from synthesis to degradation of Notch: ***(1)*** Synthesis in the endoplasmic reticulum. ***(2)*** Movement of the inactive single peptide precursor to the Golgi and cleavage by furin-like convertase (S1 cleavage). ***(3)*** Translocation into the cell membrane. Binding with Notch ligand. ***(4)*** Induction of the second cleavage (S2) by a member of metalloproteinases (ADAM) family. ***(5)*** Formation of a membrane-tethered Notch truncated (NEXT) fragment and processing in two sites (S3 and S4) by a presenilin-dependent γ-secretase complex. ***(6)*** Generation of the Notch intracellular domain (NICD), which is the active form of the Notch receptor. ***(7)*** Entrance of NICD into the nucleus and exertion of its transcriptional

activity. ***(8)*** Ubiquitination of the NICD. ***(9)*** Proteasome degradation. (Bazzoni and Bentivegna 2019)

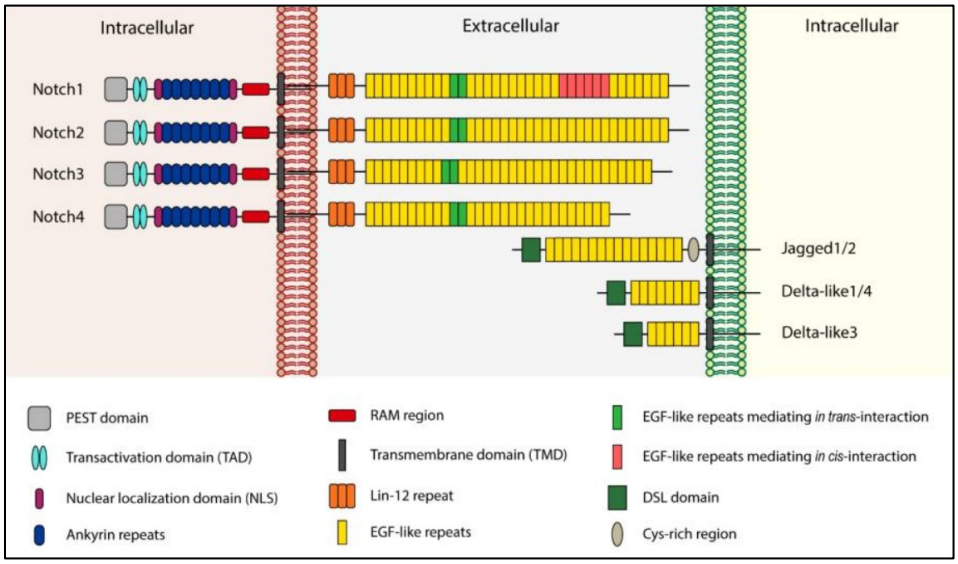


Figure 4: Structure of Notch receptors and ligands. (Figure reproduced from Bazzoni and Bentivegna 2019)

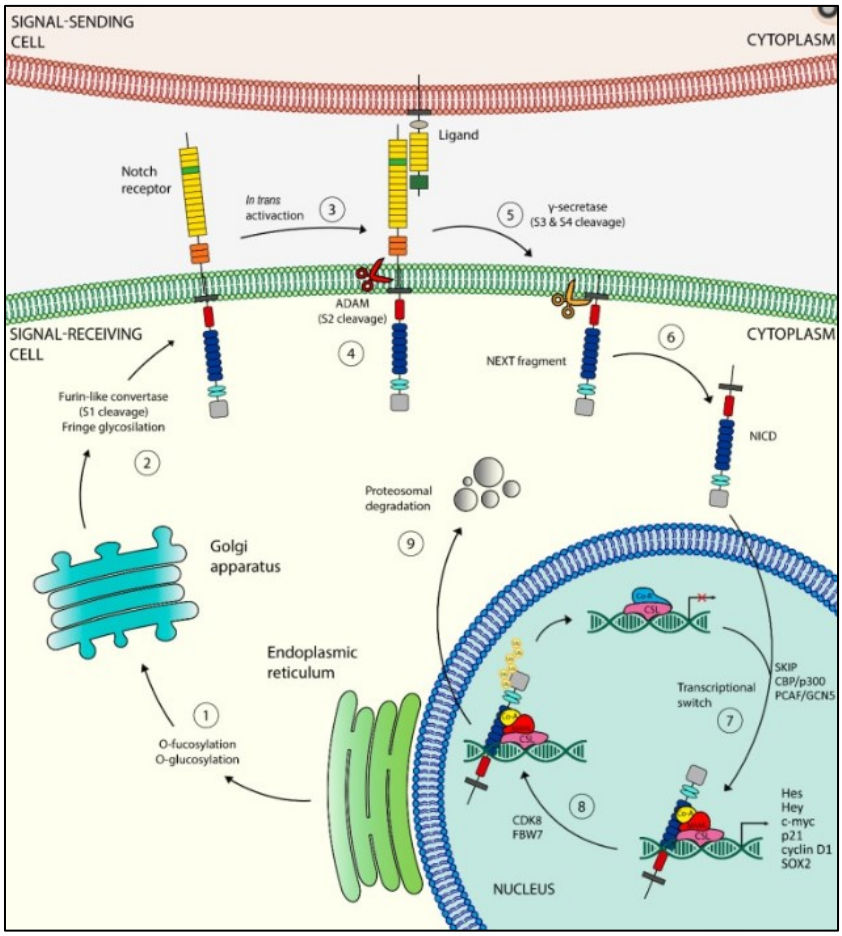


Figure 5: Schematic representation of the Notch signaling pathway. (Figure reproduced from Bazzoni and Bentivegna 2019)

5.2 Luciferase based reporter plasmids

Luciferase based reporter plasmids have been widely used as simple but powerful tools in biomedical research. The enzymes resulting after transfection of the luciferase reporter plasmids into the cells oxidize the substrates leading to emission of bioluminescence. Luciferase-based bioluminescence has played a main role in investigating biological processes, including cell trafficking, gene expression, protein-protein-interactions, tumorigenesis, and therapy. (Li, et al. 2018)

In this thesis, three luciferase based reporter plasmids (and their inducers) have been used for different biochemical investigations. TOP-NLuc (inducer: pAd), GLI-FLuc (inducer: phGli1) and CBF-GLuc (inducer: phICN1) are the reporter plasmids which generate the luciferase enzymes Nano Luciferase (NanoLuc), Firefly Luciferase (Firefly Luc) and Gaussia Luciferase (Gaussia Luc). These enzymes are responsible for generating the light signal which is the result of an oxidoreductase of their substrates Furimazine (FMZ), Luciferin (LBL) and Coelenterazine (CTZ). Biochemical researchers have come to the idea of using luciferase-luciferin reaction to generate light by watching different animals using this phenomenon.

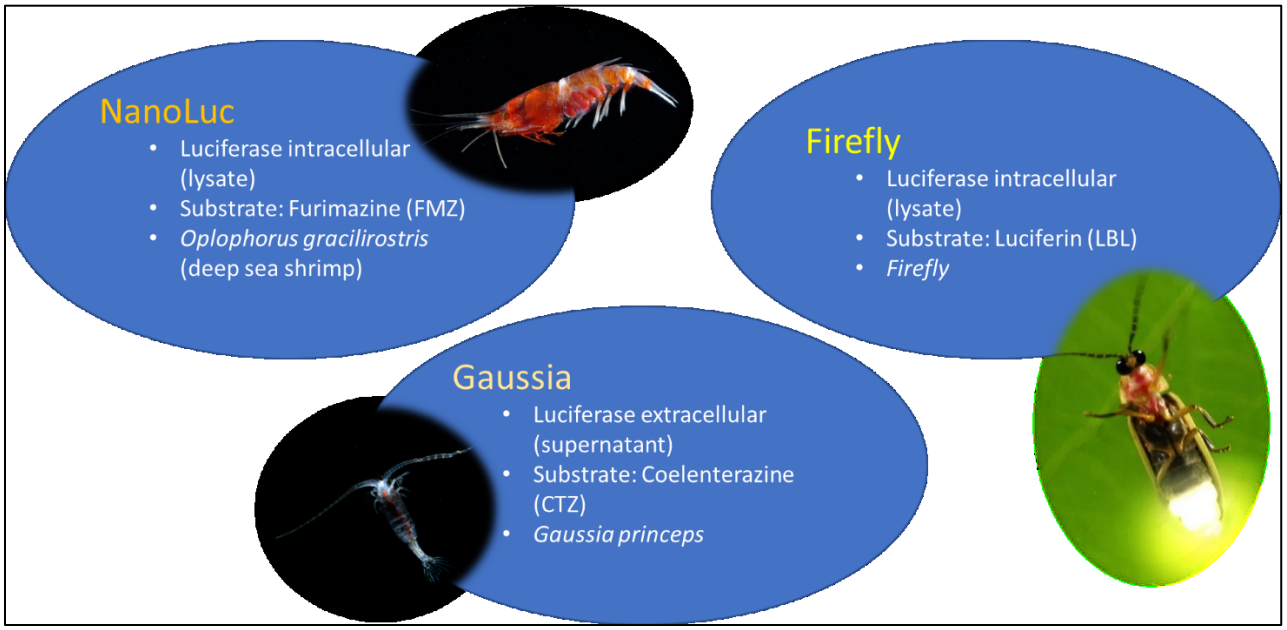


Figure 6: NanoLuc, Firefly Luc and Gaussia Luc. Figures reproduced from: (La Bioluminiscence 2017) (Bioluminiscence in Dinoflagellates 2014) (Low 2016).
Luciferase location after synthesis, substrate and animal origin of the different luciferase enzymes.

By transfecting the luciferase based reporter plasmids into cells, it is not only possible to investigate gene expression and cell trafficking. Another meaningful usage is the observation of activation or inhibition of signaling pathways through the reporter plasmids and the change of light signals which indicate a change in the regular signaling cascade of a pathway. Three pathways have been made research on through luciferase based reporter plasmids in this thesis. Wnt3a-pathway, Hedgehog-pathway and Notch-pathway can be specifically investigated through transfecting the reporter plasmids under the control of the promoters of the genes of interest. By doing this, whenever the gene of interest gets expressed, the reporter gene is also expressed and can be detected by adding the substrate and measuring the resulting light signal.

In our case, experiments from sections 6.3 (Screening of plant extracts for pathway activity) and 6.4.1 (Cross reactivity between luciferase substrates) have been performed with single reporter plasmids. The experiments described in section 7.4 (Multicistronic vector 3P-Luc for investigating crosstalk between signaling pathways) were conducted with triple reporter plasmids, where TOP-NLuc, GLI-FLuc and CBF-GLuc are combined to one plasmid called 3P-Luc plasmid. The following figures show the simplified plasmid design of the single and triple reporter plasmids.

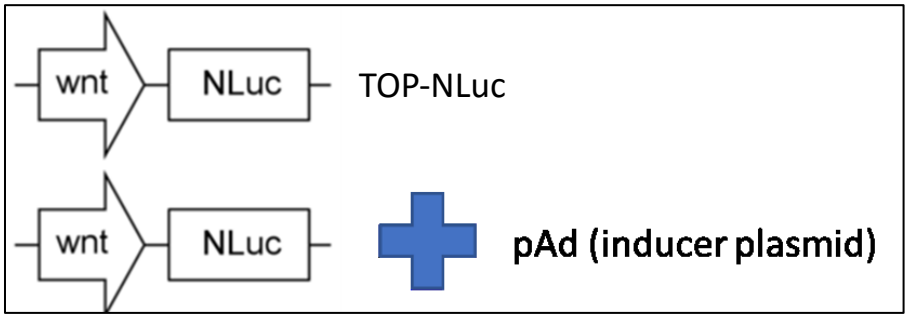


Figure 7: Single reporter plasmid for Wnt3a-pathway: TOP-NLuc and pAd inducer plasmid.

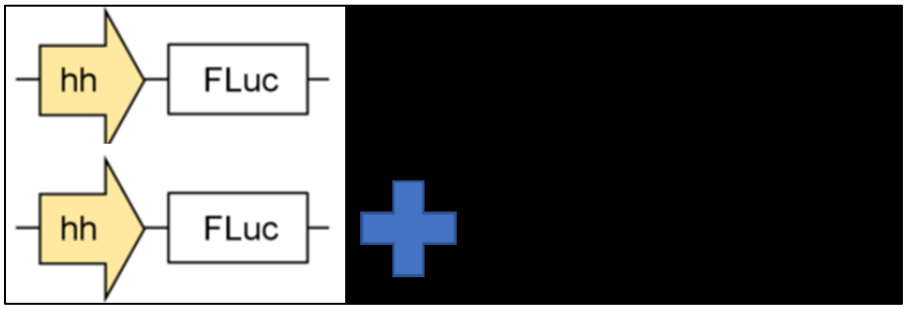


Figure 8: Single reporter plasmid for Hedgehog-pathway: GLI-FLuc and phGli1 inducer plasmid.

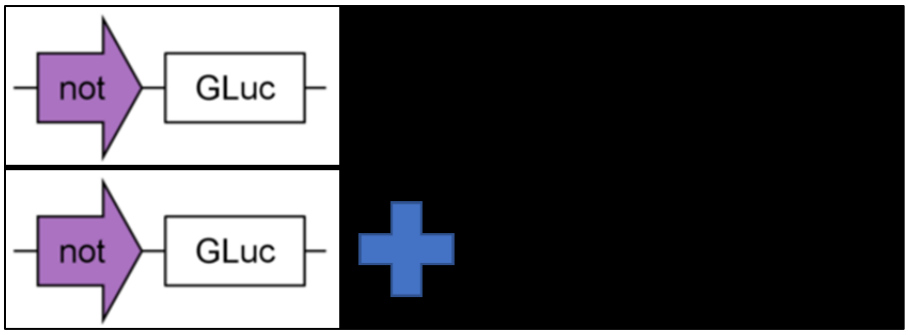


Figure 9: Single reporter plasmid for Notch-pathway: CBF-GLuc and phICN1 inducer plasmid.

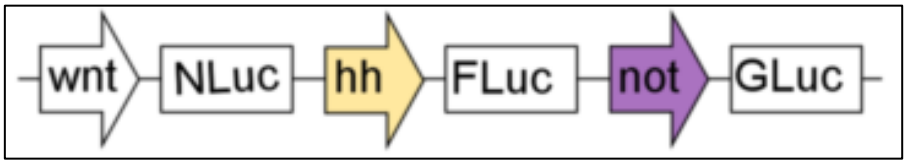


Figure 10: Triple reporter plasmid 3P-Luc for all three pathways.
All three single reporter plasmids (TOP-NLuc, GLI-FLuc and CBF-GLuc) are combined in one plasmid.

5.2.1 NanoLuc

To generate a light signal, NanoLuc is a luciferase enzyme which uses a Coelenterazine analogue as a substrate: Furimazine. In contrast to Firefly Luc, NanoLuc delivers a much brighter luminescence at 460nm wavelength (blue) without being dependent on ATP. NanoLuc has a molecular weight of 19.1kDa, which is, in comparison to other luciferase enzymes, much smaller. (Zerhouni 2015) After transcriptions, the enzyme remains cytoplasmatic.

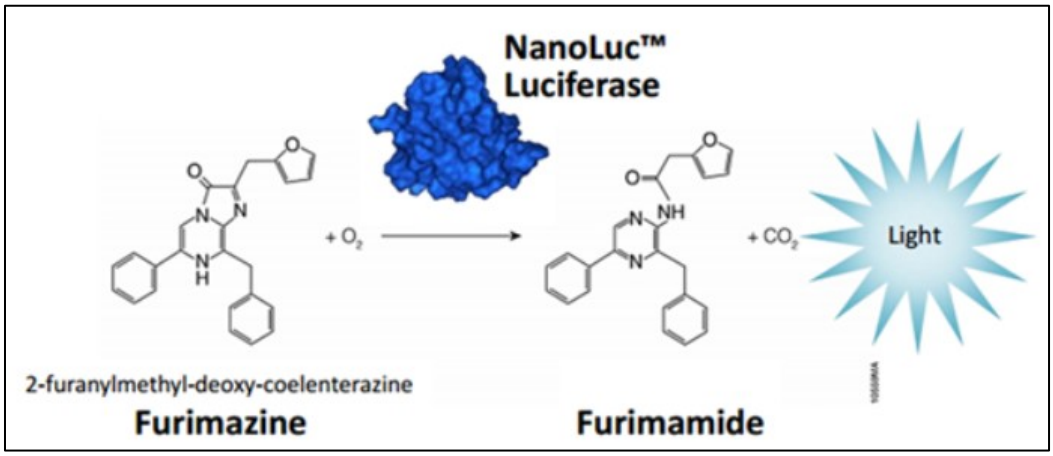


Figure 11: NanoLuc catalyzes Furimazine oxidation to Furimamide and light. (Figure reproduced from Zerhouni 2015)

The smaller size and > 150-fold increase in luminescence of NanoLuc offer several advantages over other established luciferase systems. One important benefit of these characteristics is a higher sensitivity compared to other luciferases. Not only the luciferase enzyme itself is advantageous over Firefly Luc and other luciferases, also the substrate Furimazine is

considered as more stable and low background signal producing. (England, Ehlerding and Cai 2016) For using NanoLuc as a reporter enzyme, it is necessary to transfect cells with TOP-NLuc plasmid with Wnt3a-reactive promoter. In this thesis, NanoLuc was used for investigations on the Wnt3a-pathway. To increase the light signal, the inducer pAd is co-transfected with TOP-NLuc.

5.2.2 Firefly Luc

Firefly Luc is widely used as a reporter to study gene regulation and function. Active as a monomer, it is a 62kDa protein which uses ATP and oxygen for catalyzing the oxidation of D-Luciferin to yellow-green (565nm) light and Oxyluciferin. As there is no endogenous luciferase activity in mammalian cells or tissue, the Firefly Luc is considered as being very sensitive. (Molecular Devices 2019)

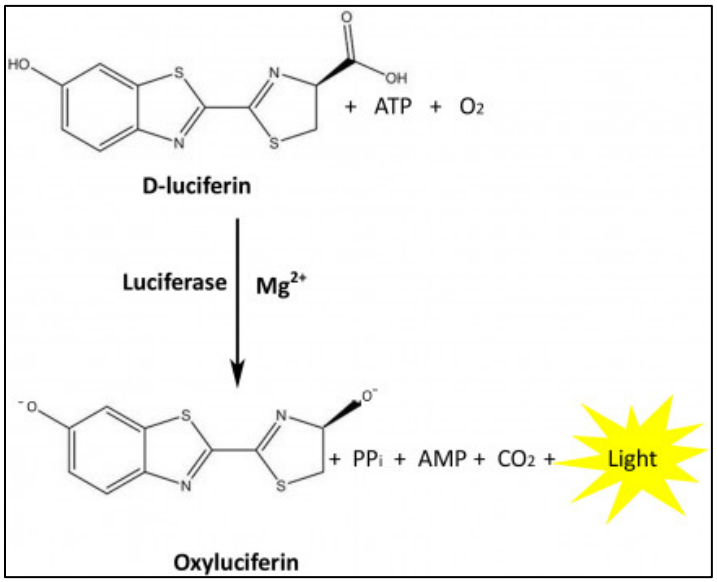


Figure 12: Firefly Luc catalyzes the oxidation of D-Luciferin to Oxyluciferin and light. (Figure reproduced from (Geno Technology, Inc. 2019).

Firefly Luc is expressed when cells are transfected with GLI-FLuc plasmid with Hedgehog-reactive promoters in order to investigate the Hedgehog-pathway activity. It is important to note that the expressed luciferase enzyme remains cytoplasmic without being secreted, which determines the method for working with Firefly Luc. The light signal can be induced when a co-transfection with phGli1 is performed.

5.2.3 Gaussia Luc

The Gaussia Luc enzyme is a 19.9kDa protein which catalyzes the oxidation of its substrate Coelenterazine producing blue luminescence. (Liu, et al. 2014) With only 185 amino acids, it is the smallest known luciferase. Through catalyzing the oxidative decarboxylation of Coelenterazine to Coelenteramide blue light at 480nm is emitted. Although the enzyme is secreted into the extracellular space, detecting Gaussia Luc expressing cells is easy due to the high sensitivity of the luciferase. (Tzertzinis, Egaña and Shea 2010)

As it shown in Figure 13, Gaussia Luc is ATP-independent and only uses oxygen for substrate conversion.

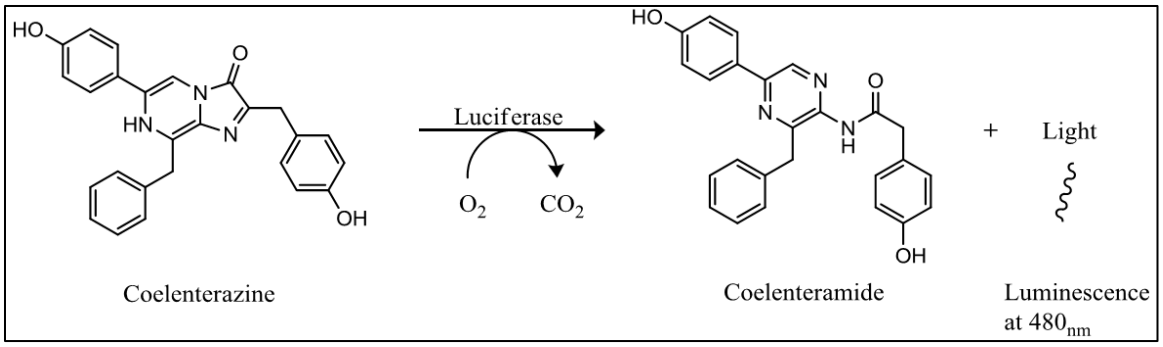


Figure 13: Gaussia Luc catalyzes the oxidation of Coelenterazine to Coelenteramide and light. (Figure reproduced from (Assay Biotechnology Company, Inc. 2013-2018)

By transfecting CBF-GLuc plasmid with Notch-reactive promoter, Gaussia Luc enzymes are expressed, with which the Notch-pathway activity is studied. For inducing the Gaussia Luc activity, CBF-GLuc is co-transfected with the inducer plasmid phICN1 (Figure 9).

