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1. Introduction

1.1. Hallmarks of cancer and cancer stem cell model

Cancer as a global healthcare problem

Globally, cancer is ranked as the second leading cause of death after circulatory disease (WHO, 2017). In 2013, 265 of 100 000 EU citizens died because of cancer (Eurostat database). The economic impact of this disease was estimated in 2010 at approximately US$ 1.16 trillion (IARC World Cancer Report 2014). These key facts show why cancer research and the development of novel therapies should be focused on, and pushed by independent research groups as well as the pharmaceutical industry.

Hallmarks of cancer

Cancer is a disease thought to be caused by dynamic changes in the genome (Bishop, J.M., and Weinberg, R.A. 1996). The genesis of a malignant neoplasia has to be regarded as a multistep process, wherein a cell acquires specific genetic alterations, which leads to proliferative advantages towards other cells (Farber 1984). Successive mutations drive clonal expansion of the cell, and as a result, tumor progression (Balmain et al. 1993). Commonly, the malignant cell and its progeny feature defects in regulatory circuits, leading to abnormal cell proliferation and homeostasis (Hanahan and Weinberg 2000). Six essential alterations in cell physiology, which are acquired stepwise during the process of tumor development, give rise to malignant growth (Hanahan and Weinberg 2000). These so called “Hallmarks of cancer” are considered to be the mandatory requirements for breaching physiological anticancer defense mechanisms, and are thus essential for tumorigenesis (Hanahan and Weinberg 2000).

The six hallmarks (Fig. 1), which represent a logical framework for simplifying the high complexity of neoplastic disease, have been extended by two capabilities (Hanahan und Weinberg 2011).

These emerging hallmarks represent “reprogramming energy metabolism”, in order to most effectively support neoplastic proliferation, and “evading immune destruction”, normally caused by leukocytes (T and B-cells, NK-cells, macrophages) (Hanahan and Weinberg 2011).