5.3 Transfection

In order to generate recombinant proteins, transfection of genetic material into cells is necessary. This is achieved with the help of chemical and non-chemical based methods. Examples for chemical based transfection constructs are liposomes, nanoparticles, polymers, and cyclodextrins, whereas electroporation and cell squeezing are interesting examples for non-chemical based transfection methods. (Kamimura, et al. 2011) (Saul, et al. 2007) Both methods mentioned use non-viral vectors, which is advantageous because they show less immunogenicity and carcinogenicity over viral vectors. On the other hand, viral vectors are less expensive to generate and show much higher transfection efficiency and expression rate. (Goswami, et al. 2019)

Using cationic polymers for transfecting plasmids is a widely spread method. These particles consist of two components: positively charged polyethyleneimine (PEI) and negatively charged nucleic acid. The load difference causes the formation of transfecting complexes, which are internalized into the cells by endocytosis. (Kircheis, et al. 2001) The following figure shows the transfection mechanism of cationic polymers:

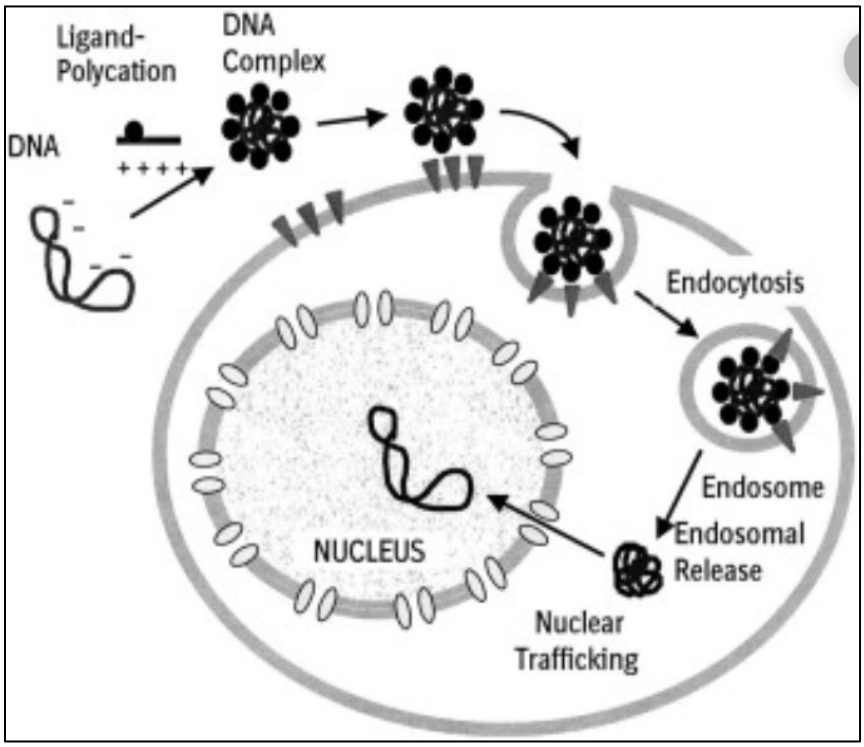


Figure 14: Transfection via cationic polymers. (Figure reproduced from Kircheis, et al. 2001)
Negatively charged DNA forms a complex with positively charged polymer, followed by endocytosis of the complex. DNA is released into the cytoplasm and transferred into the nucleus by nuclear trafficking. (Kircheis, et al. 2001)

5.4 Plant extracts relevant for Wnt3a-pathway

Considering previous research done by Sandra Milenkovic in her diploma thesis "In vitro assays for Wnt-pathway screening and splice correction", it is proven that several plant extracts have an inhibiting effect on the Wnt3a-pathway. In this thesis, her results are taken as a starting point for further research. Altogether nine plant extracts were investigated for their influence on the Wnt3a-pathway. When testing plant extracts 6 - 9, a one-tailed t-test for 25µg/ml of compound 3, 100µg/ml of compound 9 and 200µg/ml of compound 6 showed a significant decrease in Wnt3a activity. Compounds 7 and 8 had no significant effect on the Wnt3a-pathway. (Milenkovic 2019)

The following figure shows initial results from the above thesis:

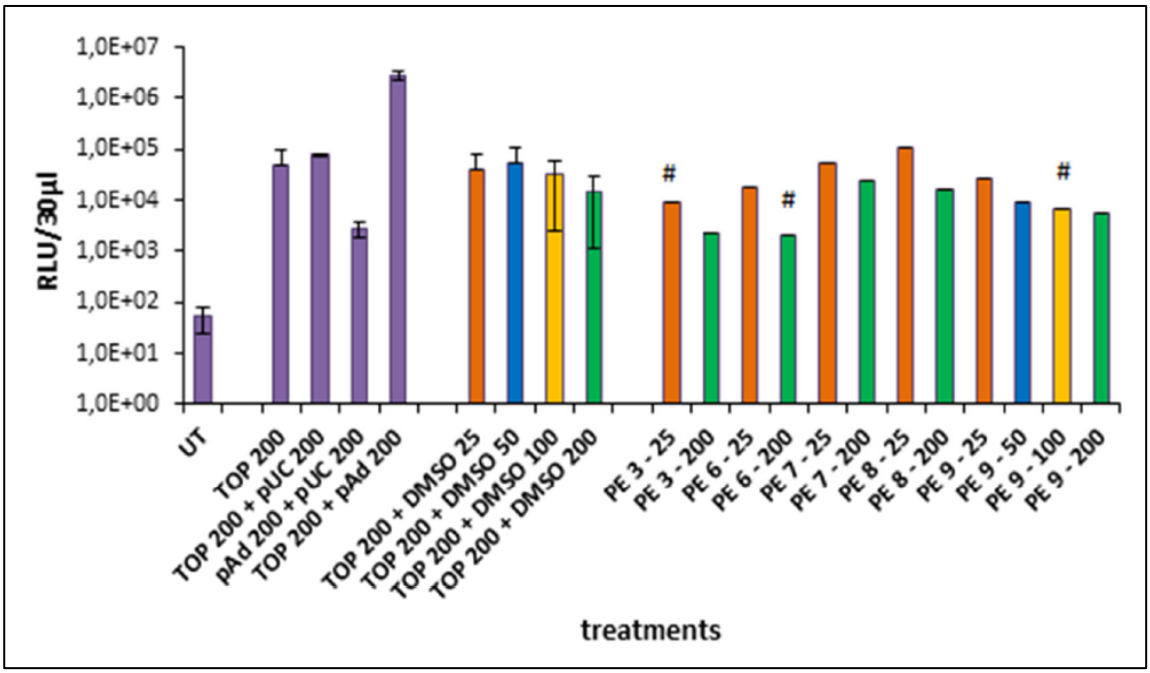


Figure 15: Screening of plant extracts for Wnt activity by NanoLuc assay. (Figure reproduced from Milenkovic 2019)

Data shown is the average with standard deviation from triplicate wells (three experiments). HEK293T cells were seeded, transfected with 200ng TOP-NanoLuc plasmid, and plant extracts 3, 6, 7, 8, and 9 were added at the concentrations 25µg/ml, 50µg/ml, 100µg/ml and 200µg/ml. As a control, DMSO was treated with the corresponding plant extract treatment (TOP200 + DMSO25; TOP200 + DMSO50; TOP200 + DMSO100 and TOP200 + DMSO200). Positive controls: Transfection with 200ng TOP-Nluc plasmid + 200ng inducer plasmid (TOP200 + pAd200) or 200ng TOP-Nluc plasmid + 200ng stuffer plasmid (TOP200 + pUC 200) or 200ng inducer plasmid + 200ng stuffer plasmid (pAd200 + pUC 200). UT = untreated. 24 hours after transfection, NanoLuc assay was performed and luminescence per well was measured. The signal marked with # shows a significant decrease in Wnt3a pathway activity (two-tailed t-test, p = 0.1; N = 9). (Milenkovic 2019)

Starting with these results, plant extract 3 and 6 are under deeper observation. Plant extract 3 is the extract of *Peucedanum ostruthium*, a plant of the *Apiaceae* family. Among the various ingredients, Coumarins are interesting components which have an effect on cancer related pathways as the Wnt3a-pathway. Osthole is a Coumarin which occurs in *Peucedanum ostruthium* at a concentration of 0.1%. Osthole has interesting characteristics regarding triple negative breast cancer (TNBC): It has been assumed that Osthole has an inhibiting ability on certain cancer cells. A recent research has shown that Osthole inhibited the growth of a panel of TNBC cells and induced apoptosis in cultured cells and in TNBC xenografts. It was also found that Osthole is capable of binding to signal transducer and activator of transcription 3 (STAT3). Moreover, Osthole shows the ability to suppress STAT3 in TNBC cells, to inhibit growth and induce cell apoptosis. On the other hand, overexpressing STAT3 in TNBC caused the reduction of the effectiveness of Osthole treatment. (Dai, et al. 2018)

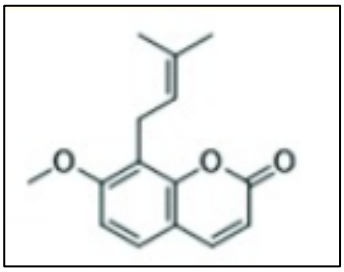


Figure 16: Chemical structure of Osthole. (Figure reproduced from Dai, et al. 2018)

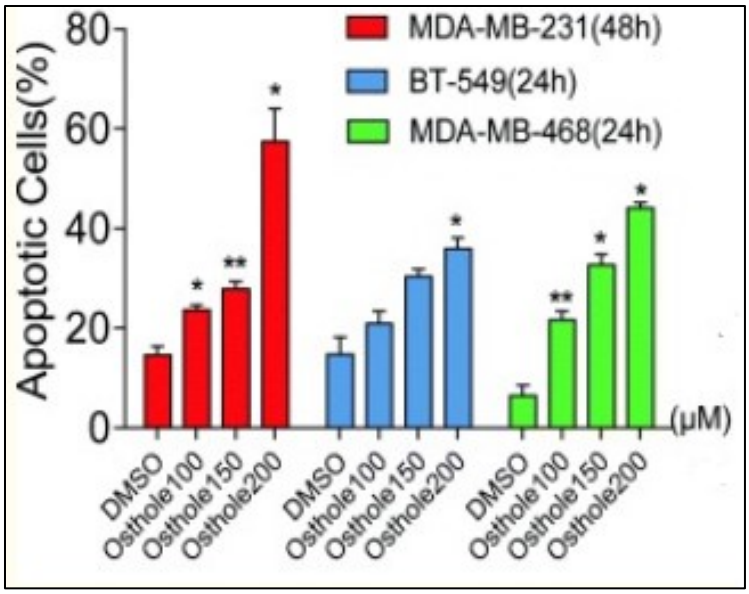


Figure 17: Amount of V/PI (= propidium iodide, staining for dead cells) stained annexin showing the percentage of apoptotic cells after Osthole treatment. (Figure reproduced from Dai, et al. 2018)

Figure 18: Peucedanum ostruthium. (Figure reproduced from (Heilpflanzenlexikon 2019)

Regarding plant extract 6, information is not disclosed due to patent issues.

5.5 Hematoxylin and Eosin staining

Hematoxylin and Eosin staining is the most common way for staining tissue to gain information about tissue structure. It is a widely used method for medical diagnosis, e.g. for diagnosing cancer in biopsies. The stain makes the general layout and distribution of cells visible and gives a general overview of the cellular structure of a tissue. This makes the differentiation between the nuclear and cytoplasmic parts of a cell very easy. (Ramulu, et al. 2013)

Hematoxylin, a natural dye derived from the logwood tree, shows its coloring ability through oxidation to Hematein. As Hematoxylin itself is a basic colorant, it colors acidic or basophilic structures blue, especially cell nuclei and the rough endoplasmic reticulum. (Ramulu, et al. 2013)

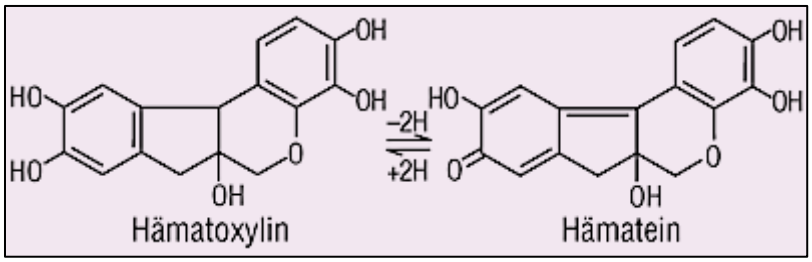


Figure 19: Chemical structure of Hematoxylin and Hematein. (Figure reproduced from (Gesundheit.de 2018)

Eosin is a synthetic acid dye and stains all acidophilic or basic (eosinophilic) structures red, especially cellular plasma proteins, mitochondria, the smooth endoplasmic reticulum, collagen and keratin. (Ramulu, et al. 2013)

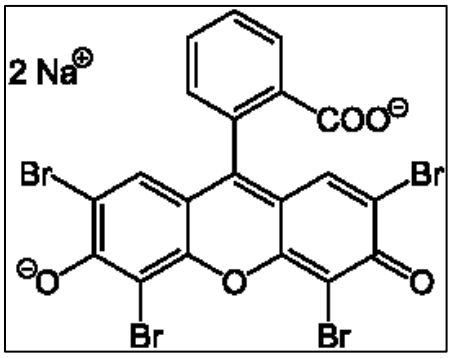


Figure 20: Chemical structure of Eosin. (Figure reproduced from (Wikipedia 2019)

5.6 CD49f expression on stem cells and cancer stem cells

Self-renewal and differentiation are characteristics that stem cells show when it comes to generating different tissues and, further, the organization of organisms. There are several categories of stem cells, depending on their capacity of differentiation: totipotent (e.g., zygote), pluripotent (e.g., embryonic stem cells), multipotent (e.g., hematopoietic stem cells), and unipotent (e.g., spermatogonial stem cells). Figure 21 shows the simplified mechanism of stem cells to differentiate to a certain cell type. (Krebsbach and Villa-Diaz 2017)

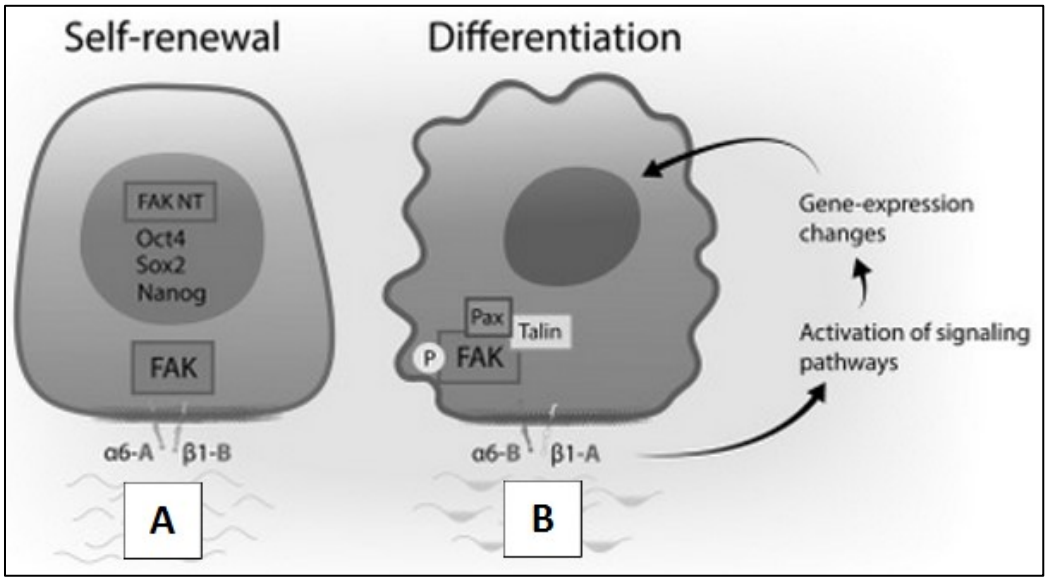


Figure 21: Simplified mechanism of cell differentiation of embryonic stem cells using integrin α6 and integrin β1. (Figure reproduced from Krebsbach and Villa-Diaz 2017)
(A) Undifferentiated ESC (=embryonic stem cell): Expression of pluripotent transcription factors Oct4, Sox2, and Nanog and integrin α6Aβ1B. This protein configuration keeps the FAK (= focal adhesion kinase) inactive and localized in the cytoplasm and the nuclei of undifferentiated cells. (B) Translation changes of integrin α6β1 to α6Bβ1A leads to recruitment to the plasma membrane, the phosphorylation of FAK, activation of signaling pathways and finally the downregulation of the pluripotent transcription factors and cell differentiation. (Krebsbach and Villa-Diaz 2017)

The diversity of stem cells makes it necessary to develop methods to identify and classify the cells through surface proteins which can serve as biomarkers for stem cells and cancer stem cells. Integrin α6, also known as CD49f, is one of the many cell surface proteins that can be considered as the only biomarker found on the surface of more than 30 different stem cell populations, including several cancer stem cells. Esophageal epithelial, hepatic, mammary epithelial prostate, hepatocellular carcinoma, prostate cancer and breast cancer stem cells are examples for CD49f positive stem cell populations. This indicates that integrin α6 plays a main role in stem cell biology and can be used as a biomarker. (Krebsbach and Villa-Diaz 2017)

Integrins are heterodimer transmembrane glycoproteins, consisting of a different number of α-and β-subunits. In mammals, there are 18 α-subunits and 8 β-subunits. Through different combinations of the α- and β-subunits, more than 24 possible heterodimer combinations can be formed, while the affinity to the extracellular matrix components like collagens, laminins, and fibronectin differs, depending on the subunit combination. This also means that integrins are fundamental for cell adhesion to the extracellular matrix and also function as a connecting element between the extracellular matrix and the cytoskeleton. (Krebsbach and Villa-Diaz 2017)

Moreover, integrins seem to have an effect on Tyrosine kinase receptors for communication between cells and the extracellular matrix. This leads to the fact that integrins play an important role for ligand activated cell mechanisms, such as cell differentiation, gene expression, motility, polarity, proliferation, shape, and apoptosis. (Krebsbach and Villa-Diaz 2017)

6 Materials and Methods

6.1 Equipment

List of equipment:

- Biological safety cabinet (Thermo Fisher Scientific)
- CellTiter-Fluor™ Cell Viability Assay kit (Promega)
- Centrifuge (Thermo Fisher Scientific)
- Centrifuge tube 15ml (CT-15) (Starlab)
- Centrifuge tube 50ml (CT-50) (Starlab)
- Counting chamber (Neubauer-improved, Paul Marienfeld GmbH & Co.KG)
- EppendorfR ResearchR plus pipettes (Sigma-Aldrich)
- Eppendorf tubes (Nerbe plus GmbH)
- Freezer (-20°C, -80°C) (Thermo Scientific)
- Incubator 37°C, 5% CO2 (Thermo Fisher Scientific)
- Inverted microscope (Motic)
- Multichannel pipette (VWR International)
- Pipette boy (VWR International)
- Pipette tips (Nerbe plus GmbH)
- Plate reader (Infinite M200 Pro, Tecan)
- Plate shaker (Eppendorf)
- Reagent reservoirs (VWR International)
- Refrigerator (4°C) (Allectric)
- Serological pipettes 5ml, 10ml, 25ml (Sarstedt)
- Syringe filters, cellulose acetate, 0,22µm (VWR International)
- TC flask T75, standard, vent. cap. (Sarstedt)
- Transparent 96-well flat-bottom microplate, sterile (Greiner)
- Vacuum aspiration system (Integra Biosciences)
- Vortex mixer (Velp Scientifica)
- Water bath (Memmert GmbH)
- White 96-well flat-bottom microplate, transparent bottom, sterile (Greiner)

List of reagents:

- CA Protein Assay Kit, Pierce TM (Thermo Scientific)
- Bovine serum albumin (Sigma-Aldrich)
- Cell line: HEK293T; #CRL-3216 (ATCC)
- Dimethyl sulfoxide (DMSO) (Sigma-Aldrich)
- Dulbecco's modified eagle's medium (DMEM) high glucose (Sigma-Aldrich)
- Dulbecco's phosphate buffered saline (DPBS) (Sigma-Aldrich)
- Fetal bovine serum (FBS) (Sigma-Aldrich)
- HEPES buffered glucose (HBG, 5 % glucose w/v, 20 mM HEPES pH 7.4), MMCT (University of Vienna)
- HEPES buffered saline (HBS, 145 mM NaCl, 20 mM HEPES pH 7.4), MMCT (University of Vienna)
- L-Glutamine 200mM (Sigma-Aldrich)
- Linear polyethylenimine (LPEI), 10kDa, MMCT (University of Vienna)
- Luciferase assay reagent buffer + luciferin (LBL)
- MilliQ-water (Sartorius)
- Nano-GloR Luciferase Assay Buffer + Substrate (Promega)
- NanoLuc purified protein (0.4mg/ml in DPBS pH 7) (Promega)
- Passive Lysis Buffer 5x (Promega)
- Penicillin-Streptomycin (Sigma-Aldrich)
- phGli1, #84922 (Addgene plasmid)
- phICN1, #17623 (Addgene plasmid)
- pUC19, #50005 (Addgene plasmid)
- pWnt3a, #26891 (Addgene plasmid)
- Sodium chloride (Applichem)
- Sodium hydroxide (Applichem)
- Trypsin-EDTA solution (Sigma-Aldrich)
- Versene (Gibco by Life Technologies)

Following plasmids were generated by Julia Maier in the course of her doctoral thesis and used for the experiments in this thesis: (Maier, et al. 2019)

- pMuLE_ENTR_CBF-GLuc_L3-L2
- pMuLE_ENTR_12GLI-FLuc_R4-R3
- pMuLE_ENTR_TOP-NLuc1.1_L5-L4
- pMuLE_EXPR_CMV-eGFP_TOP-NLuc1.1_GLI-FLuc_CBF-GLuc, 3 pathway-luciferases, 3P-Luc plasmid

List of plant extracts provided by Professor Judith Rollinger (University of Vienna):

- Plant extract 3: PeuostRDMp_2
- Plant extract 6: BurafrCEtoAc_2
- Plant extract 8: CapburHMp_2

List of materials used for histological experiments:

- Ammonium hydroxide solution (Sigma-Aldrich)
- Avidin-Biotin blocking reagent (BIO-RAD)
- Bovine Serum Albumin (Sigma-Aldrich)
- CellSens Standard (Olympus)
- coverslip 18x18 (Carl Roth)
- 3,3'-Diaminobenzidine tetrahydrochloride (Sigma-Aldrich)
- di-Sodium hydrogen phosphate dodecahydrate (AppliChem)
- Dulbecco's Phosphate Buffered Saline (Sigma-Aldrich)
- Elite PAP pen (Cedarlane Laboratories)
- Embedding station MPS/P1 (Slee medical GmbH)
- Entellan new (Merck)
- Eosin Y solution (Sigma-Aldrich)
- Ethanol denaturated >99,8% (Carl Roth)
- Ethylenediaminetetraacetic acid (Sigma-Aldrich)
- Hematoxylin solution acc to Harris (Carl Roth)
- Hydrogene peroxide 35% (Carl Roth)
- Hydrochloric acid (Sigma-Aldrich)
- microscope slide SUPERFROST (Carl Roth)

- microscope slide SUPERFROST PLUS (Thermo Fisher Scientific)
- Microtome (CUT 5062) (Slee medical GmbH)
- Olympus BX53 light microscope (Olympus)
- Potassium chloride (AppliChem)
- Potassium phosphate monobasic (Sigma-Aldrich)
- Rabbit monoclonal [EPR18124] to Integrin alpha 6 (abcam)
- Rabbit IgG Isotype (ab172730) (abcam)
- Sodium chloride (Merck/Thermo Fisher Scientific)
- tri-Sodium citrate dihydrate (VWR Chemicals)
- Triton X-100 (Sigma-Aldrich)
- Tween 20 (AppliChem)
- Vectastain® ABC kit (Vector Laboratories)
- Xylene (Carl Roth)

(These lists are derived from Milenkovic 2019 and Pichler 2019 and adapted.)

6.2 General workflow for using reporter plasmids to study signaling pathways

For studying signaling pathways it is necessary to seed the cells into a white 96-well plate, transfect them with polyplexes and read the luminescence signals after adding the substrate. The general workflow is like following:

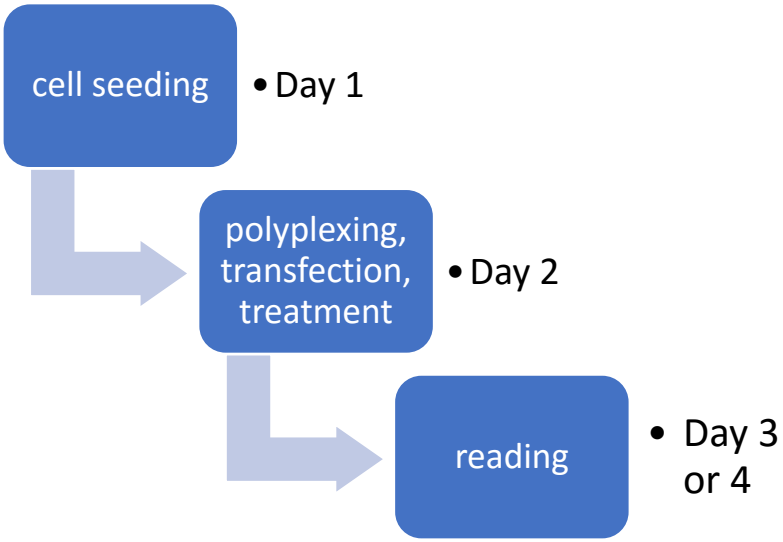


Figure 22: Workflow for using reporter plasmids.

6.2.1 Cell seeding

For cell seeding on the first day, the cells were taken out from the incubator, where they were kept in T-75 flasks. The color and turbidity of the medium were checked and the cell confluence was seen under the microscope. The medium was then removed and the bottom of the flask was gently washed with 5ml of phosphate buffered saline (PBS). For detaching the cells, 1 – 2ml of trypsin or versene (depending on the cell line) were added and the cell suspension was centrifuged. The cell pellet was then resuspended in 1ml of complete medium. The next step was counting the cells with a counting chamber. After this, the calculation was made to know which volume of cell dilution was needed to have 10,000 cells in each well. The calculated amount of complete medium was pipetted in triplicates into each well of a white 96-well plate with flat bottom and then the dilution of cell suspension was added into the wells so that the total volume of cell dilution and medium was 200µl in each well. After checking each well under the microscope, the plate was put into the incubator until the next day, where the experiment was continued.

6.2.2 Polyplexing and Transfection

On day two, the medium in the wells was removed and basal Dulbecco's modified eagle's medium (DMEM) was pipetted into the wells, while the amount of polyplexes and medium had to be 100µl in total. The polyplexes were made by flash pipetting Linear polyethyleneimine (LPEI) to the pDNA in equal amounts, while both were diluted in HEPES buffered glucose (HBG) or HBS. For calculating the right amount of LPEI, some factors have to be considered. The following equation was used for the calculation. (Taschauer, Geyer, et al. 2016)

$$m(\text{LPEI}) = \frac{m(pDNA)\ x\ molecular\ weight\ of\ one\ LPEI\ subunit\ x\ 9}{average\ molecular\ weight\ of\ a\ nucleotid} = \frac{m(pDNA)x43xN/P}{330}$$

Depending on the experiment, different amounts of pDNA were used (50ng, 100ng, 200ng, 400ng). After adding the polyplexes, the plate was incubated for 4 hours before adding 100µl of complete medium or treatment compounds to have in total 200µl of liquid in each well.

6.2.3 Reading

6.2.3.1 Luciferase

After 24 or 48 hours of incubation, the plate was taken out of the incubator. For measuring the RLU of the Firefly Luc and NanoLuc, the medium was removed with a pump and the cells were then washed with 200µl of PBS. After this, 30µl of PLB 1x (made through diluting PLB 5x with MilliQ water) were added into each well. Then the plate was taken to the shaker, where the cells were lysed by shaking for 30 minutes, speed 500rpm and room temperature. (If normalized with BCA assay: 20µl of the resulting lysate were transferred into a transparent 96-well plate.) After lysing the cells, the plate was taken to the plate reader (Infinite M200 Pro, Tecan) for the actual reading. For measuring the RLU by NanoLuc assay, the Nano-Glo Luciferase Assay Buffer and Furimazine substrate by Promega were mixed in the ratio 50:1 and kept under light protection. 40µl of this mixture were injected by the plate reader into each well.

For measuring the RLU by Firefly Luc assay, Luciferin had to be taken out of the -80°C freezer and kept under light protection. 100µl were injected into each well by the plate reader.

For measuring the RLU by Gaussia Luc assay, the cells were left unlysed and 20µl of the supernatant were transferred into another white plate and the signals were directly read from this. The substrate was made by mixing 5.07µl of CTZ aliquots, which were kept in the freezer

(-80°C) under light protection, with 3ml of PBS + 5mM sodium chloride. 50µl of this mixture were then injected into each well.

There are various possibilities to normalize the RLU values, two of them were used for the experiments in this thesis.

6.2.3.2 BCA

For normalization with BCA, 20µl of the lysate mentioned above were pipetted into a transparent plate. Then, BSA dilutions were made by mixing the following contents in the prescribed concentrations in order to normalize the light signals depending on the protein amount per well:

Sample ID	MilliQ-water [µl]	BSA 2mg/ml from Kit [µl]	**PLB 2x** [µl]	BSA (µg) / well
8A	-	50.0	50.0	20
7B	10.0	40.0	50.0	16
6C	20.0	30.0	50.0	12
5D	30.0	20.0	50.0	8
4E	40.0	10.0	50.0	4
3F	45.0	5.00	50.0	2
2G	50.0	-	50.0	-

Table 1: Modified BCA protocol (Mihalicokova and Müller 2018)

20µl of these dilutions were pipetted in triplicates starting with the lowest BSA concentration. Afterwards, 200µl of working reagent were pipetted to the dilutions and the lysate, shaken for 30 seconds (300rpm, room temperature) and incubated for 30 minutes at 37°C. As soon as the incubation time of the transparent plate was over, the plate was wrapped in aluminium foil for light protection and taken out of the incubator to cool down for 10 minutes. During this time, the plate was transported to the plate reader (Infinite M200 Pro, Tecan) and the absorption was measured at 562nm wavelength, 9nm bandwidth and 25 as a number of flashes.

The values of the BSA standard curve and lysate showed the protein amount in 20µl, whereas the RLU was measured from 10µl volume. This difference was compensated by the following calculations: The functional equation of the standard curve was used to calculate the protein amount in the total amount of 20µl lysate. The resulting value was then divided by 20 and

multiplied by 30 to know the protein amount of the whole 30µl of PLB 1x, which had been pipetted into the wells in the first place. Then the RLU of 10µl lysate was divided by 10 and also multiplied by 30. The resulting values were then divided by the corresponding values of protein amounts. The results were the normalized light units, meaning the light units per 1µg of protein.

6.2.3.3 Fluorescence

For normalizing with fluorescence, the CellTiter-Fluor™ Cell Viability Assay was conducted with the CellTiter-Fluor™ Cell Viability Assay-kit by Promega. This assay was a nonlytic, single-reagent fluorescence assay which measured the relative number of live cells in a culture population after experimental manipulation. The assay measured the protease activity within live cells and therefore served as a marker of cell viability. The live-cell protease activity was restricted to intact viable cells and was measured using a fluorogenic, cell-permeant peptide substrate called Glycyl-Phenylalanyl-Aminofluorocoumarin (GF-AFC). This substrate entered intact cells and was cleaved by a live-cell protease generating a fluorescent signal (Aminofluorocoumarin) proportional to the number of living cells. (Promega 2007-2017)

The kit used for the experiments in this thesis had the following contents: 50ml assay buffer and 5 x 10µl substrate. As both components are stored at -20°C, they had to be thawed in the water bath, vortexed and spun. Then 10µl of substrate were added to 2ml of buffer to create a 5x dilution. The resulting reagent was sensitive and had to be protected from light, therefore wrapped in aluminium foil and stored at 4°C until it was used. Before using the reagent, the plate with the transfected cells needed to be prepared as follows: Firstly, the supernatant was transferred into a transparent 96-well plate. Secondly, 100µl PBS were pipetted into each well of the white 96-well plate. Then 20µl of the CTF reagent were pipetted into each well. The plate was wrapped in aluminium foil and put into the incubator for 2.5 hours. During this period of time, the preparations for measuring the RLU were done. After the incubation, the total volume of 120µl (PBS + CTF reagent) was transferred into a black 96-well plate and taken to the plate reader to measure the fluorescence. Here, two types of settings could be chosen: optimal gain, or manual gain. With optimal gain, the plate reader measured the fluorescence of all wells and optimized the signals. The results were average values and therefore they did not vary much. With manual gain, the fluorescence of every single well was measured and shown without optimization. Additional settings: excitation wavelength = 380nm, emission wavelength = 505nm, excitation bandwidth = 9nm and emission bandwidth = 20nm.

After the fluorescence and the RLU were measured, the normalization was conducted by dividing the RLU values by the fluorescence values of the corresponding wells.

6.3 Screening of plant extracts for pathway activity

For screening plant extracts, HEK293T cells were used for all the experiments. The plant extracts were kindly provided by Univ.-Prof. Dr. Judith Maria Rollinger (University of Vienna) and previously examined by Sandra Milenkovic, a former diploma student. Two plant extracts showed an influence on the Wnt3a-pathway, these were plant extract 3 (PeuostRDMp_2) and plant extract 6 (BurafrCEtoAc_2). In this thesis, they were examined to find out, if they have a significant influence, not only the Wnt3a-pathway, but also on the Hedgehog- and Notch-pathways. Plant extract 8 (CapburHMp_2) was used as a control. For all these experiments the seeding, transfecting and reading procedure was conducted as described in section 6.2. The plant extracts were dissolved in DMSO.

6.3.1 Effect on Wnt3a-pathway

The experiment started on day 1 with seeding 10,000 HEK293T cells into a white 96-well plate with flat bottom, as described in section 6.2.1. On the second day, the medium was aspirated and 90µl of pure DMEM were added into each well, 80µl into the wells, where the plasmid concentration was 400ng. 10µl of TOP-NLuc polyplexes (200ng) were added into the wells as it is shown in Figure 23. As a control, the first triplicate was left untreated (UT) and only 10µl of HBG were added to them. Two co-transfections in two concentrations (200ng and 400ng) were added as positive controls. The components were TOP-NLuc with pAd (100ng + 100ng or 200ng + 200ng) and TOP-NLuc with pUC19 (100ng + 100ng or 200ng + 200ng). Four hours after the transfection, the compounds were mixed in the concentrations shown below and 100µl of these mixtures were added to the cells.

- For 200µg/ml: 8µl compound + 392µl complete DEMEM = 400µl →for 1 triplicate
- For 100µg/ml: 4µl compound + 396µl complete DEMEM = 400µl → for 1 triplicate
- For 50µg/ml: 2µl compound + 398µl complete DEMEM = 400µl → for 1 triplicate
- For 25µg/ml: 2µl compound + 798µl complete DEMEM = 800µl → for 1 triplicate

For the DMSO controls, the same pattern is used for mixing the concentrations 25, 50, 100 and 200µg/ml.

For UT, co-transfections and TOP-NLuc 200ng, 100µl of complete DMEM were added.

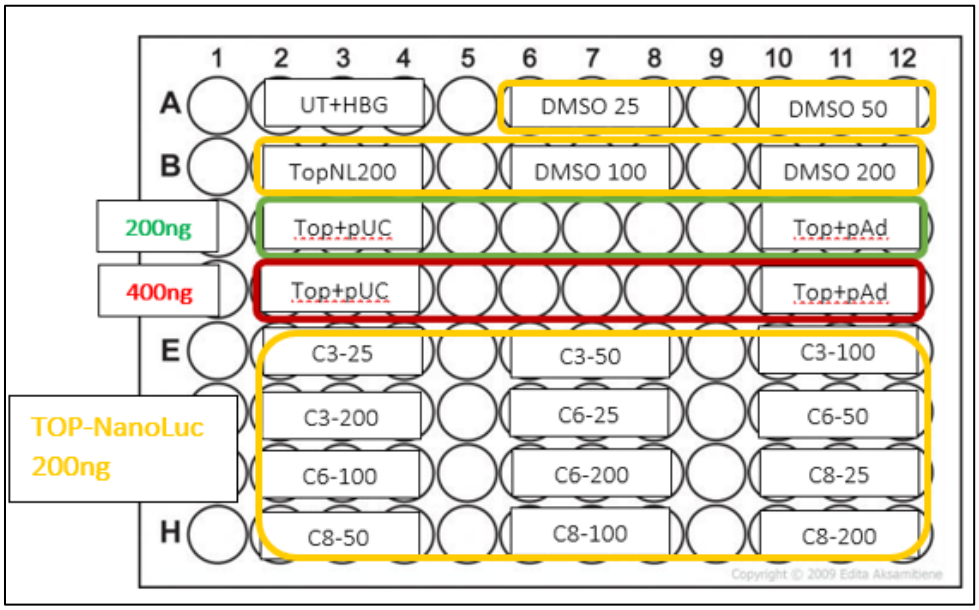


Figure 23: Transfection pattern for investigating compound 3 and 6 on Wnt3a-pathway with compound 8 as a control.

On day 3, the reading was performed as it is described in section 6.2.3. and the values were normalized with BCA. For generating the RLU, the Nano-Glo Luciferase Assay Buffer and Furimazine substrate by Promega were mixed in the ratio 50:1. 40µl of the resulting Nano-Glo reagent were injected by the plate reader into each well to create the light signals. This experiment was repeated 3 times.

6.3.2 Effect on Hedgehog- and Notch-pathways

Here, only compound 3 was tested on the Hedgehog- and Notch- pathways. The cell seeding on day 1 was performed as mentioned in section 6.2.1. On day 2, polyplexes were made to examine the Hedgehog-pathway with Firefly Luc and the Notch-pathway with Gaussia Luc. In fact, two experiments were combined to one because the incubation time after transfection is 48 hours for both luciferase assays. The plasmids used and the transfection pattern are shown in the following figure:

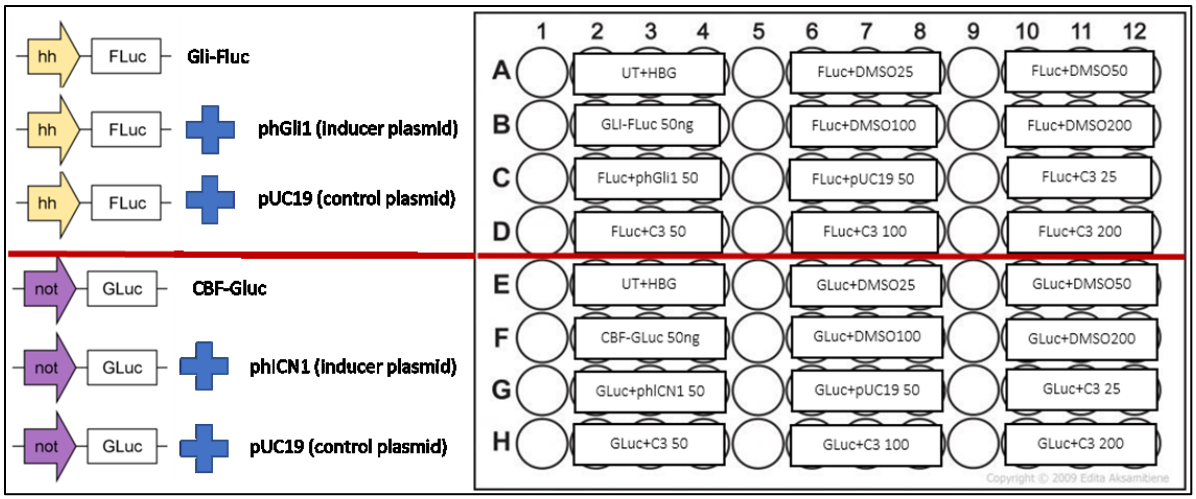


Figure 24: Plasmids and transfection pattern for investigating compound 3 on Hedgehog- and Notch-pathways.

Like in the previous experiment, where compounds 3 and 6 were examined on the Wnt3a-pathway in section 6.3.1., the same medium amounts and plasmid concentrations for UT + HBG, reporter plasmid + DMSO and reporter plasmid + compound were used. The only difference lied in the concentrations of the co-transfections. Here, only 25ng of reporter plasmid + 25ng of inductor or pUC19 were used, which makes a total plasmid amount of 50ng. Except for this difference, the same procedure was conducted like in section 6.3.1. for transfection and adding the compounds after four hours of incubation. Two experiments were performed differently, the first one was read after 24 hours and normalized with BCA assay. For the second experiment, after 48 hours of incubation the plate was prepared for the reading and normalization with CellTiter-Fluor™ Cell Viability assay, like it is shown in Figure 25.

For measuring the Firefly Luc activity, LBL was taken out of the -80°C freezer and thawed at room temperature under light protection. 100µl of the LBL were added by the injector into each well to generate the RLU.

For measuring Gaussia Luc activity, 5.07µl of CTZ aliquots were taken out of the -80°C and added to 3ml of PBS + 5mM sodium chloride. 50µl of the resulting mixture were injected to each well to generate a light signal from the luciferase enzymes, which were secreted in the supernatant.

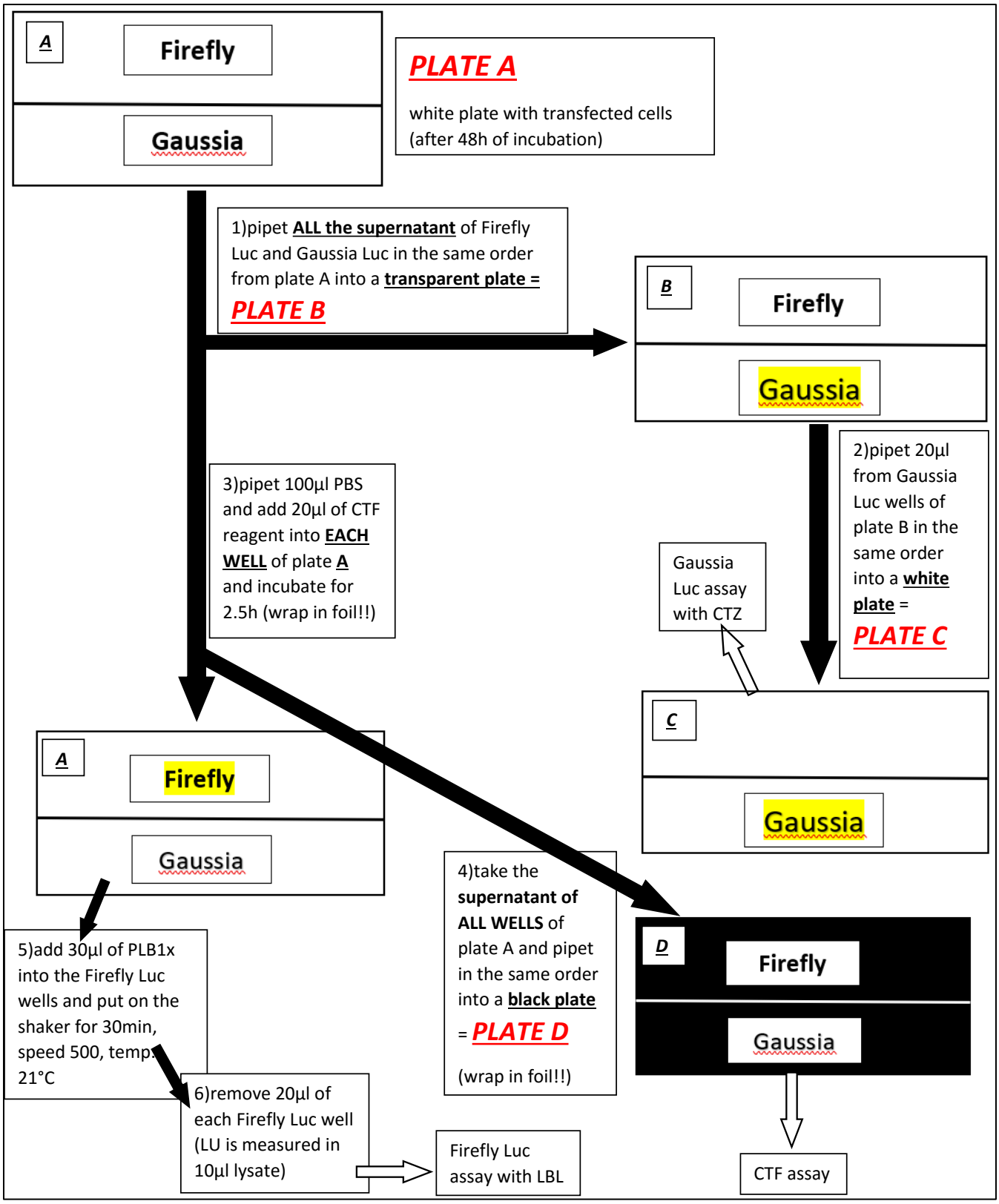


Figure 25: Reading the screening experiment for investigating compound 3 on Hedgehog- and Notch-pathways.

6.4 Luciferase reporters for pathway activity

6.4.1 Cross reactivity between luciferase substrates

As mentioned in the introduction, the signaling pathways and their luciferase assays needed to be investigated more deeply. The experiments were supposed to examine if there is a cross reactivity between the substrates Furimazine and Coelenterazine. The idea behind this experiment was to find out, whether NanoLuc gives signals with the substrate of Gaussia Luc and vice versa. Therefore, two plates were transfected, each of them had two identical sets of transfections. Two sets of transfections per plate were important because one set had to be read with Coelenterazine and one set had to be read with Furimazine to show if a NanoLuc assay would generate signals with Coelenterazine and if a Gaussia Luc assay would generate signals with Furimazine. If this was verified, it would mean that there is a cross reactivity between the two luciferases and that the results of experiments, which are carried out with both luciferase assays, are affected by this cross reactivity. The expected locations of the luciferase enzymes shown in the following figure were an important indication for planning and implementing the experiment strategies.

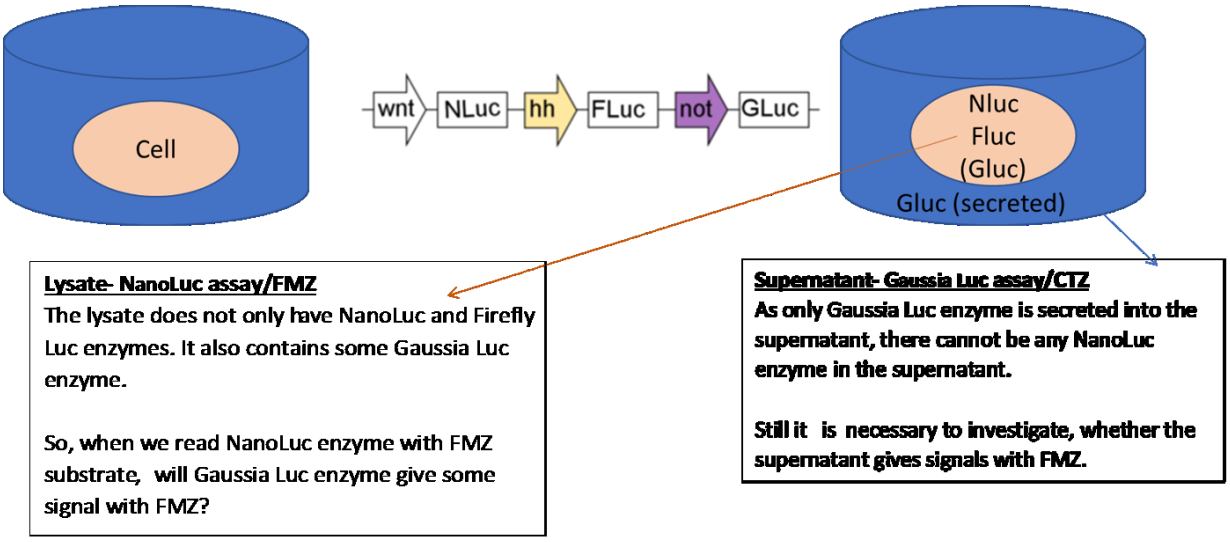


Figure 26: Expected secretion location of NanoLuc and Gaussia Luc enzymes after transfection with 3P-Luc plasmid.

Therefore, HEK293T cells were seeded into a white 96-well plate (10,000 cells per well in triplicates) on day 1. On day 2, the polyplexing and transfection were conducted. The transfection pattern looked like this:

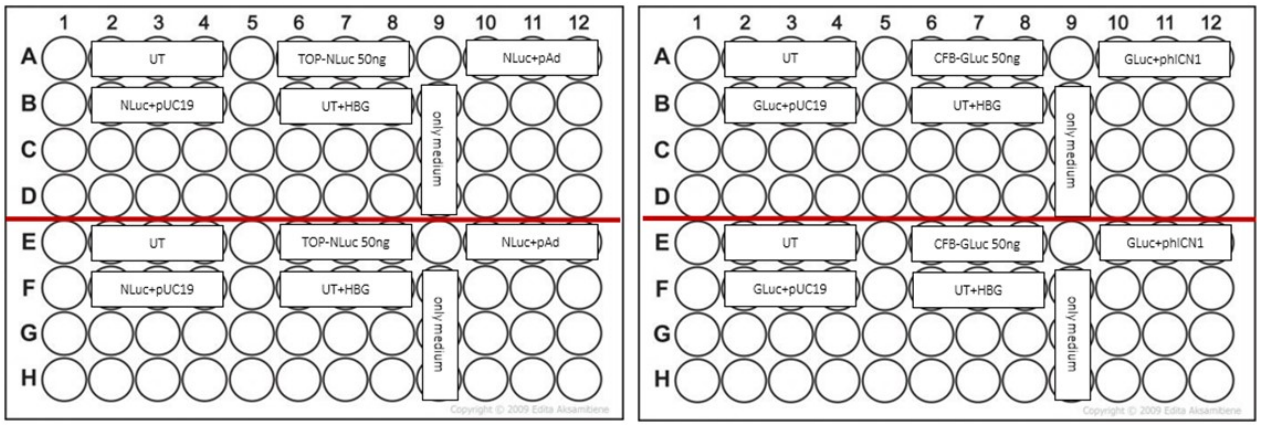


Figure 27: NanoLuc plate and Gaussia Luc plate for investigating the cross reactivity between Furimazine and Coelenterazine.

UT = untreated; UT + HBG = untreated + HBG. Both plates show a double set of transfections.