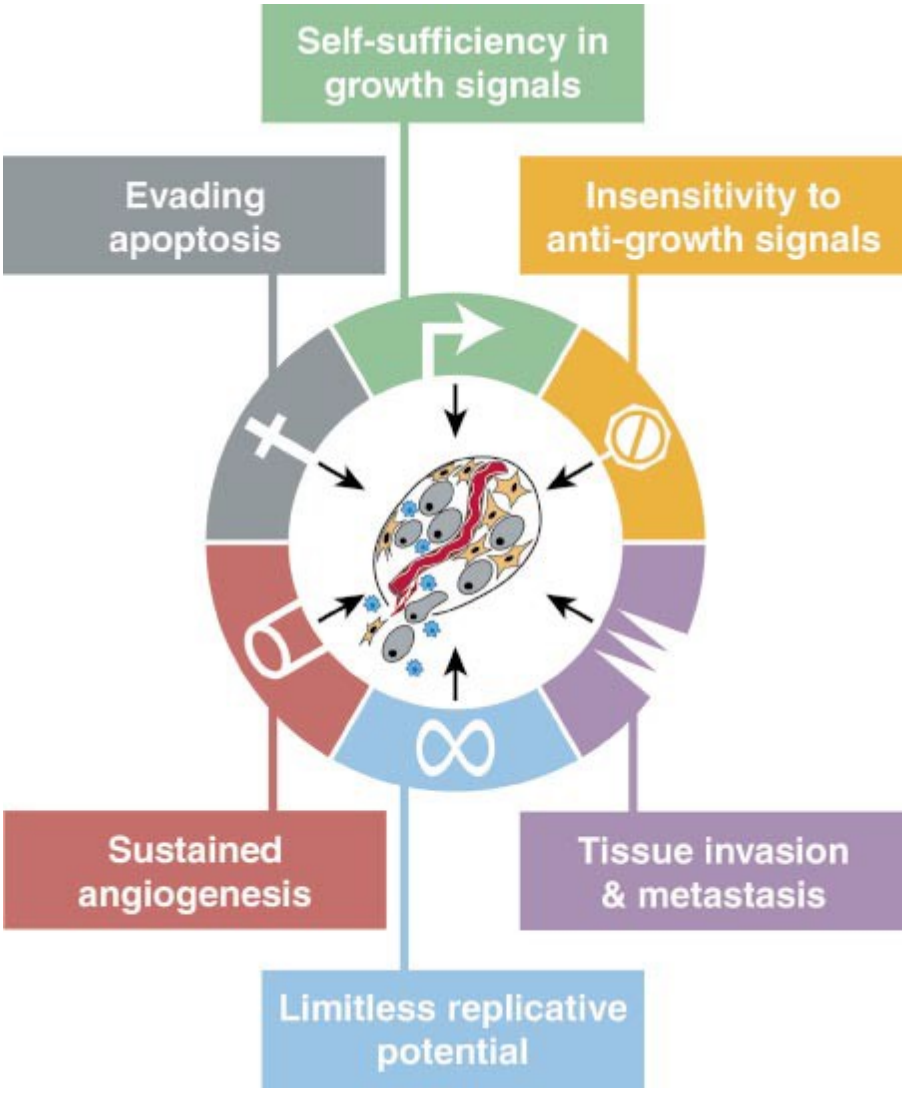

Figure 1. The six “Hallmarks of cancer”. In the context of this work, it will be focused on growth signal aberrations. “Reprogramming energy metabolism” and “evading immune destruction” have been added 2011 as emerging hallmarks. Figure from Hanahan and Weinberg 2000.

Cancer stem cell (CSC) model

The theory of CSCs came up, as a subpopulation of malignant cells with special abilities was identified, isolated and used in functional assays (Cho and Clarke 2008). These cells showed the ability to efficiently seed new tumors upon inoculation into immunodeficient mice, as they were capable of self-renewal and differentiation (Cho and Clarke 2008). These two capabilities are normally related to stem cells found in proliferative tissues such as bone marrow and skin or intestinal epithelium, leading to the term cancer stem cells for this subpopulation (Clevers 2011). Originally discovered in leukemia (Karamboulas and Ailles 2013; McCulloch 1983), CSCs have later also been found in solid tumors such as colon, lung and breast cancer (Chen et al. 2013).

Aside from their special functional capabilities, CSCs can be distinguished from other cells in the tumor due to surface markers (Fig. 2) normally found on non-malignant cells (Al-Hajj and Clarke 2004). Sets of surface markers combined with techniques such as flow cytometry allow sorting of marker-positive and marker-negative subpopulations (Clevers 2011).

Marker	Expression in healthy tissue	Marks cancer stem cells in
CD19	Broadly on B lymphocytes	B cell malignancies
CD20	Broadly on B lymphocytes	Melanoma
CD24	Broadly on B cells; neuroblasts	Pancreas/lung cancer, negative on breast cancer
CD34	Hematopoietic and endothelial progenitors	Hematopoietic malignancies
CD38	Multiple stages of B and T cells	Negative on AML
CD44	Broadly on many tissues	Breast/liver/head and neck/ pancreas cancer
CD90	T cells, neurons	Liver cancer
CD133	Proliferative cells in multiple organs	Brain/colorectal/lung/liver cancer

Figure 2. Surface markers which are used to sort CSCs from a tumor bulk. Various clusters of differentiation (CDs) are normally found in healthy tissues, but have also been identified as markers for CSCs. Figure from Clevers 2011.

Nevertheless, the existence of CSCs is still controversially discussed (Tirino et al. 2013). There are technical and logistical challenges in isolating and identifying CSCs, especially regarding assays to confirm stem-cell like properties (Tirino et al. 2013). Therefore, an alternative name for this cell population, which draws the focus away from the stem cell similarity, is “tumor-initiating cells” (Reya, Tannishtha, et al. 2011).