50ng TOP-NLuc plasmid were transfected in all triplicates except for the untreated wells. For co-transfections as controls, 25ng pAd or pUC19 were used together with 50ng TOP-NLuc. Also, 50ng CBF-GLuc were transfected in all the triplicates except for untreated controls. 25ng of phGli1 or pUC19 were co-transfected with 50ng CBF-GLuc as controls. To measure the background signal, one triplicate of each set of transfections was only treated with the same media and reagents and read with the same parameters without any cells.

After the transfection was done, the plates were incubated for 4 hours at 37°C and then the wells are filled up to 200µl of complete medium.

After 24 hours of incubation, the plates were read. Here, the workflow differed from the general workflow for the reading of the screening experiments. The resulting RLU signals were normalized with CellTiter-Fluor™ Cell Viability assay and the experiment was repeated twice.

6.4.1.1 Detailed workflow

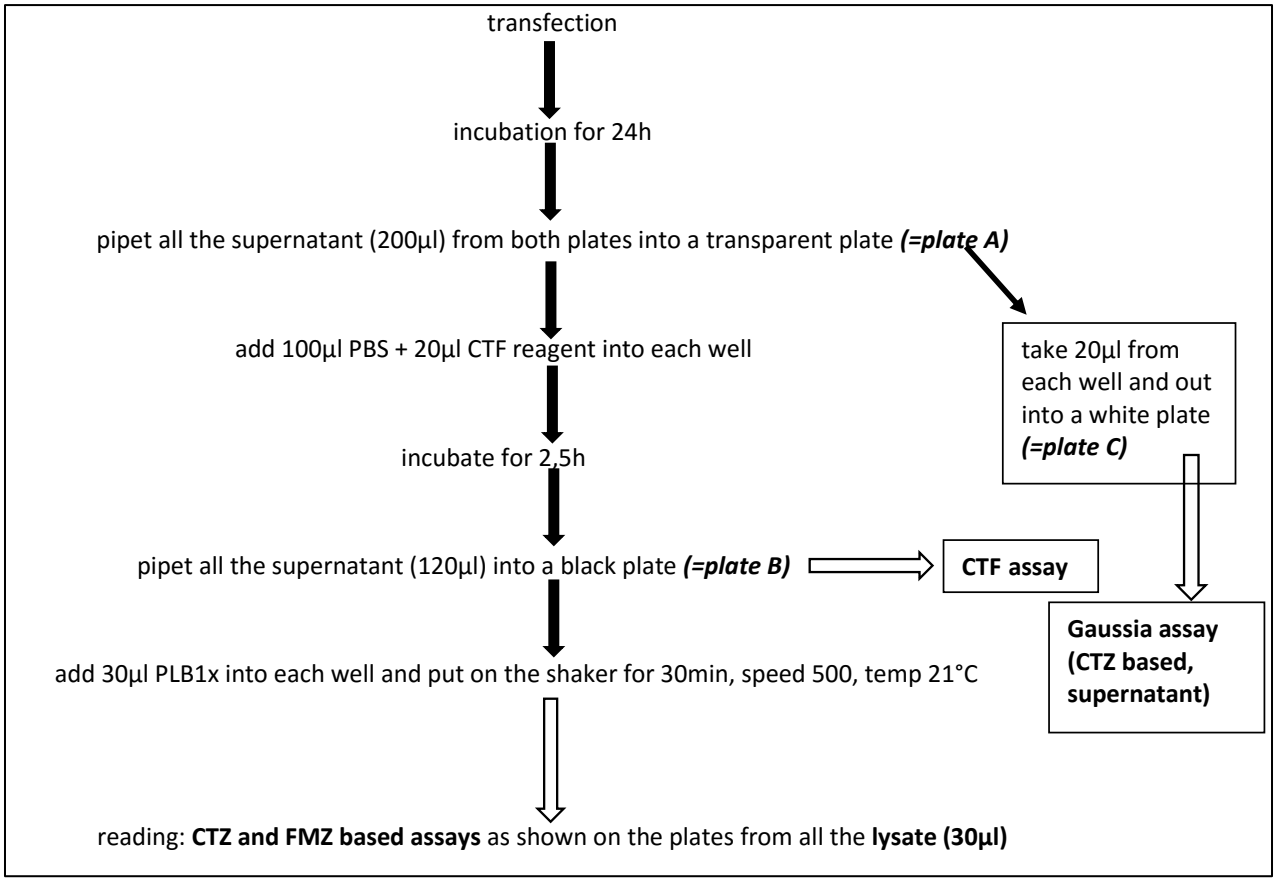


Figure 28: Preparation steps for reading the cross reactivity between Furimazine and Coelenterazine.

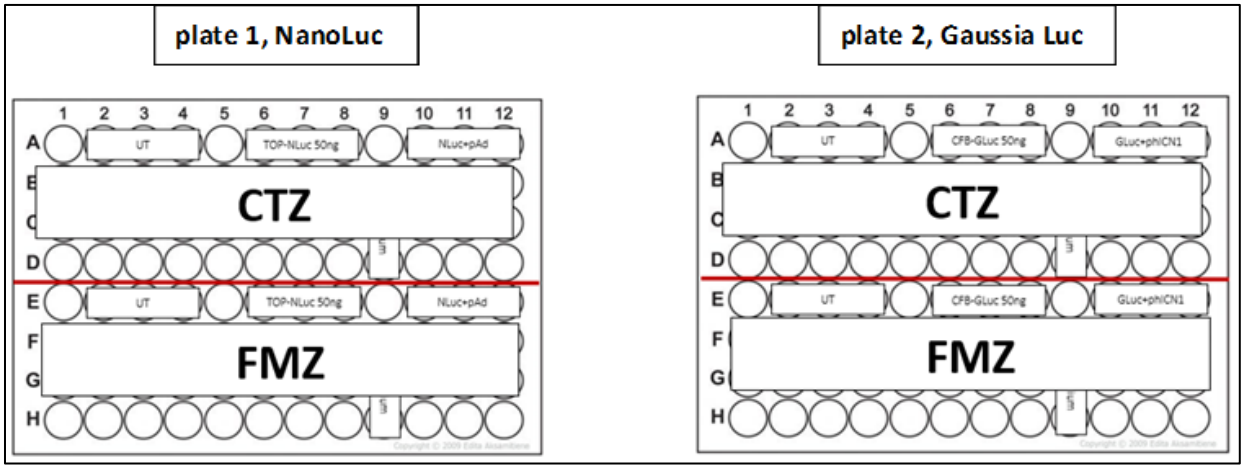


Figure 29: Reading pattern for both plates.

The first set of transfections is read with Coelenterazine, the second set is read with Furimazine.

6.4.2 Multicistronic vector 3P-Luc for investigating crosstalk between signaling pathways

To gain a deeper understanding for all three signaling pathways (NanoLuc, Firefly Luc, Gaussia Luc), it was necessary to investigate whether there is a crosstalk between the signaling pathways and if there is a cross induction between the inducers. Therefore, the multicistronic vector 3P-Luc was used. This figure shows the components of the 3P-Luc plasmid:

Figure 30: 3P-Luc plasmid.
Components: promoter for Wnt3a-pathway (TOP-NLuc), promoter for Hedgehog-pathway (GLI-FLuc), promoter for Notch-pathway (CBF-GLuc).

Two types of experiments needed to be done. The first experiment (A) was designed in a way to show whether the inducer for NanoLuc promoter also induces the other two promoters (GLI-FLuc and CBF-GLuc). The second experiment (B) was a combination of two experiments to show the induction of phGli1 and phICN1 of the other two, non-according promoters.

The reason for separating the experiments the different incubation time after transfection. For NanoLuc, the incubation time after transfection is 24 hours, whereas for Gaussia Luc and Firefly Luc, 48 hours of incubation are prescribed.

The following figure shows the components of the 3P-Luc plasmid and the experiment design with which the experiments were planned to investigate the cross induction:

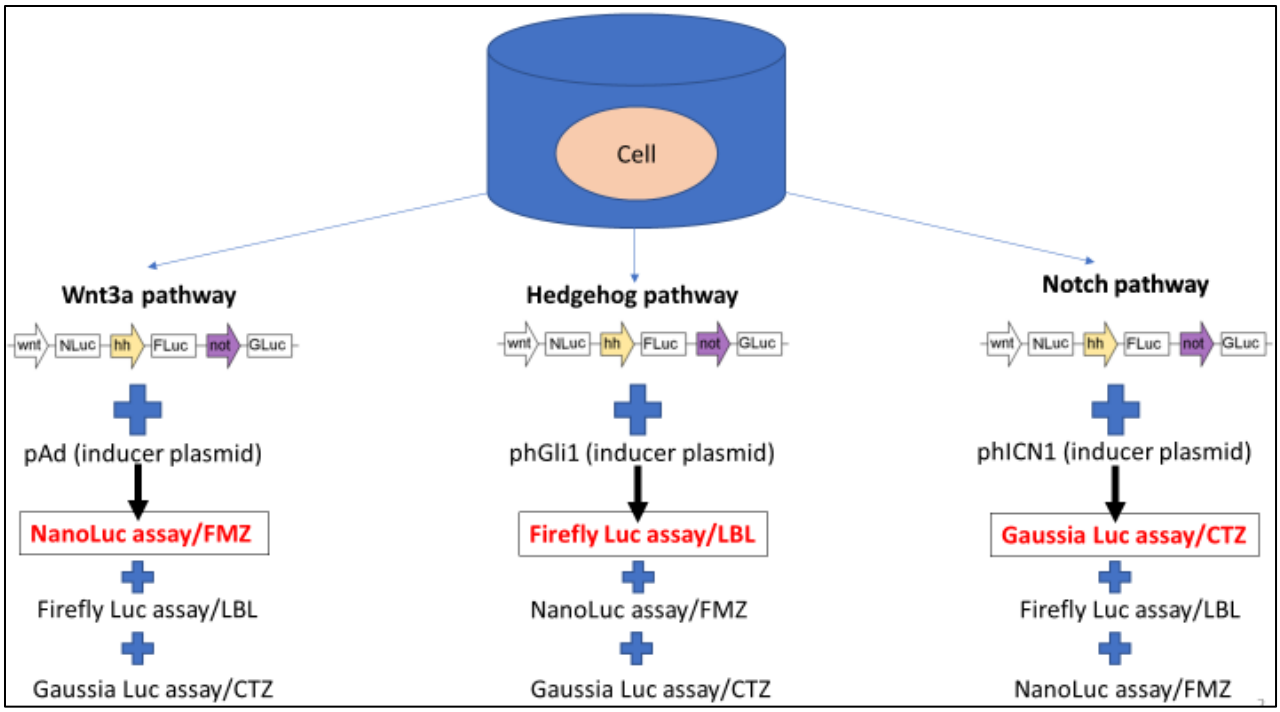


Figure 31: Concept of investigating the cross induction between the signaling pathways with 3P-Luc plasmid.

For all three experiments, 10,000 cells were seeded into two white 96-well plates (plate 1 = NanoLuc assay plate; plate 2 = Firefly Luc assay plate) on day 1. The detailed procedure for this is explained in section 6.2.1. Regarding the transfection pattern on day 2, it differed according to whether experiment A or B was performed.

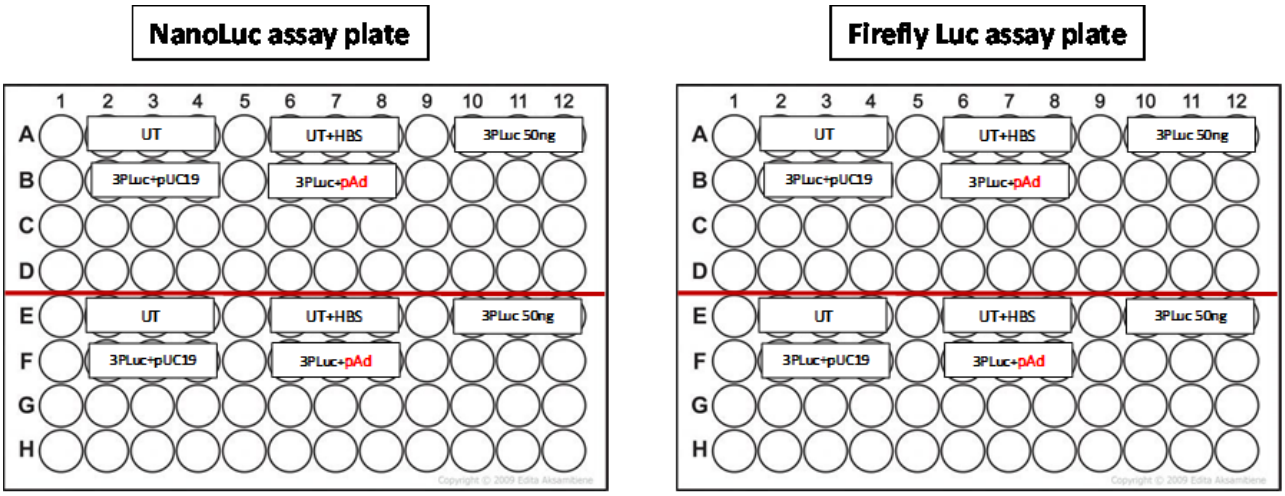


Figure 32: Transfection pattern for investigating the induction of GLI-FLuc and CBF-GLuc by pAd (Experiment A).

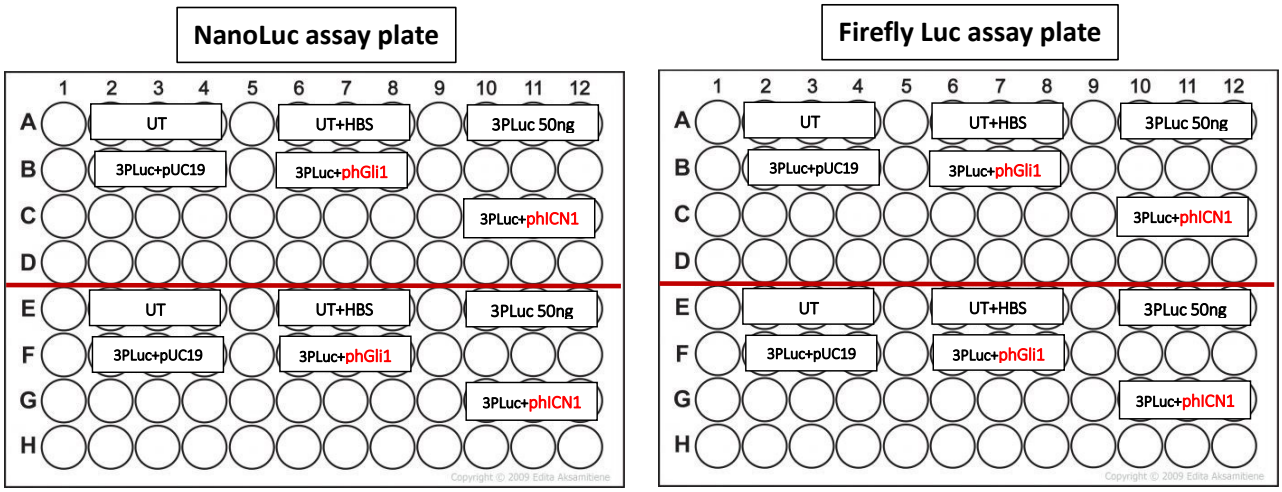


Figure 33: Transfection pattern for investigating the induction of TOP-NLuc and CBF-GLuc/ GLI-FLuc by phGli1 and phICN1 (Experiment B).

Experiment A and B consisted of two plates with four identical sets of transfections. Each of them had one triplicate with untreated cells as a control, one triplicate with untreated cells and HBS as a control and one triplicate with 50ng of 3P-Luc. The significant difference between experiments A and B was the co-transfections. In experiment A, the co-transfection was a combination of 50ng of 3P-Luc and 25ng of pAd in all four sets of transfections in addition to the co-transfection of 50ng 3P-Luc and 25ng pUC19 as a control. In experiment B, three co-transfections were needed. The first one was the co-transfection with 50ng of 3P-Luc and 25ng of phGli1 to show, whether an induction of TOP-NLuc and CBF-GLuc will occur. The second co-transfection contained 50ng of 3P-Luc and 25ng of phICN1, where the change in signal of TOP-NLuc and GLI-FLuc was on focus. The last co-transfection was a control, consisting of 50ng 3P-Luc and 25ng stuffer plasmid pUC19. Four hours after the transfection, complete medium was added to the wells to make 200µl of total volume in each well. After 24 hours, the plates were taken out of the incubator and were prepared for the reading.

Figure 34 shows the detailed preparation steps for both, experiments A and B. They were carried out separately and the signals were normalized with CellTiter™-Fluor Cell Viability assay. Both experiments were performed twice.

6.4.2.1 Detailed workflow

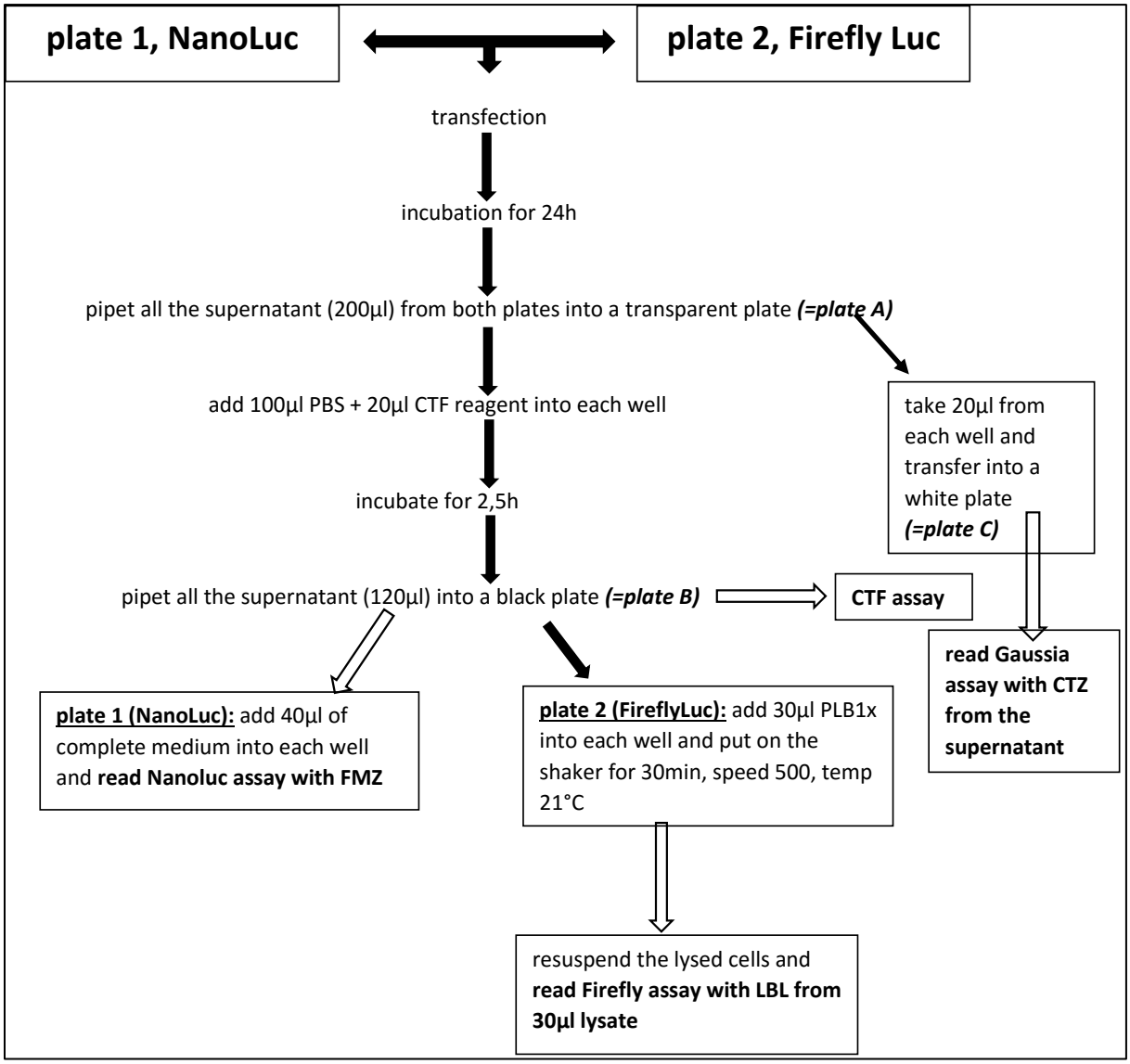


Figure 34: Preparation steps for reading the experiments A and B.

Figure 34 shows that four different assays were performed to examine the cross induction between the pathways. The first assay was the Gaussia Luc assay with Coelenterazine as a substrate, measured from the supernatant. The second assay was the NanoLuc assay, using Furimazine as a substrate. This assay was not read from the lysate, although NanoLuc is synthesized and remains cytoplasmic. The reason for this is that it was necessary to stick to the same workflow prescribed in the paper, for which these experiments were needed. Therefore, 40µl of complete medium were added to the wells of plate 1, from which the NanoLuc assay was performed. The third assay was the Firefly Luc assay, which was read with LBL from the lysate. As it is seen in the figure, reading the fluorescence from the black plate was performed to normalize the RLU, which was the fourth assay for this experiment.

6.5 Histological methods and characterization of tumor tissue

Histological experiments were necessary in order to localize the CD49f proteins, which are over expressed on the surface of cancer cells. By localizing the CD49f on cancerous murine lung tissue, further research could be done on the topic, whether a widely spread surface protein like the CD49f can be used to improve the transfection efficiency of polyplexes carrying nucleic acids for cancer treatment. Therefore, mice were intravenously injected with triple-negative breast cancer cells of the 4T1 cell line, resulting in the formation of lung cancer. Then, polyplexes carrying the reporter plasmids for Firefly Luc were applied through intratracheal aerosolization, while the LPEI of the polyplexes was linked to a CD49f peptide by a PEG chain. The aim of this research is to see, whether the linking CD49f peptide has an increasing impact on the nucleic acid delivery, as this surface protein is over expressed on the generated cancerous lung tissue. Measuring the Firefly Luc signals via bioluminescence imaging shows the efficiency of the transfection, which is proved to be significantly increased by linking the CD49f peptides to the LPEI of the polyplexes. (Taschauer, Polzer, et al. 2019)

6.5.1 Hematoxylin and Eosin staining

For staining the tissue with Hematoxylin and Eosin, a number of preparation steps were necessary. The following figure shows the three basic steps after prewarming the slides for two hours at 55°C in the drying chamber: tissue hydration, staining, tissue dehydration.

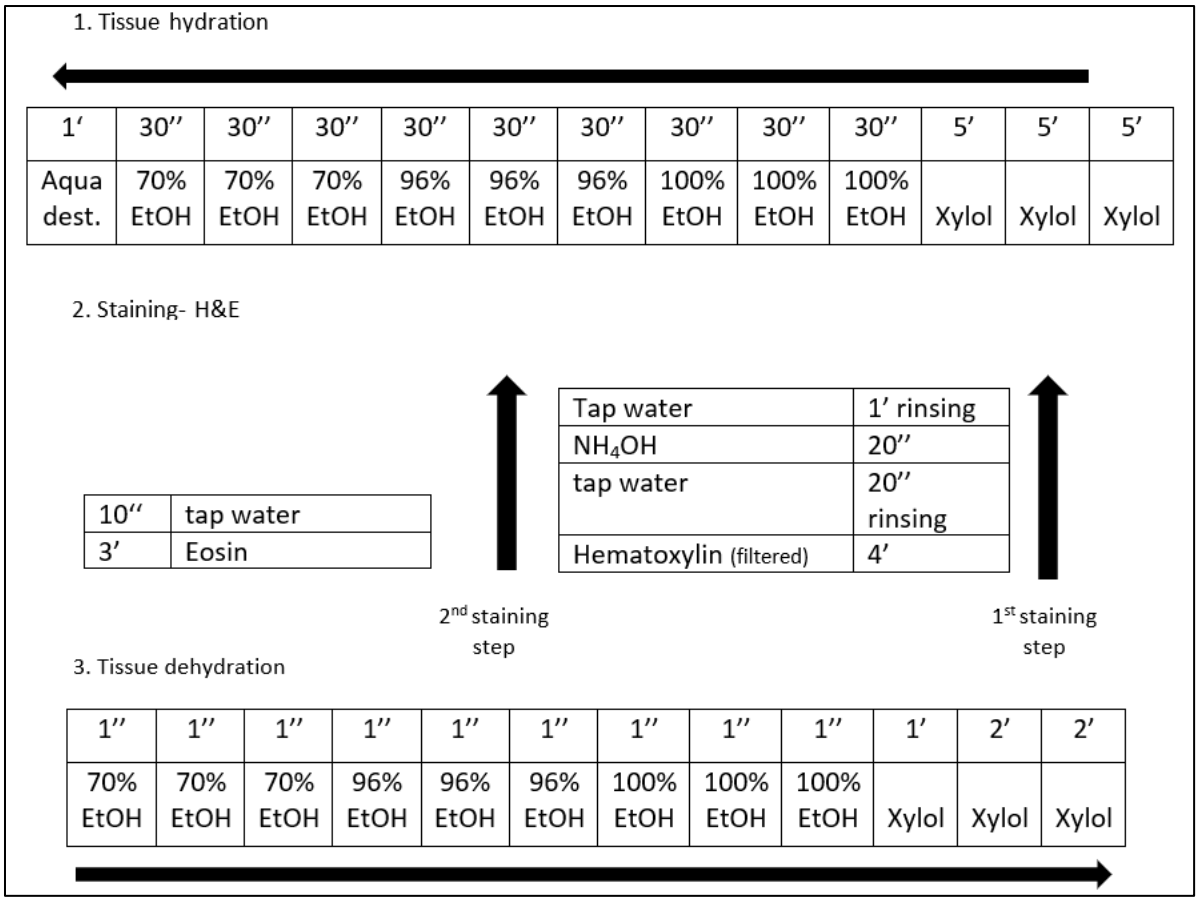


Figure 35: General workflow for Hematoxylin and Eosin staining. (Schönhofer and Pichler 2018)

The tissue hydration was done by passing the slides through liquids with successively increasing hydrophilicity. The first station was Xylene, the second one is a row of ethanol with a gradually decreasing percentage. The last station was distilled water, where the slides were incubated for 1 minute. As soon as the hydration step was done, the actual staining was performed. For this step, Hematoxylin needed to be filtered before every staining to remove crystals which are formed through oxidation of Hematoxylin to Hematein. Depending on the tissue, the incubation time in Hematoxylin differed. In this case, lung tissue was stained, where 4 minutes would have made the staining too intense. Therefore, the slides were incubated in Hematoxylin for only 2 minutes and 30 seconds. The next step was rinsing the slides in tap water for 20 seconds and a short incubation for further 20 seconds in a dilution of Ammonium in distilled water. After 1 minute of rinsing in tap water, the slides were incubated in Eosin for 2 minutes and 30 seconds. Again, the prescribed time of 3 minutes would have generated an overstaining and therefore it was shortened by half a minute. After rinsing with tap water, the dehydration step started with the lowest percentage of ethanol, which increased successively. The last step was the incubation in Xylene, after which a coverslip was stuck on the slides by using Entellan.

6.5.2 Immunohistochemistry for CD49f expression

As soon as the tumor region was found through the Hematoxylin an Eosin staining, more sections of the same region were made and immunohistochemistry was performed to specifically stain the tumor cells through the CD49f, which is expressed on the surface of the cells. For this intention, six sections of the tissue to be investigated were needed. As a negative control, six sections of a non-tumorous lung tissue were used and stained in parallel with the tumorous tissue, so in total, 6 slides with two sections each were used for one experiment. Each of the six sections had an important purpose for the experiment, as it can be seen in Figure 36.

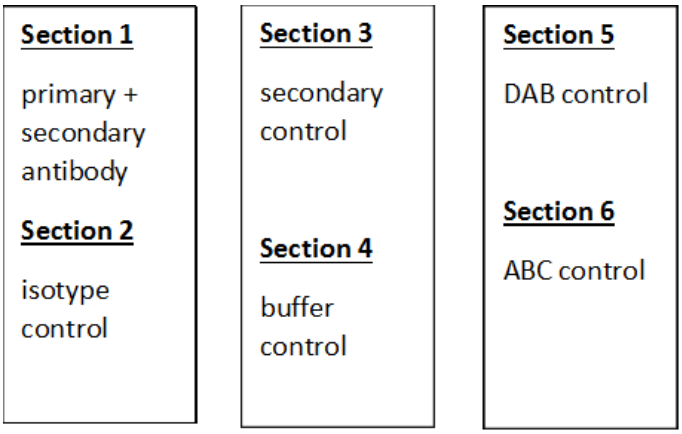


Figure 36: Schematic view of the slides used for IHC.

The CD49f targeted antibody staining started in day 1 with prewarming the sections at 55°C in the drying chamber for 2 hours. Then, the sections were deparaffinized with Xylene, ethanol and distilled water, as it was done for the Hematoxylin and Eosin staining in section 6.5.1 with the only difference that the sections were kept in the distilled water for only 30 seconds. The next step was putting the slides back to back into the HIER jar, which is filled with TRIS-EDTA buffer (+0,05% Tween 20, pH 9). The jar was boiled for 30 minutes in the silicon oil bath at 131°C and then left at room temperature to cool down for 30 minutes. After washing the slides 3 times in PBS washing buffer, the PAP pen was used to create a hydrophobic area around the tissues to avoid leaks when pipetting the reagents to the tissues. The slides were put into the staining chamber and the diluted blocking serum (1 drop of blocking serum of the Vectastain® ABC kit + 3333µl of 2% BSA/PBS) was added to the sections, followed by an incubation time of 30 minutes. The blocking serum was drained and the slides were washed 3 times with PBS washing buffer. Afterwards, the endogenous Avidin and Biotin were blocked by adding 1 – 2 drops of Avidin solution (BIORAD) to each section and after 15 minutes of incubation and the washing step, the same amount of Biotin solution (BIORAD) was added to the tissues, followed

by another 15 minutes of incubation. The last step before incubating for 24 hours at +4°C was draining the Biotin followed by washing 3 times in PBS washing buffer and adding the primary antibody, which is a Rabbit Monoclonal antibody to Integrin α6 (= CD49f), to section number 1 (Figure 36). The Rabbit IgG Isotype was pipetted to section number 2 (= isotype control, Figure 36) in the same concentration as the primary antibody (1:500). The other sections were treated with 2% BSA/PBS buffer.

On the next day, the primary antibody and the isotype were drained. After three washing steps with PBS washing buffer, the slides were incubated in 0.3% H_2O_2 for 15 minutes to block endogenous peroxidase. After washing the slides in PBS washing buffer, the secondary antibody, a Goat-Anti-Rabbit Biotinylated Secondary Antibody, was pipetted on the sections number 1, 2 and 3 (Figure 36), the other sections were treated with 2% BSA/PBS buffer. After 30 minutes, the secondary antibody and buffer were drained and the prepared ABC reagent from the Vectastain® ABC kit was used to generate the Avidin-Biotin-complex by incubating all the sections except for section number 5 (= DAB control, Figure 36) with the ABC reagent. Then, the color is generated (Figure 37) by adding DAB to all the sections, except for section number 6 (= ABC control, Figure 36). DAB was left on the tissues for approximately 1.5 minutes until the staining was intense enough. This could be checked under the CellSens Standard (Olympus) microscope. Whenever the staining was well to observe under the microscope, the slides were put into a jar filled with distilled water to stop the color generating reaction between DAB and the Avidin-Biotin-complex. Counterstaining was performed by incubating the slides in Hematoxylin for approximately 3 minutes. The slides were rinsed with tap water and coverslips were stuck on them with Entellan.

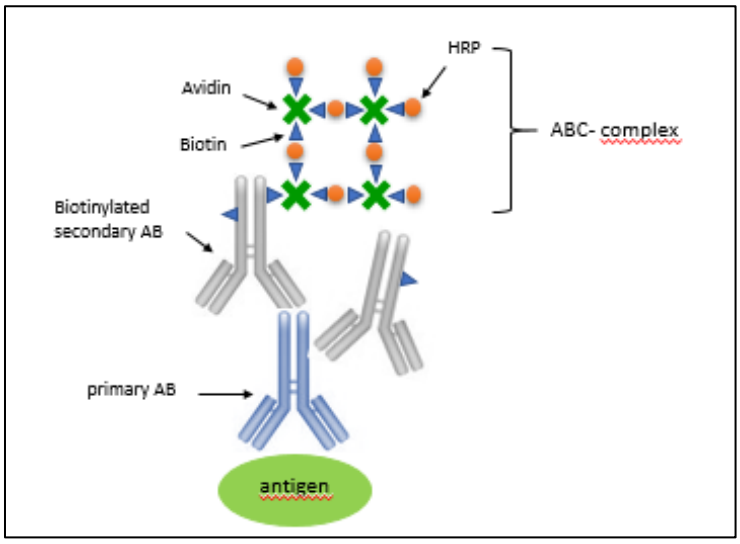


Figure 37: Detection system using Avidin and Biotin complex. (Figure reproduced from (Pichler 2019)

7 Results

7.1 Effect of plant extracts on Wnt3a-pathway

Based on initial work, plant extracts number 3, 6 and 8 were selected for investigating their effect on the Wnt3a-pathway (and further on the Hedgehog- and Notch-pathways). To see the effect on the Wnt3a-pathway, compound 3 or 6 or 8 was added to TOP-NLuc transfected cells at different concentrations and NanoLuc assay was performed as a read out of Wnt3a activity and compared with corresponding solvent control.

As it can be seen in Figure 38, the Wnt3a-pathway is inhibited by compound 3 and 6, as compound 3 and 6 show a lower normalized RLU in comparison to the respective DMSO control. Surprisingly, compound 6 shows a significant inhibition as of a lower concentration (100µg/ml) than compound 3. The U-test and the two-tailed t-test (P = 0.01) showed a statistically significant difference between the respective DMSO control and the compounds 3 and 6 at concentration of 200µl/ml for compound 3 and 100µl/ml and 200µl/ml for compound 6. As compound 8 is used as a negative control, the normalized RLU signals are similar to the solvent control and no signal decrease is taking place.

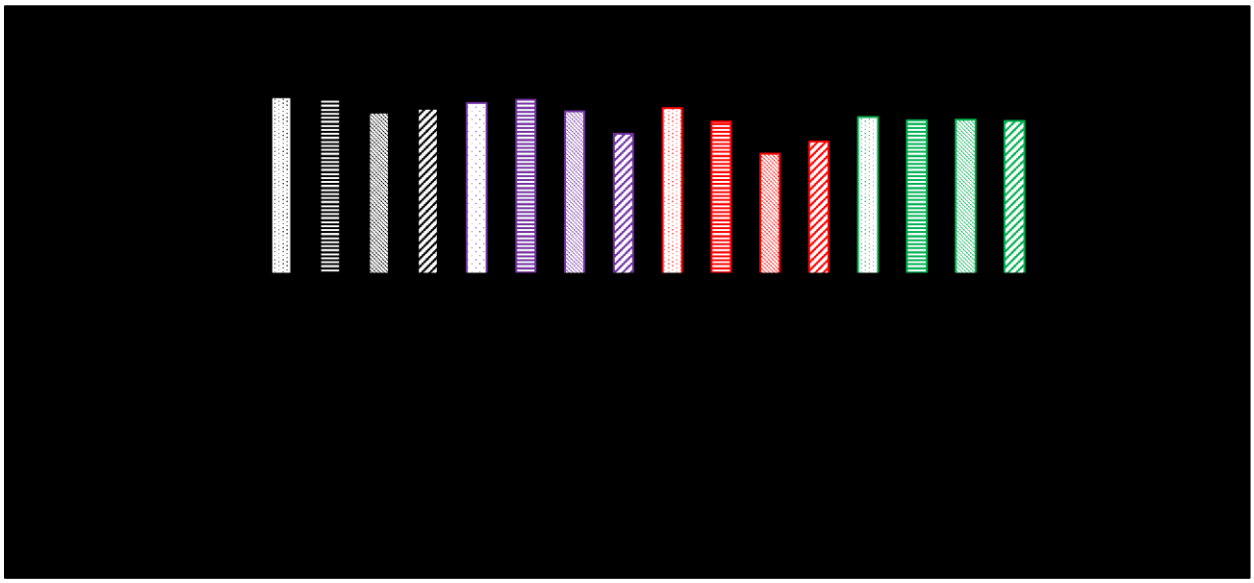


Figure 38: Effect of compounds 3, 6 and 8 on Wnt3a pathway.
UT + HBG = untreated + HBG; C3/ C6/ C8= compound 3/ compound 6/ compound 8; SLVNT CTRL = solvent control (DMSO); CTRL/INDUCER = pUC (stuffer inducer plasmid)/ pAd (inducer plasmid); shown data is the average of 3 experiments. HEK293T cells were seeded (10,000 per well) and transfected with TOP-NLuc 200ng or a co-transfection with TOP-NLuc 100ng + inducer plasmid pAd 100ng or + stuffer inducer plasmid pUC19 100ng as a control was performed on the next day. Four hours after transfection, the cells were treated with either DMSO or compound 3 and 6 in different concentrations

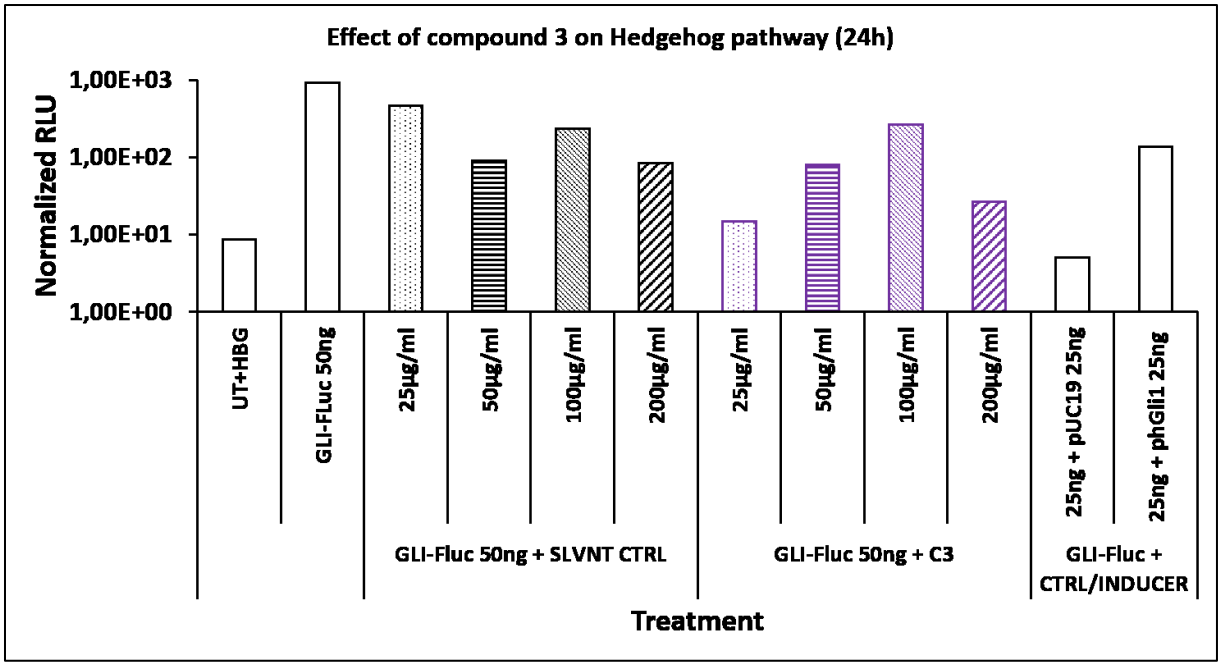
(25, 50, 100, 200µg/ml). Compound 8 was used in the same concentrations as a negative control. Reading was performed after 24 hours and the data was normalized with BCA assay. Compound 3 (200µl/ml) and compound 6 (100 and 200µl/ml) show a significant decrease in Wnt3a activity (two-tailed t-test; P = 0.01; N = 9).

7.2 Effect of plant extracts on Hedgehog- and Notch- pathways

Further, the extracts were examined on the Hedgehog- and Notch- pathways. The goal was to see, if there is a significant effect on the pathways, as it was previously proved for the Wnt3a-pathway. As both, compound 3 and 6, have shown a significant inhibition, compound 3 was picked to carry out the experiments for investigating the effect on the Hedgehog- and Notch-pathways after treating for 24h and 48h.

The following figures show the results of two single experiments. GLI-FLuc + DMSO controls are supposed to be compared to the GLI-FLuc + compound 3 in the corresponding concentrations. The comparison between 50ng of GLI-FLuc + compound 3 to the DMSO controls show that there is no significant impact on the Hedgehog-pathway. This can be due to the very little amount of plasmids used. The insufficient effect of the plasmids due to the little plasmid amount is clearly seen in the co-transfection of GLI-FLuc with the inductor plasmid phGli1. Here, only 25ng of each plasmid were used, which resulted in a poor induction of GLI-FLuc.

(A)



(B)

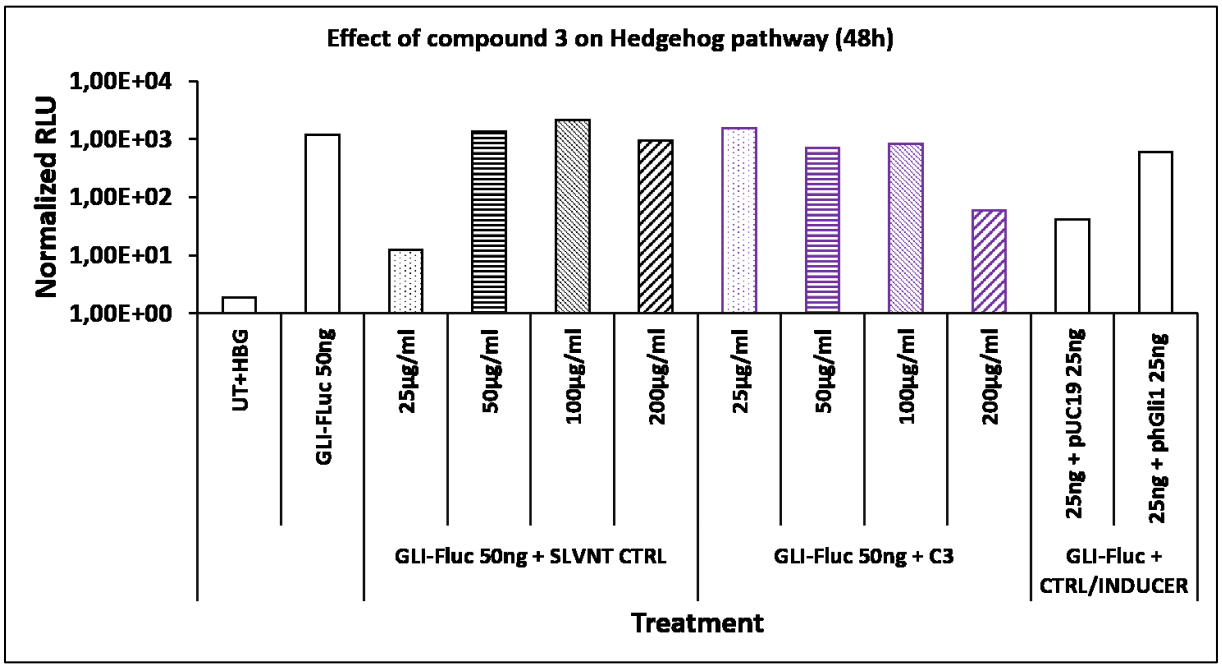
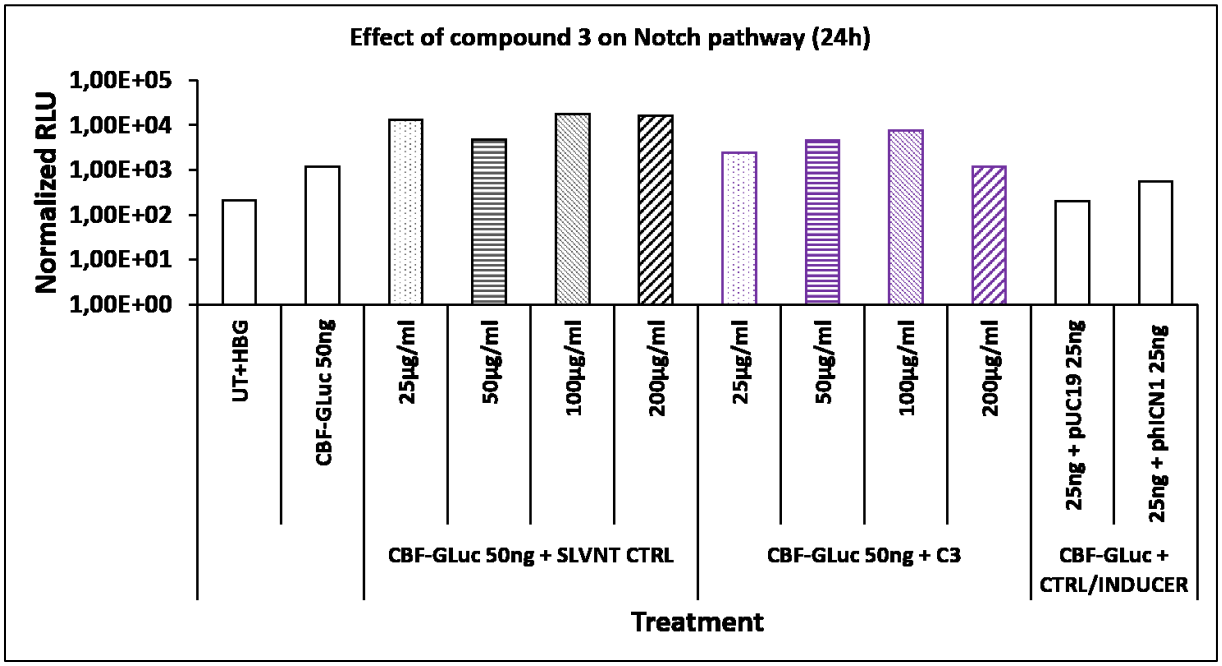


Figure 39: Effect of compound 3 on Hedgehog pathway.

UT + HBG = untreated + HBG; C3 = compound 3; SLVNT CTRL = solvent control (DMSO); CTRL/INDUCER = pUC (stuffer inducer plasmid)/ phGli1 (inducer plasmid); shown data in each diagram is the result of one experiment. HEK293T cells were seeded (10,000 per well) and transfected with GLI-FLuc 50ng or a co-transfection with GLI-FLuc 25ng + inducer plasmid phGli1 25ng or + stuffer inducer plasmid pUC19 25ng as a control was performed on the next day. Four hours after transfection, the treatment with either DMSO or compound 3 was done in the concentrations 25, 50, 100 and 200µg/ml. Reading was performed (A) after 24 hours and normalized with BCA assay. (B) after 48 hours and normalized with CellTiter-Fluor™ Cell Viability assay.

Considering the following graphs, higher signals can be seen in the Gaussia Luc assays than in previous Firefly Luc assays. Generally speaking, Gaussia Luc assays give higher signals than Firefly Luc assay, which leads to the assumption that even if the plasmids for the Gaussia Luc assay are used in a small amount, the results are more reliable and better to interpret. The fact that the signals are higher in the Gaussia Luc assays makes the comparison between the values with CBF-GLuc 50ng + DMSO and CBF-GLuc 50ng + compound 3 in the corresponding concentration easier than in the previous experiment. Here, a reduction in signal when treated with compound 3 can be noticed, but the decrease in pathway activity is statistically not significant. Still, the plasmid amount of 50ng or 25ng in the co-transfections makes it difficult to make a sure conclusion about the significance of the impact on the Notch-pathway.