Nowadays, there are two models (Fig. 3) which are proposed to explain tumor growth and heterogeneity (Beck and Blanpain 2013). In the stochastic model, all tumor cells are considered as equipotent (Beck and Blanpain 2013). In the CSC model, a hierarchic organization, as we can find it in normal tissues containing stem cells, is proposed (Beck and Blanpain 2013). Currently, neither the stochastic model nor the CSC model can be regarded as completely wrong or right. Both are an attempt to explain tumor growth and heterogeneity.

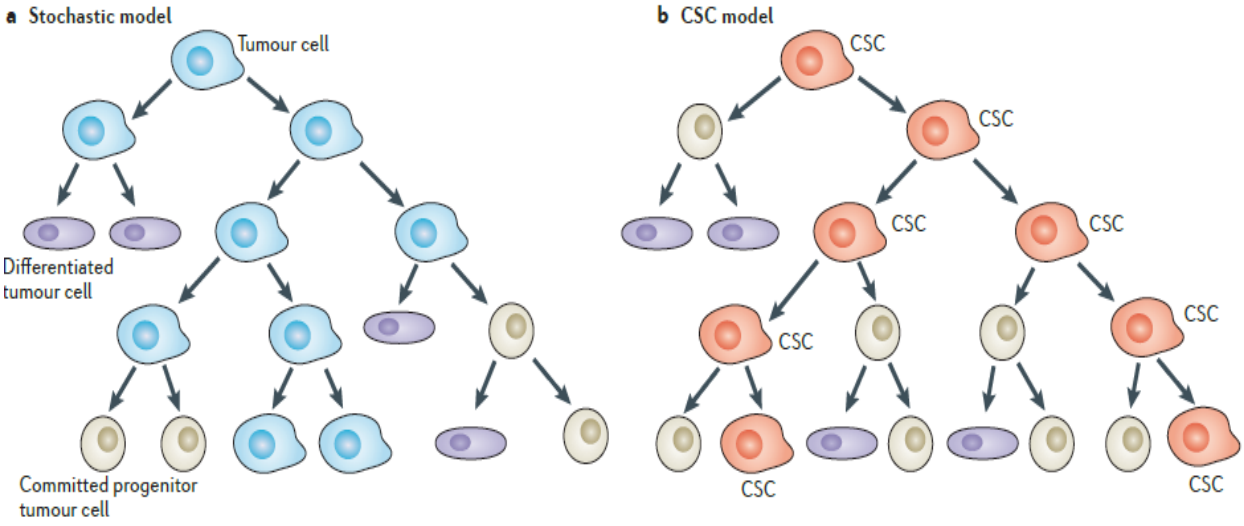

Figure 3. Models of tumor growth. **a)** In this model, any tumor cell type is able to form a new tumor. To fuel tumor growth a part of the tumor cells proliferative stochastically whereas other cells differentiate, which leads to heterogeneity. **b)** Just a few cells of the whole tumor bulk have the ability to initiate a tumor and drive progression as they have the capability for long-term self-renewal. These cells divide asymmetrically, producing an identical daughter cell and a more differentiated cell. Committed progenitors with limited proliferative potential arise. They generate the vast majority of tumor bulk and can then terminally differentiate (figure from Beck and Blanpain 2013).

The importance of CSCs in the progression of the primary tumor is getting clearer, whereas the role of CSCs in metastases can still be regarded as obscure (Dalerba und Clarke 2007). There is growing evidence that CSC are connected to metastases, as a study from Hermann et al. (2007) showed that metastases derived from pancreatic tumors contained a subpopulation of migrating CSC from the primary tumor. Furthermore, Odoux et al. (2008) showed, by analyzing colon cancer and corresponding liver metastases of patient samples, that CSCs could not only be found in the primary tumor, but also in metastases.