(A)



(B)

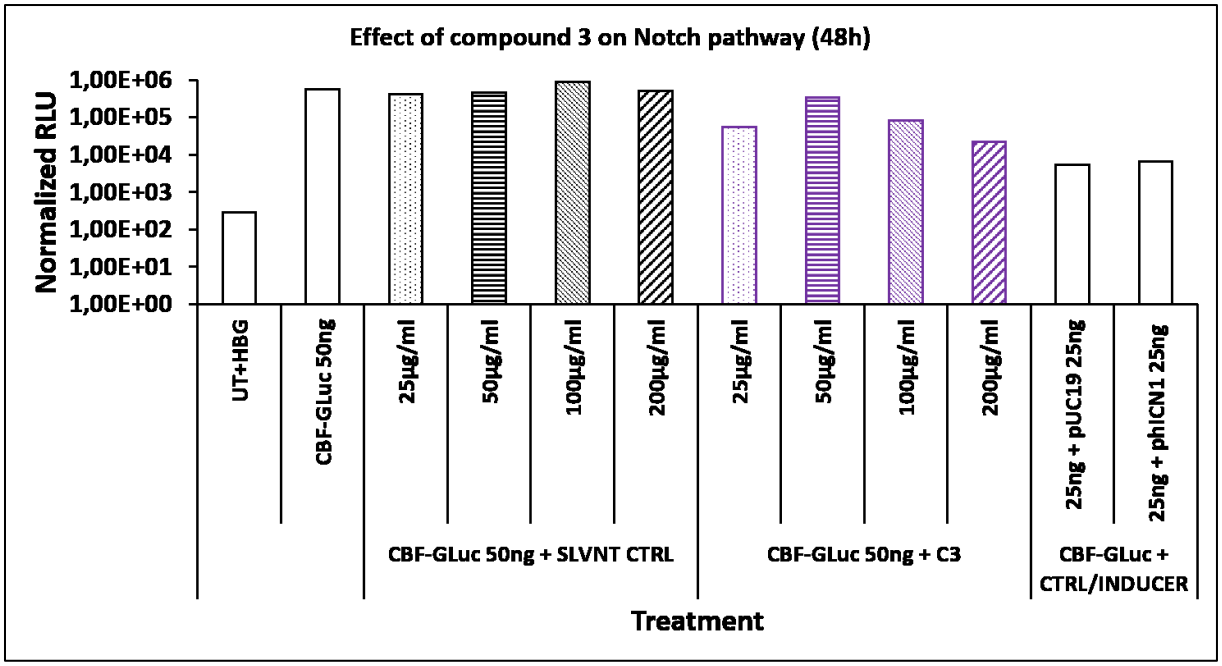


Figure 40: Effect of compound 3 on Notch pathway.

UT + HBG = untreated + HBG; C3 = compound 3; SLVNT CTRL = solvent control (DMSO); CTRL/INDUCER = pUC (stuffer inducer plasmid)/ pICN1 (inducer plasmid); shown data in each diagram is the result of one experiment. HEK293T cells were seeded (10,000 per well) and transfected with CBF-GLuc 50ng or a co-transfection with CBF-GLuc 25ng + inducer plasmid phICN1 25ng or + stuffer inducer plasmid pUC19 25ng as a control was performed on the next day. Four hours after transfection, the treatment with either DMSO or compound 3 was done in the concentrations 25, 50, 100 and 200µg/ml. Reading was performed (A) after 24 hours and normalized with BCA assay. (B) after 48 hours and normalized with CellTiter-Fluor™ Cell Viability assay.

In conclusion, it could not be proved that compound 3 at a concentration of 200µg/ml has an inhibiting effect on neither Hedgehog-, nor Notch- pathway. Due to the fact that these experiments need some more optimization and repetition (as they are single experiments), there is a lot more to explore on this topic. Still, the results of these experiments can be taken as a good starting point for more research.

7.3 Cross reactivity between luciferase substrates

When Gaussia Luc and NanoLuc enzymes are present within the same cell, there is the possibility of a crosstalk between Furimazine and Coelenterazine. The objective was to answer two questions: Can the NanoLuc enzyme use Coelenterazine as substrate, although Furimazine is the main substrate for NanoLuc? Can Gaussia Luc enzyme use Furimazine as substrate, although Coelenterazine is the main substrate for Gaussia Luc? For answering these questions, the experiments were planned and performed as it is explained in section 6.4.1.

In the following figure (Figure 41) NanoLuc based plasmids show the signal level of the untreated cells, which means that there is no NanoLuc enzyme in the supernatant that could use Coelenterazine. As expected, the Gaussia Luc based plasmids show a high signal with Coelenterazine, which verifies the assumption that Gaussia Luc enzymes are mainly secreted into the supernatant and use Coelenterazine as a substrate.

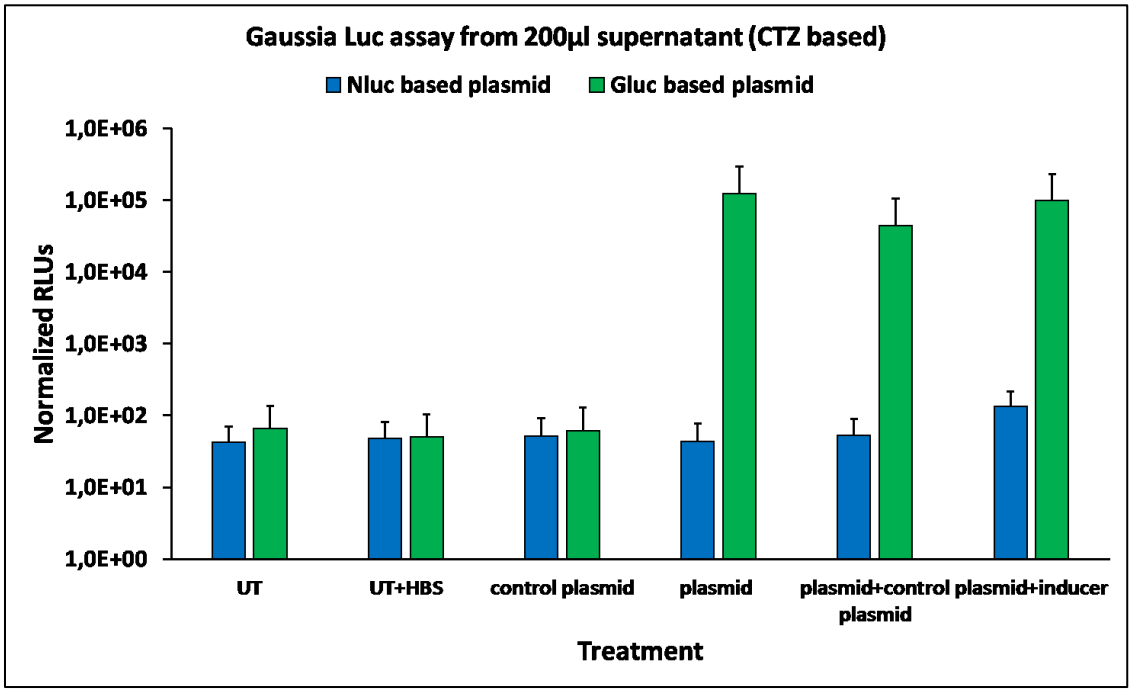


Figure 41: Normalized RLUs from 200µl supernatant by a Coelenterazine based Gaussia Luc assay.

UT = untreated; UT + HBS = untreated + HBS; control plasmid = pUC19; plasmid = TOP-NLuc or CBF-GLuc; plasmid + control plasmid = TOP-NLuc or CBF-GLuc + pUC19; plasmid + inducer = TOP-NLuc + pAd or CBF-GLuc + phICN1; shown data is the average of 2 experiments; values are multiplied by 1000. HEK293T cells were seeded (10,000 per well) and transfected, after 24 hours the reading was performed from the supernatant with Coelenterazine. RLU was normalized with CellTiter-Fluor™ Cell Viability assay.

Figure 42 shows the results for reading the lysate with Coelenterazine. Untreated, buffer control treated, and control plasmid treated cells show a very low signal, which was expected. As for the NanoLuc based plasmid values, one can see that the NanoLuc enzyme in the lysate reacts with Coelenterazine, which indicates a cross reactivity. When the focus is shifted to the Gaussia Luc based plasmid values, it is shown that a part of the synthesized Gaussia Luc enzymes is present in the lysate and reacts with Coelenterazine.

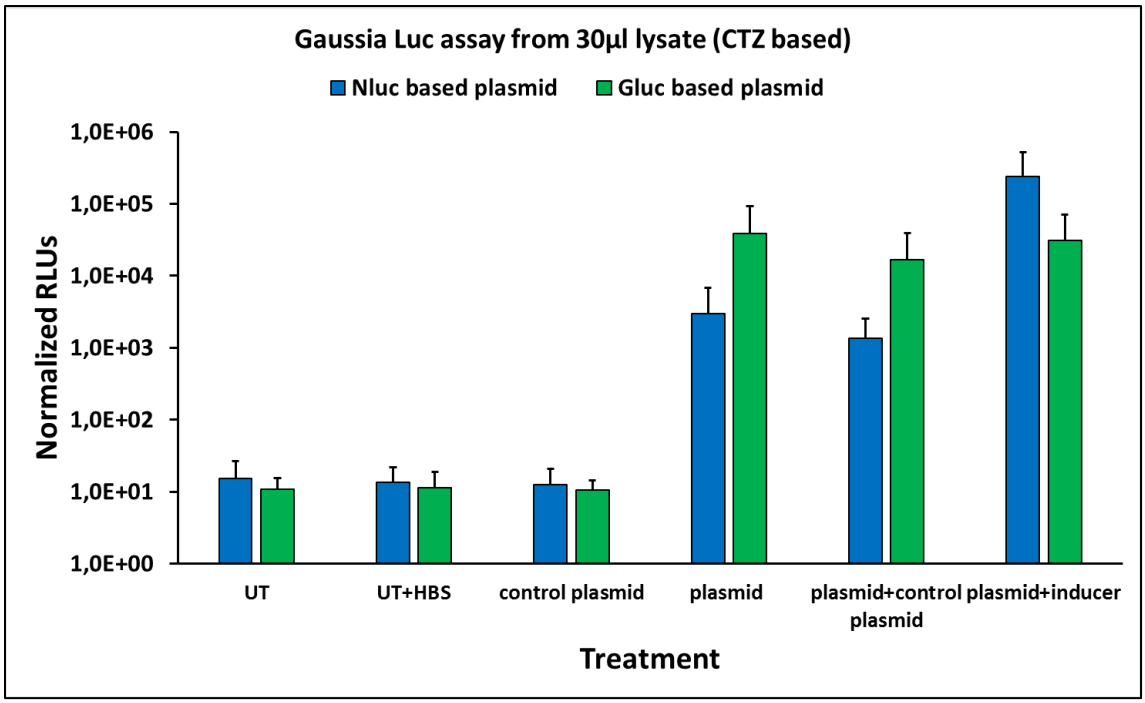


Figure 42: Normalized RLUs from 30µl lysate by a Coelenterazine based Gaussia Luc assay.
UT = untreated; UT + HBS = untreated + HBS; control plasmid = pUC19; plasmid = TOP-NLuc or CBF-GLuc; plasmid + control plasmid = TOP-NLuc or CBF-GLuc + pUC19; plasmid + inducer = TOP-NLuc + pAd or CBF-GLuc + phICN1; shown data is the average of 2 experiments; values are multiplied by 10,000. HEK293T cells were seeded (10,000 per well) and transfected, after 24 hours the reading was performed from the lysate with Coelenterazine. RLU was normalized with CellTiter-Fluor™ Cell Viability assay.

As expected, the following figure shows the typical signal pattern of the NanoLuc based plasmids when read from the lysate with Furimazine. The results of this experiment also show that even if there is a small amount of Gaussia Luc enzymes present in the lysate, it has no reactivity with Furimazine. Regarding the values for UT, UT + HBS and control plasmid for Gaussia Luc based plasmid, it is questionable, why the signal is lower than the UT and UT + HBS control values of the NanoLuc based plasmid values.

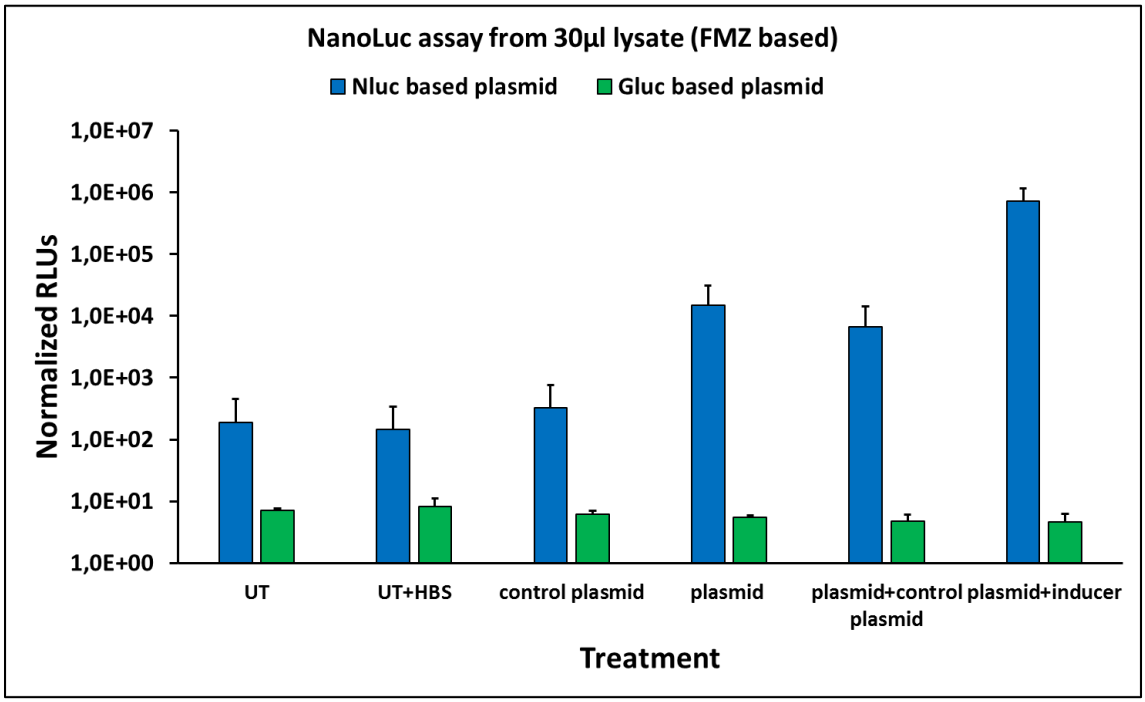


Figure 43: Normalized RLUs from 30µl lysate by a Furimazine based NanoLuc assay.
UT = untreated; UT + HBS = untreated + HBS; control plasmid = pUC19; plasmid = TOP-NLuc or CBF-GLuc; plasmid + control plasmid = TOP-NLuc or CBF-GLuc + pUC19; plasmid + inducer = TOP-NLuc + pAd or CBF-GLuc + phICN1; shown data is the average of 2 experiments; values are multiplied by 10,000. HEK293T cells were seeded (10,000 per well) and transfected, after 24 hours the reading was performed from the lysate with Furimazine. RLU was normalized with CellTiter-Fluor™ Cell Viability assay.

Concluding the investigation on cross reactivity between luciferase substrates, it is proven that Gaussia Luc enzymes do not use Furimazine as a substrate when read from the lysate, even if Gaussia Luc enzymes are partly kept in the lysate. Hence, there cannot be any cross reactivity with the NanoLuc signals. Furthermore, one can clearly see that NanoLuc enzymes do not react with Coelenterazine when read from the supernatant. This means that there is no cross reactivity here as well. The only cross reactivity detected in this experiment is that NanoLuc enzymes show a high signal when read with Coelenterazine from the lysate. Considering that the paper for which these experiments were performed, there is no possibility that the results are distorted by a cross reactivity of the luciferase substrates, because the reading in the paper experiments was only performed from the supernatant with Coelenterazine.

7.4 Multicistronic vector 3P-Luc for investigating crosstalk between signaling pathways

As not only the cross reactivity between luciferase substrates needed to be examined for the 3P-Luc reporter plasmid, the cross induction between the pathways was the second aspect, which needed to be investigated. Towards this, whether the inducer plasmids pAd (inducer for TOP-NLuc), phGli1 (inducer for GLI-FLuc) and phICN1 (inducer for CBF-GLuc) have an inducing activity on the non-corresponding reporter plasmids, cross-induction experiments were planned. Figure 31 shows the design of the experiments, where the 3P-Luc plasmid played a central role. The sets of transfections shown in Figure 32 and Figure 33 with the 3P-Luc plasmid were implemented to investigate the cross induction between Wnt3a-, Hedgehog- and Notch- pathways with one single experiment design. The statistics used to interpret the results was calculated by https://www.socscistatistics.com/.

7.4.1 Wnt3a inducer

7.4.1.1 Effect of Wnt3a inducer on Wnt3a-pathway activity

The results of the following experiment are as expected: Reading the signals of the co-transfection of 3P-Luc + pAd (compared to 3P-Luc alone) with Furimazine shows an induction of Wnt3a-pathway compared to the 3PLuc values. The untreated controls show equally low values as expected.

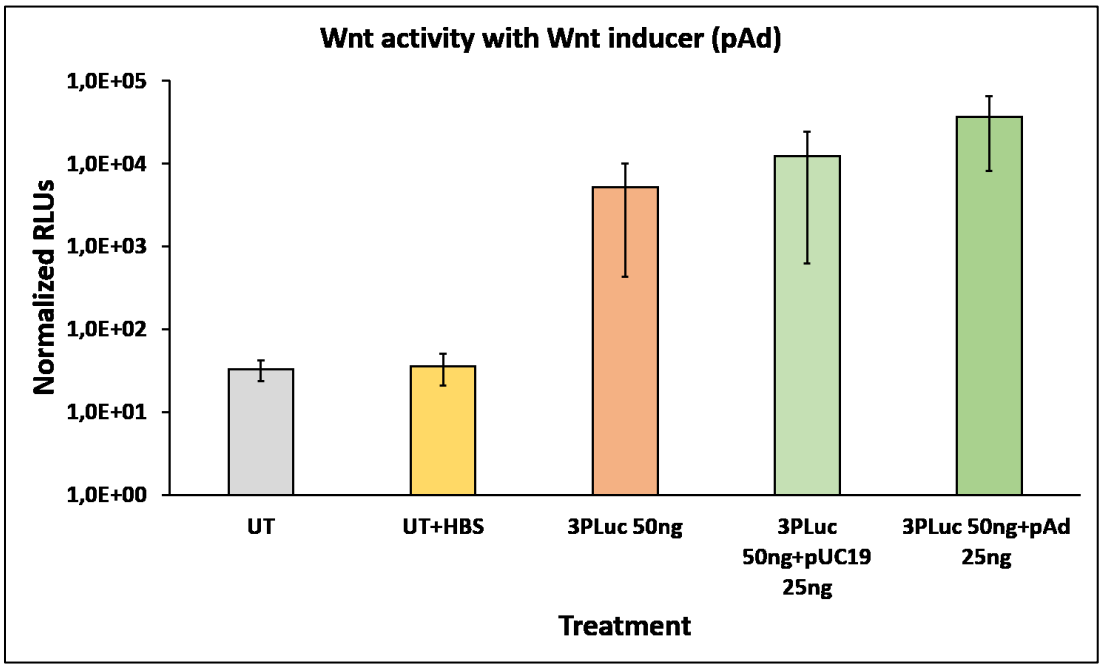


Figure 44: Wnt3a activity with Wnt3a inducer (pAd).

UT = untreated; UT + HBS = untreated + HBS; shown data is the average of 2 experiments; values are multiplied by 100,000. HEK293T cells were seeded (10,000 per well) and transfected with 3P-Luc 50ng/ 3P-Luc 50ng + pUC19 25ng/ 3P-Luc 50ng + pAd 25ng. The assays were read after 24 hours and normalized with CellTiter-Fluor™ Cell Viability assay.

7.4.1.2 Effect of Wnt3a inducer on Hedgehog-pathway activity

Co-transfecting 3P-Luc with Wnt3a inducer (pAd) but investigating Hedgehog activity via Firefly Luc assay shows that there is a cross induction between the pathways, as there is a significant increase in the signal of Firefly (Hedgehog activity), when pAd is co-transfected with 3P-Luc. A two-tailed t-test for 2 independent means was performed and the increase in signal for 3P-Luc + pAd in comparison to 3P-Luc was significant for both experiments (P = 0.01; N = 6).

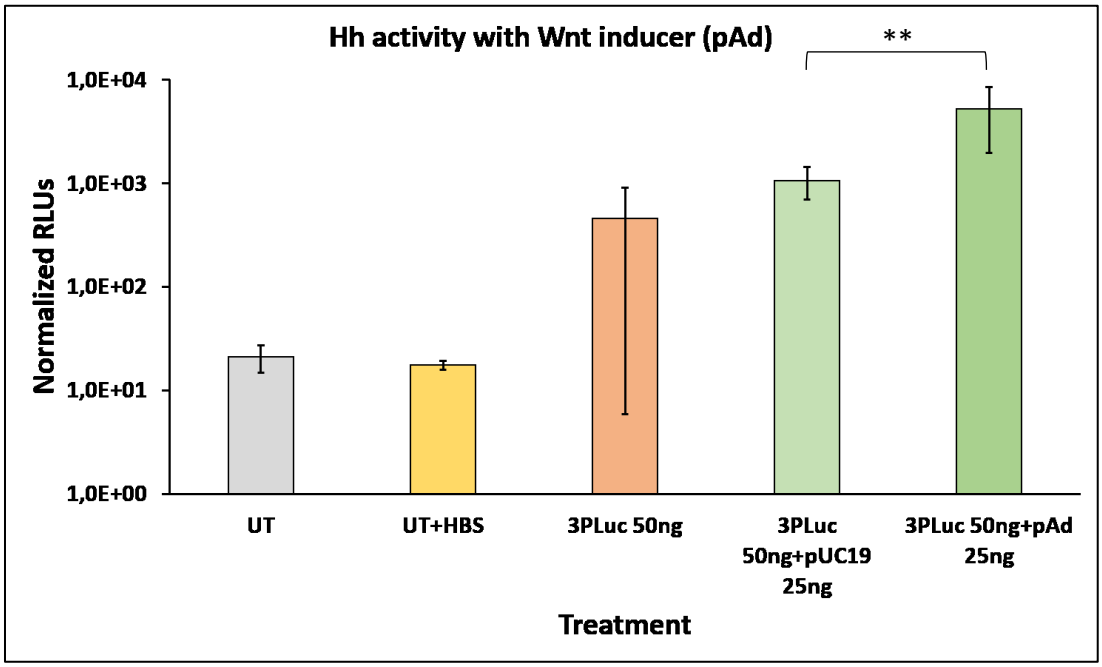


Figure 45: Hedgehog activity with Wnt3a inducer (pAd).
UT = untreated; UT + HBS = untreated + HBS; shown data is the average of 2 experiments; values are multiplied by 100,000. HEK293T cells were seeded (10,000 per well) and transfected with 3P-Luc 50ng/ 3P-Luc 50ng + pUC19 25ng/ 3P-Luc 50ng + pAd 25ng. The assays were read after 48 hours and normalized with CellTiter-Fluor™ Cell Viability assay. Cross induction is significant (two-tailed t-test for 2 independent means, P = 0.01; N = 6).

7.4.1.3 Effect of Wnt3a inducer on Notch-pathway activity

When pAd is co-transfected with 3PLuc and a Gaussia Luc assay is carried out, it can be investigated whether the Wnt3a inducer has an inducing effect on the Notch-pathway activity. The following graphs show a significant induction (based on a two-tailed t-test for 2 independent means; P = 0.01; N = 12).