CSC are more resistant to various commonly used chemotherapeutic treatments and therefore have the ability to trigger tumor relapse after therapy (Dick 2008). Thus, their efficient eradication in a therapeutical setting is mandatory (Dick 2008). Mechanisms which lead to a high survival capability of CSCs include for example expression of ABC drug pumps, resistance to DNA damage, and an enhanced expression of proteins with antiapoptotic properties (Clevers 2011). Most anticancer agents target cells with a high cell division rate (Tirino et al. 2013). In contrast, CSCs divide slowly and can remain quiescent for extended periods which contributes to therapy failure (Tirino et al. 2013). In consequence, this subpopulation of cells has attracted much attention in research and drug development (Chen et al. 2013).

Hence, developing analytical systems, for example reporter constructs, which enable us to selectively identify CSCs faster and easier, should be a priority. This promises a deeper insight into the mechanisms behind these malignancies, especially regarding tumor initiation, progression, metastasis formation and recurrence after therapy.

1.2. Developmental pathways in cancer

Physiological role and relevance in cancer

Cancer research has shown that malignancies hold aberrations in various signaling pathways, especially in those which normally play an important role in tissue homeostasis and embryonic development (Takebe et al. 2015). These include signal transduction via the evolutionarily conserved Notch, Hedgehog and Wnt pathways (Takebe et al. 2015). As these three pathways are involved in the determination of stem cell fate, their contribution to the progression of cancer stem cells is discussed (Al-Hajj and Clarke 2004), explaining their relevance in understanding tumor biology (Takebe et al. 2015). Considered clinically, deregulation of these pathways lead e.g. to a poorer prognosis for patients e.g. in non-small cell lung cancer (Huang et al. 2008) and breast cancer (Liu et al. 2010).

Notch

Notch signaling (Fig. 4) represents a path for short range communication between two adjacent cells (Gu et al. 2012). In mammals, the notch system consists of four transmembrane receptors, Notch 1-4 (Uyttendaele et al. 1996), and 5 ligands called as Jagged proteins (JAG1 and JAG2) and delta-like proteins (DLL1, DLL3 and DLL4) (Karamboulas and Ailles 2013).

The role of Notch signaling in cancer is at present not fully understood (Espinoza and Miele 2013). The pathway is considered to contribute to tumorigenesis on the one hand by inhibiting differentiation and apoptosis of cells, and on the other hand by promoting cell proliferation (Espinoza and Miele 2013).