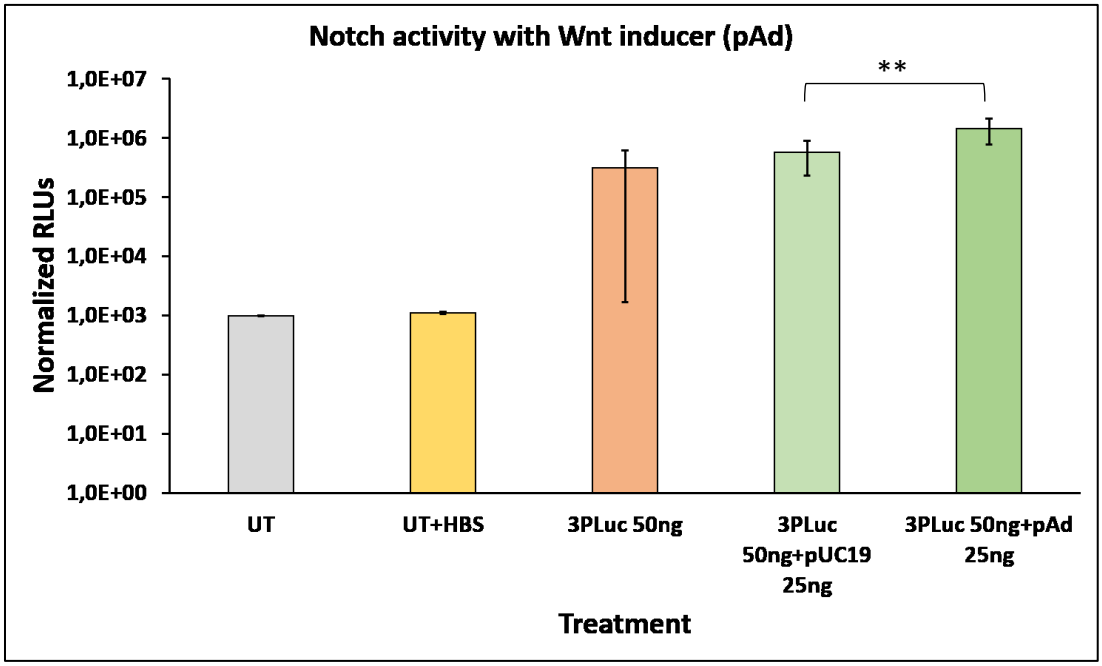


Figure 46: Notch activity with Wnt3a inducer (pAd).
UT = untreated; UT + HBS = untreated + HBS; shown data is the average of 4 experiments; values are multiplied by 100,000. HEK293T cells were seeded (10,000 per well) and transfected with 3P-Luc 50ng/ 3P-Luc 50ng + pUC19 25ng/ 3P-Luc 50ng + pAd 25ng. The assays were read after 48 hours and normalized with CellTiter-Fluor™ Cell Viability assay. Cross induction is significant (two-tailed t-test for 2 independent means, P = 0.01; N = 12).

Concluding the results for the three pathways with Wnt3a-inducer, it could be detected that pAd is not only inducing the Wnt3a-pathway but Hedgehog-pathway and Notch-pathway are also inducted by pAd, which is an indication that a cross induction exists.

7.4.2 Hedgehog inducer

7.4.2.1 Effect of Hedgehog inducer on Wnt3a-pathway activity

Wnt3a-pathway activity measured in a NanoLuc assay with Furimazine shows an insignificant increase with the Hedgehog inducer (phGli1) (based on a two-tailed t-test for 2 independent means; P = 0.01; N = 6).

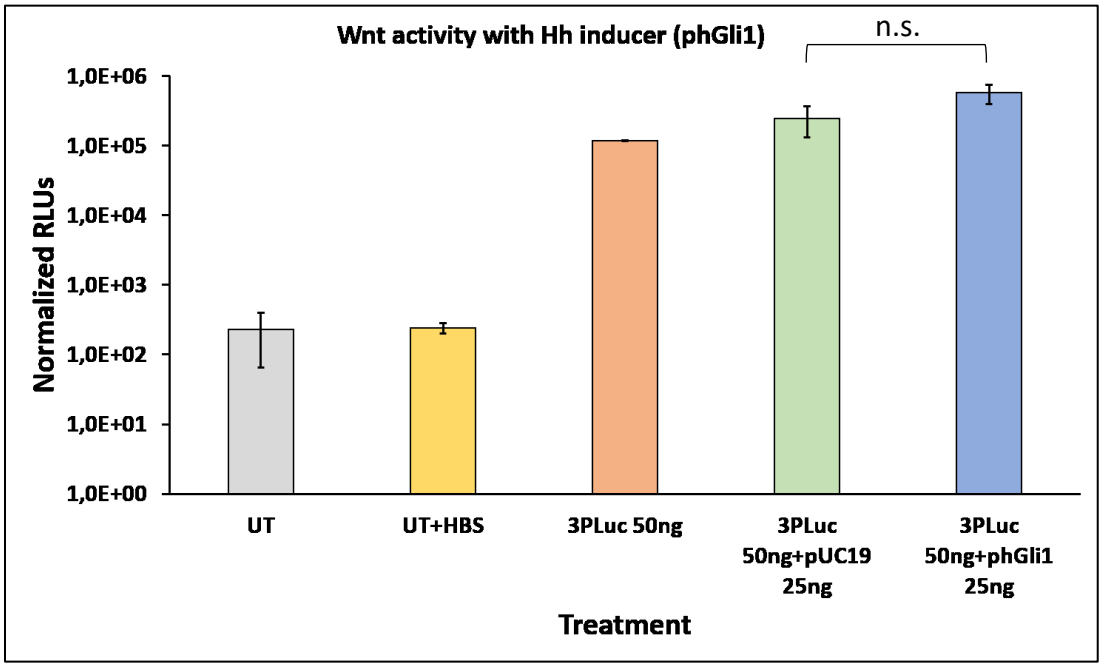


Figure 47: Wnt3a activity with Hedgehog inducer (phGli1).
UT = untreated; UT + HBS = untreated + HBS; shown data is the average of 2 experiments; values are multiplied by 100,000. HEK293T cells were seeded (10,000 per well) and transfected with 3P-Luc 50ng/ 3P-Luc 50ng + pUC19 25ng/ 3P-Luc 50ng + phGli1 25ng. The assays were read after 24 hours and normalized with CellTiter-Fluor™ Cell Viability assay. Cross induction is insignificant (two-tailed t-test for 2 independent means, P > 0.01; N = 6).

7.4.2.2 Effect of Hedgehog inducer on Hedgehog-pathway activity

As expected, the induction of the Hedgehog-pathway (read via Firefly assay) takes place in response to Hedgehog induction.

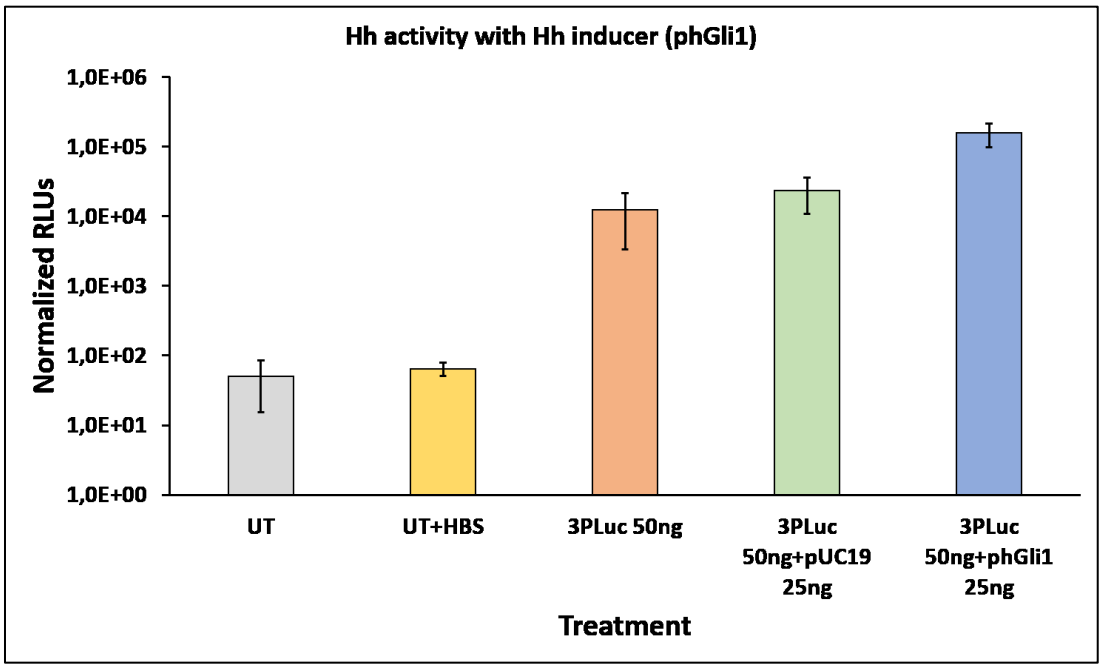


Figure 48: Hedgehog activity with Hedgehog inducer (phGli1).
UT = untreated; UT + HBS = untreated + HBS; shown data is the average of 2 experiments; values are multiplied by 100,000. HEK293T cells were seeded (10,000 per well) and transfected with 3P-Luc 50ng/ 3P-Luc 50ng + pUC19 25ng/ 3P-Luc 50ng + phGli1 25ng. The assays were read after 48 hours and normalized with CellTiter-Fluor™ Cell Viability assay.

7.4.2.3 Effect of Hedgehog inducer on Notch-pathway activity

The following graph shows that Notch-pathway activity (read via Gaussia Luc assay) is significantly induced when 3PLuc is co-transfected with the Hedgehog inducer (phGli1) based on a two-tailed t-test for 2 independent means; P = 0.001; N = 12).

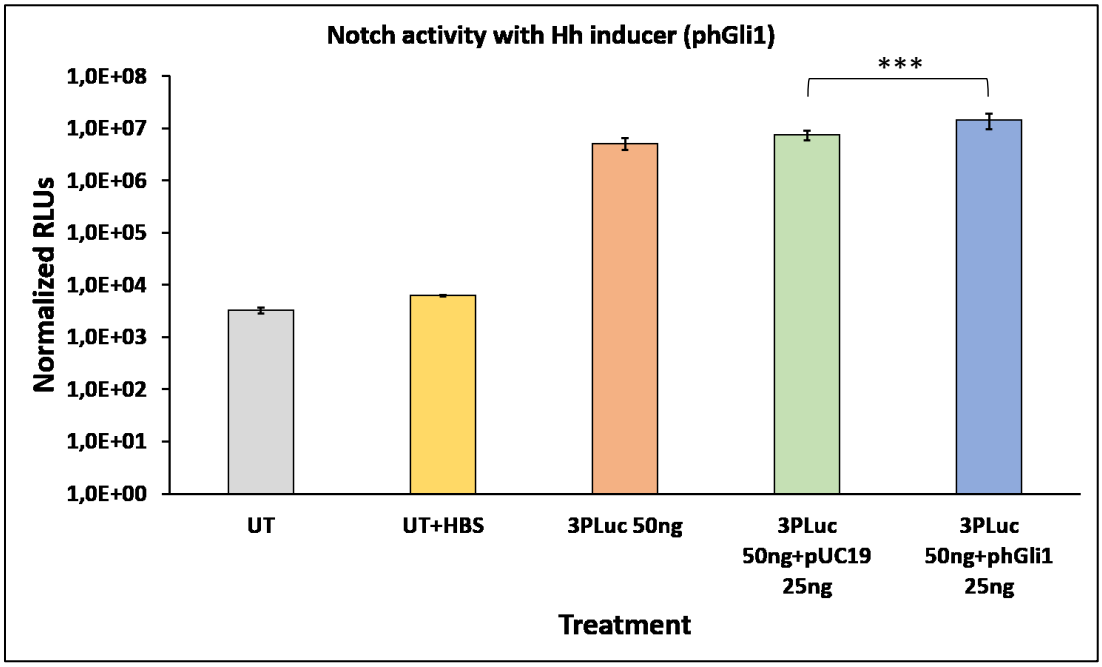


Figure 49: Notch activity with Hedgehog inducer (phGli1).
UT = untreated; UT + HBS = untreated + HBS; shown data is the average of 4 experiments; values are multiplied by 100,000. HEK293T cells were seeded (10,000 per well) and transfected with 3P-Luc 50ng/ 3P-Luc 50ng + pUC19 25ng/ 3P-Luc 50ng + phGli1 25ng. The assays were read after 48 hours and normalized with CellTiter-Fluor™ Cell Viability assay. Cross induction is significant (two-tailed t-test for 2 independent means, P = 0.001; N = 12).

Summarizing the experiment results on investigating the ability of phGli1 to induce Wnt3a- and Notch- pathways in addition to the Hedgehog-pathway, the past graphs make the cross induction of the Notch-pathway visible, whereas the Wnt3a-pathway is not being induced by the Hedgehog inducer.

7.4.3 Notch inducer

7.4.3.1 Effect of Notch inducer on Wnt3a-pathway activity

The following graph shows whether the Notch inducer (phICN1) induces the Wnt3a-activity (read via NanoLuc assay). The results show a statistically insignificant induction (based on a two-tailed t-test for 2 independent means; P = 0.01; N = 6). Cross induction between the Notch-inducer and the Wnt3a-pathway can be excluded.

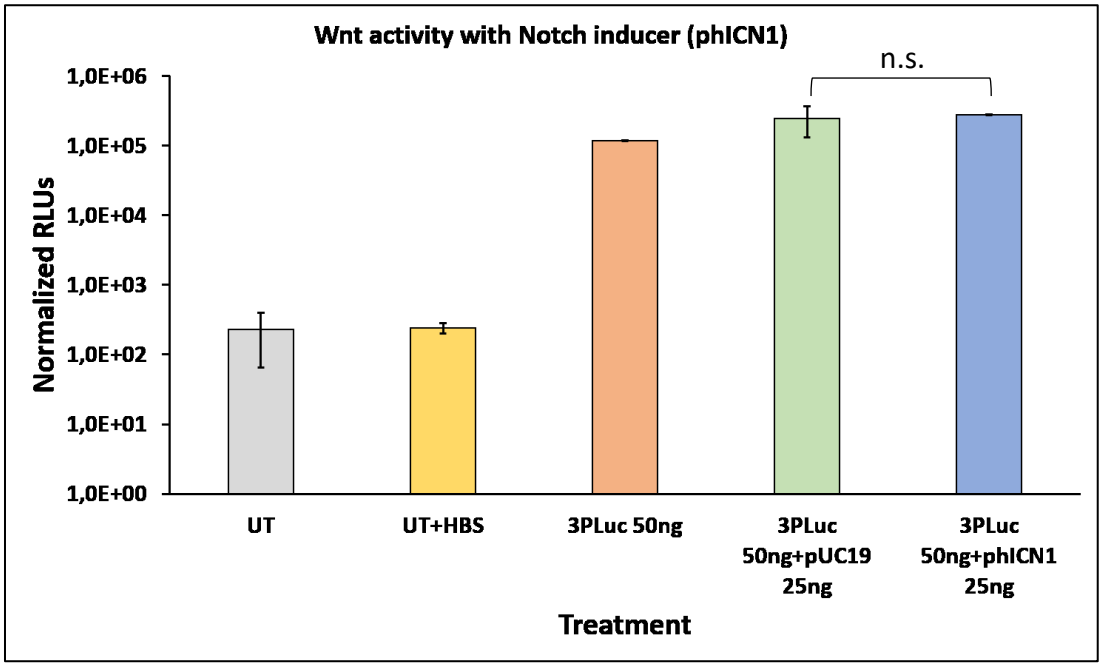


Figure 50: Wnt3a activity with Notch inducer (phICN1).

UT = untreated; UT + HBS = untreated + HBS; shown data is the average of 2 experiments; values are multiplied by 100,000. HEK293T cells were seeded (10,000 per well) and transfected with 3P-Luc 50ng/ 3P-Luc 50ng + pUC19 25ng/ 3P-Luc 50ng + phICN1 25ng. The assays were read after 24 hours and normalized with CellTiter-Fluor™ Cell Viability assay. Cross induction is insignificant (two-tailed t-test for 2 independent means, P > 0.05; N = 6).

7.4.3.2 Effect of Notch inducer on Hedgehog-pathway activity

Derived from the following graph, the Hedgehog-pathway (read via Firefly assay) is not significantly affected by the Notch-inducer (phICN1) (based on a two-tailed t-test for 2 independent means; P = 0.01; N = 6).

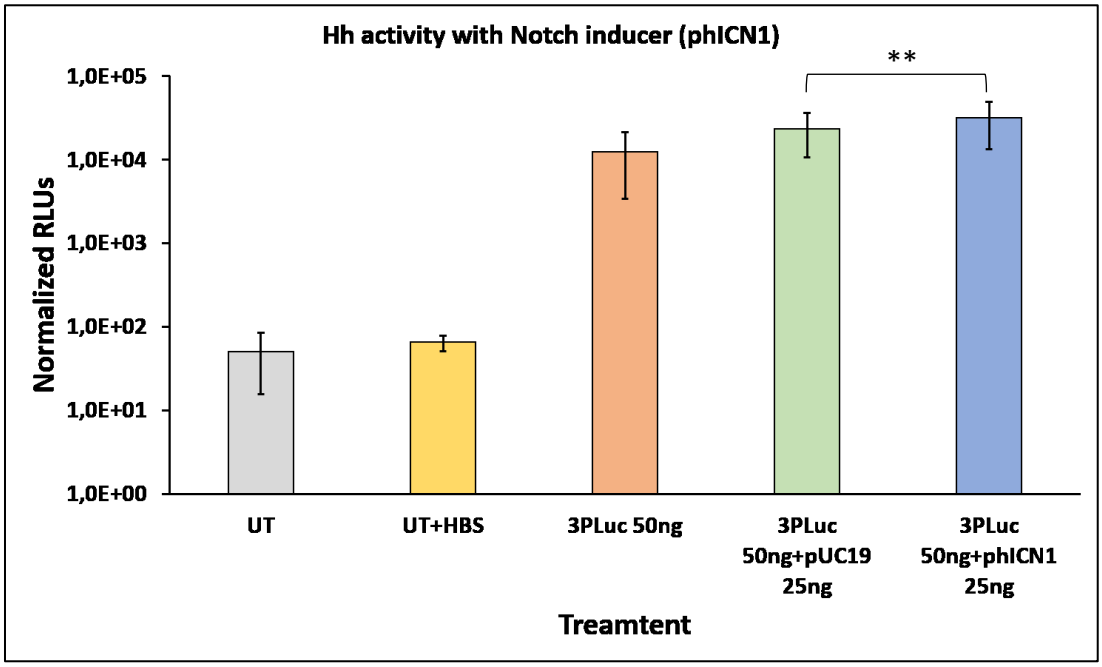


Figure 51: Hedgehog activity with Notch inducer (phICN1).
UT = untreated; UT + HBS = untreated + HBS; shown data is the average of 2 experiments; values are multiplied by 100,000. HEK293T cells were seeded (10,000 per well) and transfected with 3P-Luc 50ng/ 3P-Luc 50ng + pUC19 25ng/ 3P-Luc 50ng + phICN1 25ng. The assays were read after 48 hours and normalized with CellTiter-Fluor™ Cell Viability assay. Cross induction is insignificant (two-tailed t-test for 2 independent means, P = 0.01; N = 6).

7.4.3.3 Effect of Notch inducer on Notch-pathway activity

The expected induction of Notch-pathway (read via Gaussia Luc assay) through Notch inducer (phICN1) is shown in the following graph.

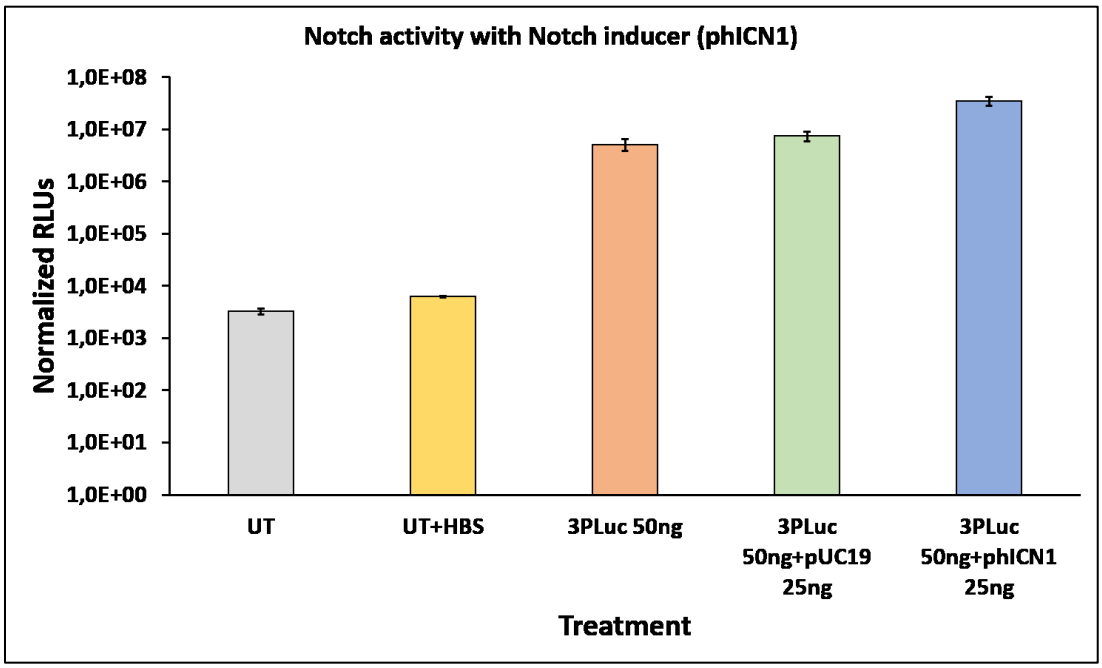


Figure 52: Notch activity with Notch inducer (phICN1).
UT = untreated; UT + HBS = untreated + HBS; shown data is the average of 4 experiments; values are multiplied by 100,000. HEK293T cells were seeded (10,000 per well) and transfected with 3P-Luc 50ng/ 3P-Luc 50ng + pUC19 25ng/ 3P-Luc 50ng + phICN1 25ng. The assays were read after 48 hours and normalized with CellTiter-Fluor™ Cell Viability assay.

To sum up the results which are presented in this thesis concerning the cross induction of the three pathways, one can see that a cross induction exists. The ability of pAd, the Wnt3a inducer, to induce the other two pathways is proven by the experiments and statistics showed that the induction is significant. Still, the induction of the Wnt3a-pathway caused by pAd is more powerful than for the other two pathways. The Hedgehog inducer also does not only induce the corresponding pathway, but also the Notch-pathway. The interesting part is that phICN1 seems to be very specific for the Notch-pathway and does not induce neither of the other two pathways, as it can be seen in Figure 50 and Figure 51. It will be interesting to find out why pAd and phGli1 have a multi-inducing ability and in contrast to this, why phICN1 is so specific? Seen from the opposite perspective, the Notch-pathway is induced by all three inducers, while this is not true for the Wnt3a- and Hedgehog- pathways. This is interesting because according to the results presented in this thesis, one can assume that the inducer plasmid for the Notch-pathway is very specific, whereas the pathway of this inducer is unspecific and can be induced by other inducer plasmids. Another important point is that the Wnt3a pathway seems to be induced only by its corresponding inducer (pAd). This indicates a specificity of this pathway. The following figure illustrates the conclusion of the results more lucidly:

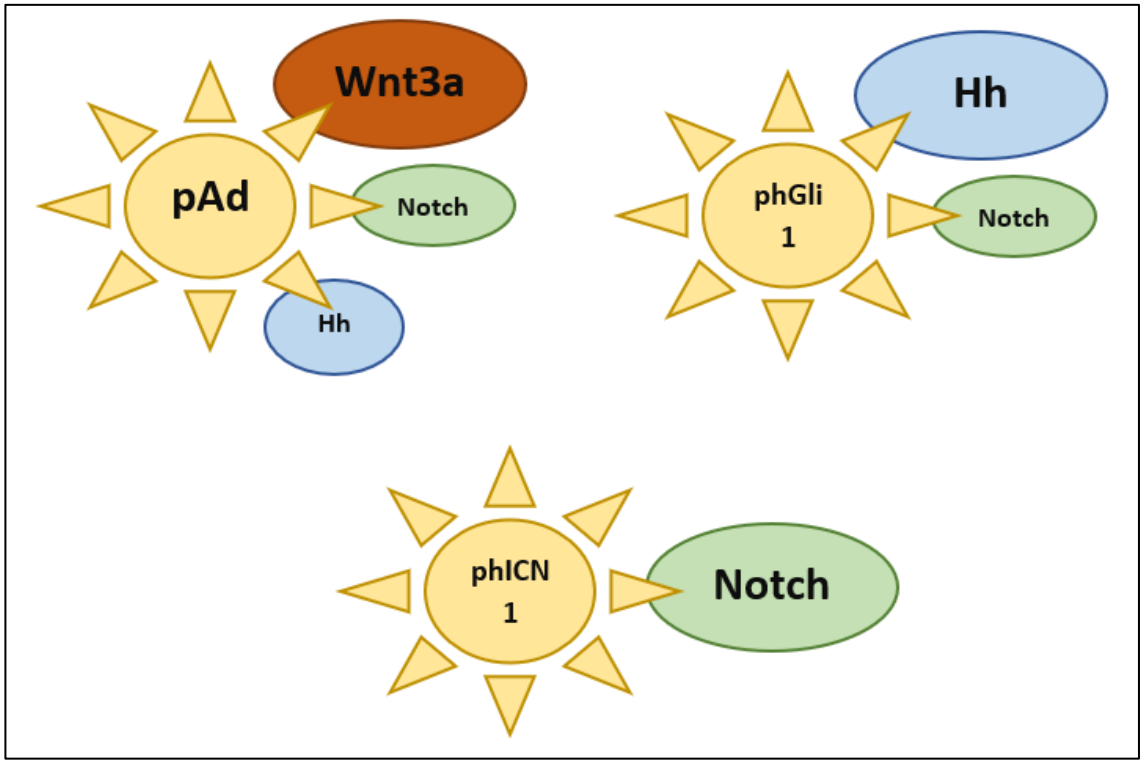


Figure 53: Schematic representation of cross induction between signaling pathways.

7.5 Immunohistochemistry on CD49f targeted lung tissue

For examining the expression of CD49f on tumor tissue of mice lungs, immunohistochemistry was performed on 3 different samples. Each sample was sectioned and stained with Hematoxylin and Eosin to find the tumor region. More sections were made to target the CD49f molecules with a primary Rabbit monoclonal antibody and Goat-Anti-Rabbit biotinylated secondary antibody after blocking the Avidin- and Biotin- reagent parts of the tissue. By treatment with Avidin, Biotin and DAB, the detection is performed through brown color development. The results of each experiment show 6 sections treated with antibodies/ buffer (for controls) and 1 section with Hematoxylin and Eosin staining.

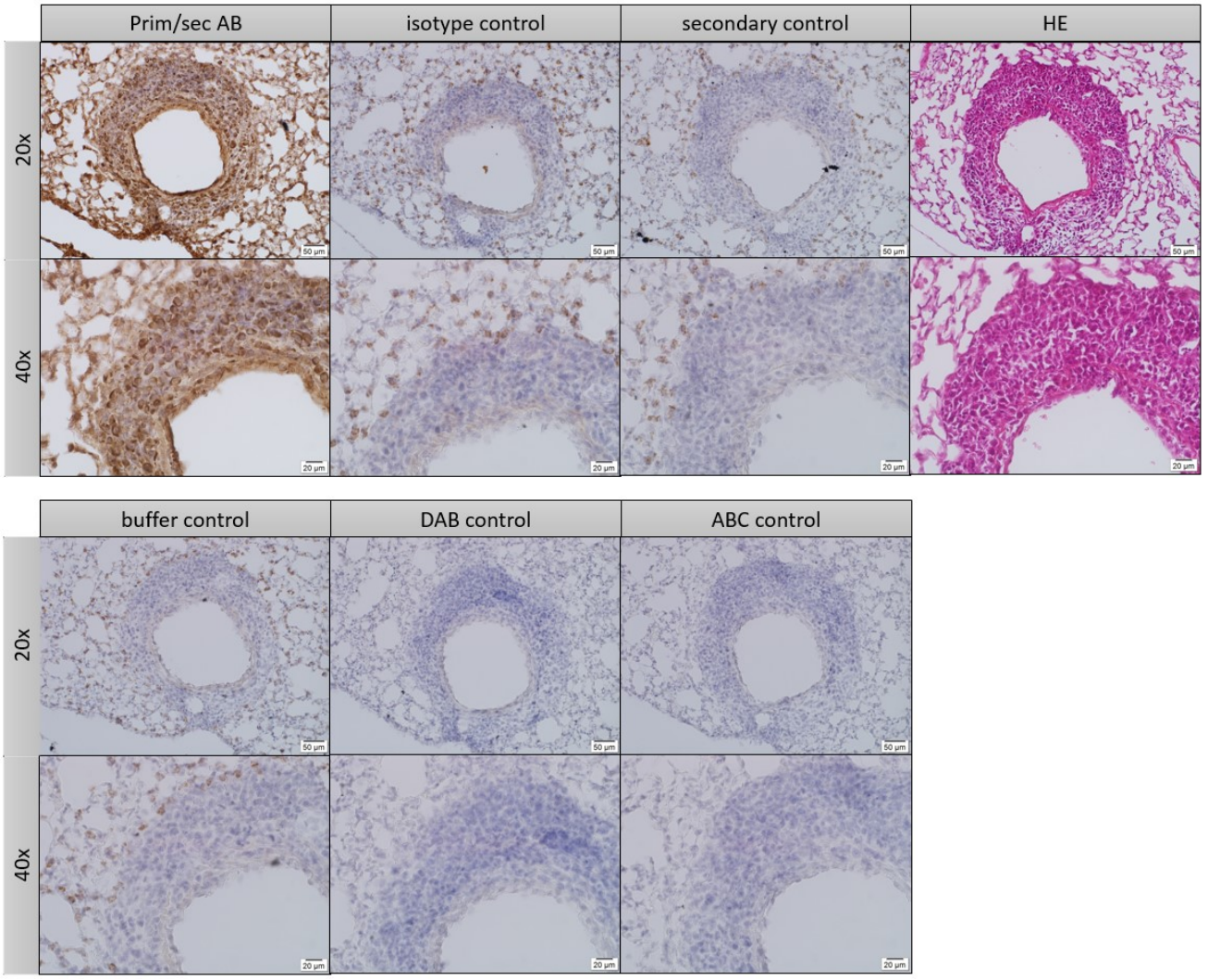


Figure 54: Experiment1: IHC and HE on Lu_MCT0097_PEG.
Positive CD49f signal is visible. Isotype/ secondary/ buffer/ DAB/ ABC controls are negative. HE staining makes the tumor region visible. Scale bars: 50µm (20x), 20µm (40x).

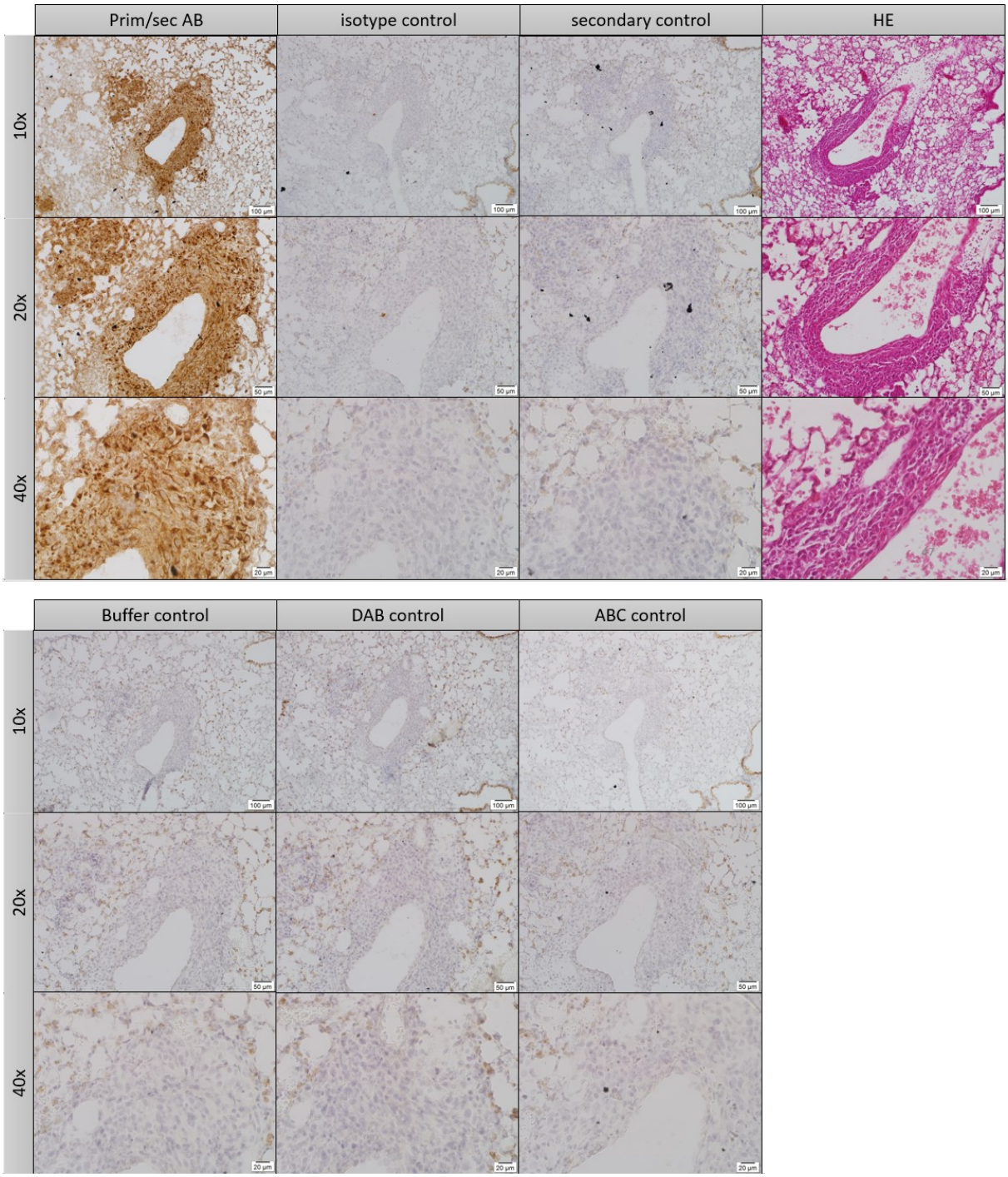


Figure 55: Experiment 2: IHC and HE on MCT_0086_CD49f_targeted.
Positive CD49f signal is visible. All the controls are negative. HE staining makes the tumor region visible. Scale bars: 100µm (10x), 50µm (20x), 20µm (40x).

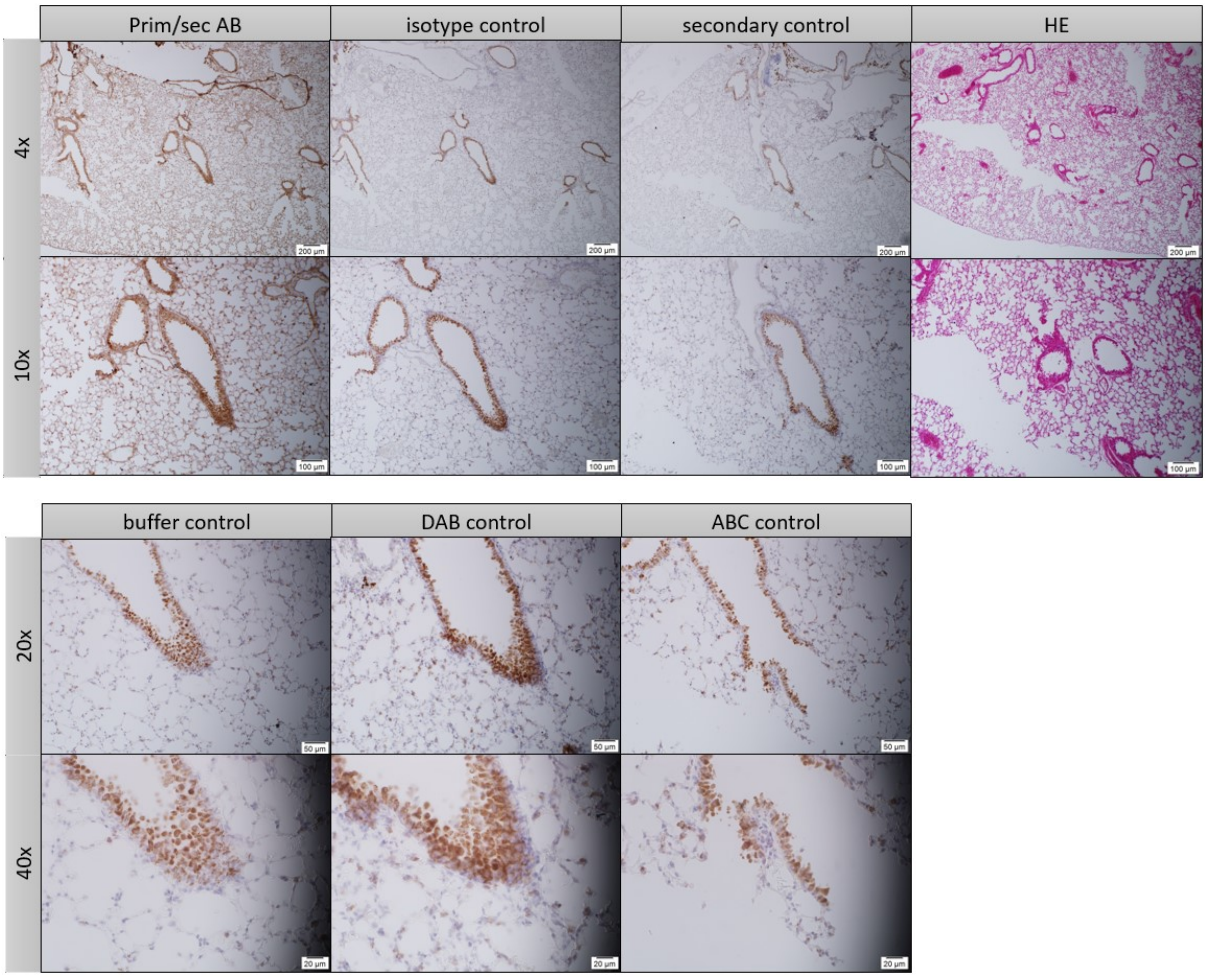


Figure 56: Experiment 3: IHC and HE on Lu_MCT0099_PEG.
Positive CD49f signal is visible. All the controls are negative. HE staining makes the tumor region visible. Scale bars: 50µm (20x), 20µm (40x).

It is visible that all three experiments show a specific CD49f staining when treated with primary antibody, secondary antibody, ABC and DAB. Especially experiment 2 has a high importance as it is used for further research on tumor lesions development in lungs after aerosol mediated gene delivery. (Taschauer, Polzer, et al. 2019)

8 Discussion

This diploma thesis summarizes the attempts to study several different subjects which are focused on in the laboratory of Macromolecular Cancer Therapeutics. To make a general separation, two main areas are represented in this thesis: in vitro investigation of signaling pathways through transfecting cells with nanoparticles and characterizing the expression of the surface protein CD49f by using immunohistochemistry.

Investigating the influence of plant extracts 3, 6, and 8 on signaling pathways

The intention to investigate the extracts of *Peucedanum ostruthium* and *"plant A"* (identity not disclosed) was the continuation of the experiments carried out by the former diploma student Sandra Milenkovic. Out of 9 different plant extracts, she discovered that plant extracts 3 and 6 have a significant decreasing effect on the Wnt3a-pathway activity. (Milenkovic 2019) This study included transfecting 10,000 HEK293T cells per well with polyplexes, consisting of LPEI and TOP-NLuc or LPEI and co-transfection of TOP-NLuc with pAd as a positive control and TOP-NLuc with DMSO as a negative control. Also, TOP-NLuc was co-transfected with pUC19 as negative control for the co-transfection. Adding the compounds at different concentrations, dissolved in DMSO and diluted with HBS, after an incubation of 4 hours and reading the light signals on the next day by performing a NanoLuc assay showed a difference between the DMSO controls and the compound-treated cells.

To take a deeper look into these results, the goal in this thesis was to study the reproducibility of the earlier experiments and also do additional investigations. Therefore, the experiment was performed three times. Again, HEK293T cells were seeded on the first day, transfected with the polyplexes on the second day and treated with the compounds or DMSO in the concentrations 25, 50, 100 and 200µg/ml (diluted with HBS). On the third day, the NanoLuc assay was performed by lysing the cells and adding Furimazine by the plate-reader-injector to generate the RLUs, which were then normalized with BCA assay. To our expectations, the results were verified and the significant inhibition of the Wnt3a-pathway could be proven at the concentration of 200µg/ml of plant extract 3 and 100µg/ml and 200µg/ml of plant extract 6. Thinking of the possibilities that are opened by this research, one can say that (at least for plant extract 3) Osthole (and other Coumarins) in the extract of Peucedanum ostruthium can

be used for cancer therapy, or at least be the subject of further cancer-related treatment research, as the inhibition of the Wnt3a-pathway is associated with less cell division.

To continue the research on plant extract 3, it was further investigated for its effect on Hedgehog-pathway and Notch-pathway, as the results for the Wnt3a-pathway had shown a significant decrease in the pathway activity. Therefore, it was planned to investigate one of the compounds which had previously shown a significant effect. Therefore, HEK293T cells were seeded on day 1, treated on day 2, and on day 3 a combined Gaussia Luc and Firefly Luc assay were performed with Coelenterazine and LBL and normalized with BCA assay. As the results had shown poor signal, it was decided to change the workflow into reading after 48 hours and normalizing with CellTiter-Fluor™ Cell Viability assay. The idea behind changing the incubation time after treatment from 24 hours to 48 hours was that Gaussia Luc and Firefly Luc assays are supposed to be read after 48 hours if not mentioned otherwise in the protocol. By reading after 48 hours slight decrease in the Hedgehog and Notch pathways were noticeable when treated with 200µg/ml of compound 3. Still, these were single experiments and definitely need more repetition and optimization in terms of plasmid amount because the reason for the low signals in both experiments could be the amounts of plasmids (50ng reporter plasmid or 25ng reporter plasmid with 25ng inducer plasmid for co-transfections). Especially in the Gaussia Luc assays in both experiment workflows a reduction in signal is detected for the transfected cells with the highest concentration of compound 3 (200µg/ml). With this, one can assume that the repetition of these experiments can verify the assumption that, in addition to the Wnt3a-activity, plant extract 3 also reduces the Hedgehog- and Notch-activity.

Cross reactivity between luciferase substrates

The cross reactivity between the two substrates Furimazine and Coelenterazine was the next aim in the present diploma thesis. In multi-luciferase assays, like performing both NanoLuc and Gaussia Luc assays in one experiment, investigating the cross reactivity between the substrates of these assays is very important. Knowing that NanoLuc enzymes are not secreted into the supernatant, almost no signal was expected from the supernatant read with Coelenterazine. The important question was to find out whether the small amount of Gaussia Luc, which remains cytoplasmic, would give a signal when read with Furimazine from the

lysate and vice-versa, whether NanoLuc in the lysate gives signals when read with Coelenterazine.

The plan of performing the experiments was based on the idea to combine the NanoLuc assay, where the signals are read from the lysate, with the Gaussia Luc assay, where the enzymes are secreted into the supernatant. Another important point was to perform the protocol-based NanoLuc and Gaussia Luc assays and additionally perform the reading with the non-corresponding substrate. On that basis, HEK293T cells were seeded into two plates on day 1. The next day, the transfection took place, where each plate had two identical sets of transfections (NanoLuc based plasmids and Gaussia Luc based plasmids). Reading and normalizing the signals on the third day was performed by taking the supernatant of all wells and transferring them into a transparent plate. 20µl of each well were taken to read with Coelenterazine through Gaussia Luc assay. The cells of both plates were lysed and each, NanoLuc plate and Gaussia Luc plate, was read with Furimazine and Coelenterazine. The normalization was done with CellTiter-Fluor™ Cell Viability assay. The experiment was repeated twice.

Looking at the results, firstly, Gaussia Luc based plasmids do not interfere in the Furimazine based assay from the lysate. Secondly, NanoLuc based plasmids have a reactivity in a Coelenterazine based assay from the lysate, which means that a cross reactivity exists. However, the reading with Coelenterazine performed for the 3P-Luc paper is from the supernatant, where NanoLuc has shown that it does not interfere. In other words, a cross reactivity would be problematic if the Gaussia Luc assay was read from the lysate, where NanoLuc enzymes also use Coelenterazine as a substrate.

Cross induction between signaling pathways

Another important aspect to be investigated for multi-reporter plasmids was the cross induction of the three signaling pathways Wnt3a, Hedgehog and Notch when the cells are transfected with the 3P-Luc plasmid. The question, whether the inducer plasmids of each reporter plasmid are also inducing the other two reporter plasmids, had to be answered. Therefore, two experiments were designed. The goal of the first experiment was to find out whether the inducer of the Wnt3a-pathway reporter plasmid TOP-NLuc, pAd, induces the reporter plasmids of the other two pathways. The second experiment was a combination of two experiments. Here, the cross induction of phGli1, the Hedgehog-inducer, and phICN1, the

Notch-inducer, were investigated. Each of the experiments was carried out with HEK293T cells and repeated twice.

For investigating the ability of the Wnt3a-inducer (pAd) to induct the other two pathways, two plates were seeded on the first day, transfected with the 3P-Luc plasmid and a co-transfection of 3P-Luc with pAd (Wnt3a-inducer) on the second day. On day 3, the reading was performed: NanoLuc assay with Furimazine from the unlysed cells (in 40µl of complete medium), Firefly Luc assay with LBL from the lysate, Gaussia Luc assay with Coelenterazine from the supernatant and CellTiter-Fluor™ Cell Viability assay for normalization. By performing all three assays (NanoLuc, Firefly Luc, Gaussia Luc) together with CellTiter-Fluor™ Cell Viability assay in one experiment, where the inducer plasmid for one pathway is under examination, the results will show the effect of the Wnt3a inducer on all three pathways and the results can be normalized with cell viability.

For the second experiment, where the Hedgehog- and Notch- inducers were investigated on cross induction, a combined experiment design was made. As both experiments need 48 hours of incubation after transfection, the best way to examine both was to co-transfect the cells with 3P-Luc + Hedgehog inducer (phGli1) and 3P-Luc + Notch inducer (phICN1), from which we would verify or falsify the cross induction ability of both inducer plasmids.

Again, the HEK293T cells were seeded into two plates on the first day. On the second day, the transfection was performed and the reading was conducted on day 4. The same procedure was done (NanoLuc assay from unlysed cells, Firefly Luc assay from lysate, Gaussia Luc assay from supernatant, cell viability assay for normalization) like in the experiment for investigating the cross induction of the Wnt3a inducer pAd.

The results delivered from these experiments show that a cross-induction does exist between the three pathways, when the 3P-Luc plasmid is transfected as a reporter plasmid. It is interesting to see that the Wnt3a inducer increases the activity of all three pathways. Vice-versa, the Wnt3a pathway seems to be very specific to its inducer plasmid, as its activity is not significantly increasing by any other inducer than its own inducer plasmid pAd. The Hedgehog inducer also does not inly increase the Hedgehog-activity, but also the Notch-activity. The only inducer that has a very high specificity is the Notch-inducer phICN1. Here, a significant signal increase is only measured with the Notch-pathway. In lights of pathway-induction ability, the Notch-pathway is very unspecific its activity is increased by all three inducers. It would be an

interesting approach to try to find out why this is the case and what makes each pathway differently specific to the inducer plasmids?

Immunohistochemistry on CD49f targeted lung tissue

As CD49f plays a central role as a biomarker for cancer stem cells, the goal of the experiments in this thesis was to target the CD49f in tumorous lung tissues of mice, where the CD49f is expressed. This was approached by, firstly, making 6 sections of the fixed lung tissue, performing a Hematoxylin and Eosin staining to find the tumor region and finally, immunohistochemistry was performed to specifically localize the surface protein CD49f. In addition to the 6 sections of tumorous tissue, 6 sections of healthy lung tissue were stained in parallel as a negative control.

Performing the immunohistochemistry was a procedure which started with prewarming the slides in the drying chamber for 2 hours at 55°C. The next step was dehydration and boiling at 131°C in a silicon oil bath. After that the biotin-blocking (Vectastain® ABC kit) step was performed, followed by incubating with Avidin and Biotin (Vectastain® ABC kit) for 15 minutes each, the next step was adding the primary antibody (Rabbit Monoclonal antibody to Integrin α6, abcam) on section number 1 or isotype (Rabbit IgG) on section number 2 (Figure 36). Both have to be diluted to generate the same concentration (1:500). On the next day the sections were incubated in 0.3% H_2O_2 for 15 minutes. The sections for the staining with primary + secondary antibody (section number 1), the isotype control (section number 2) and the secondary control (section number 3) were then treated with the secondary antibody (Goat-Anti-Rabbit Biotinylated Secondary Antibody) and incubated for 30 minutes. After draining this, ABC-reagent was pipetted to the sections and then DAB was added to start the brown color development.

The results of the experiments were positive and the expression of the CD49f surface protein was specifically localized. There is some background staining visible, which still does not interfere with the CD49f staining result.

9 List of figures

10 References

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