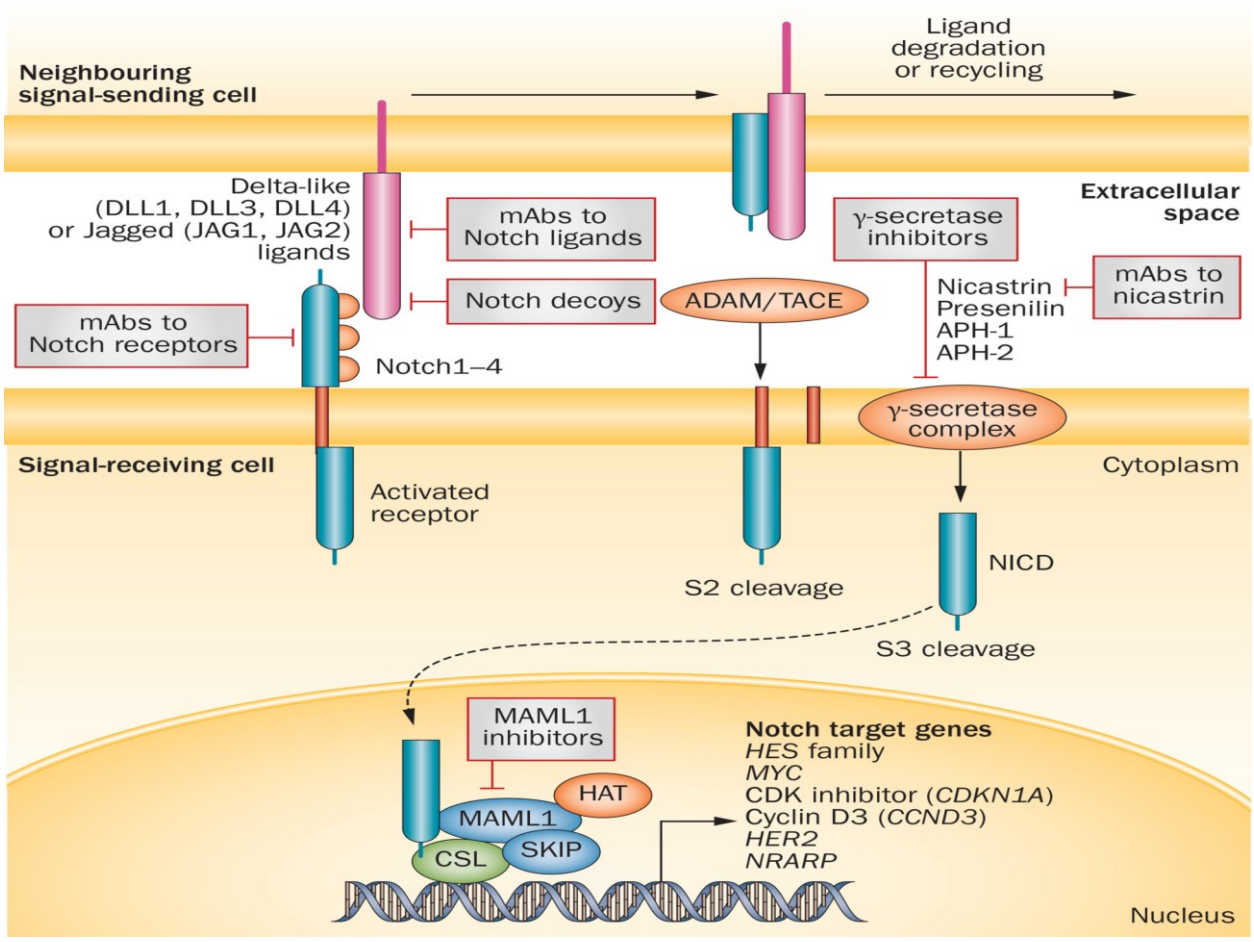

Figure 4. Canonical Notch signaling pathway. The signal-sending cell offers a transmembrane ligand (DLLs and JAGs) to a transmembrane receptor (Notch 1-4; different tumors express different notch receptors) on a signal-receiving cell. Ligand-receptor-interaction leads to a two-step proteolytic cleavage of the receptor. The metalloproteinase ADAM, which is also called tumor necrosis factor-α converting enzyme (TACE), is activated and catalyzes the first step of the cleavage (extracellular domain). Afterwards, γ-secretase attacks the intracellular rudiment of the receptor, which leads to the release of a fragment that can interact with nuclear factors and therefore regulate target gene expression (figure from Takebe et al. 2015).

Hedgehog

In mammals, there are three proteins which activate Hedgehog signaling (Fig. 5): sonic hedgehog (shh), desert hedgehog (dhh) and indian hedgehog (ihh). Sonic hedgehog is best characterized (Athar et al. 2006). It is involved in the genesis of the brain and spinal cord but also the eye, the limbs and craniofacial structures, and is therefore one of the most essential signaling pathways in embryonic development (Ming et al. 1998). Thus, aberrations in this pathway cause several clinical disorders ranging from congenital malformations to sporadic tumors (Ming et al. 1998).

Watkins, D. Neil, et al. (2003) could show an important role of this pathway in the regeneration of airway epithelium after induced damage, but also in carcinogenesis, as they could demonstrate high activation of the Hedgehog signaling in small-cell lung cancer. Furthermore, loss-of-function mutations in the Hedgehog pathway have been described in medulloblastoma, basal cell carcinoma and rhabdomyosarcoma (Ng and Curran 2011). In conclusion, aberrant Hedgehog signalling contributes to cancer proliferation, survival and angiogenesis (Takebe et al. 2015).

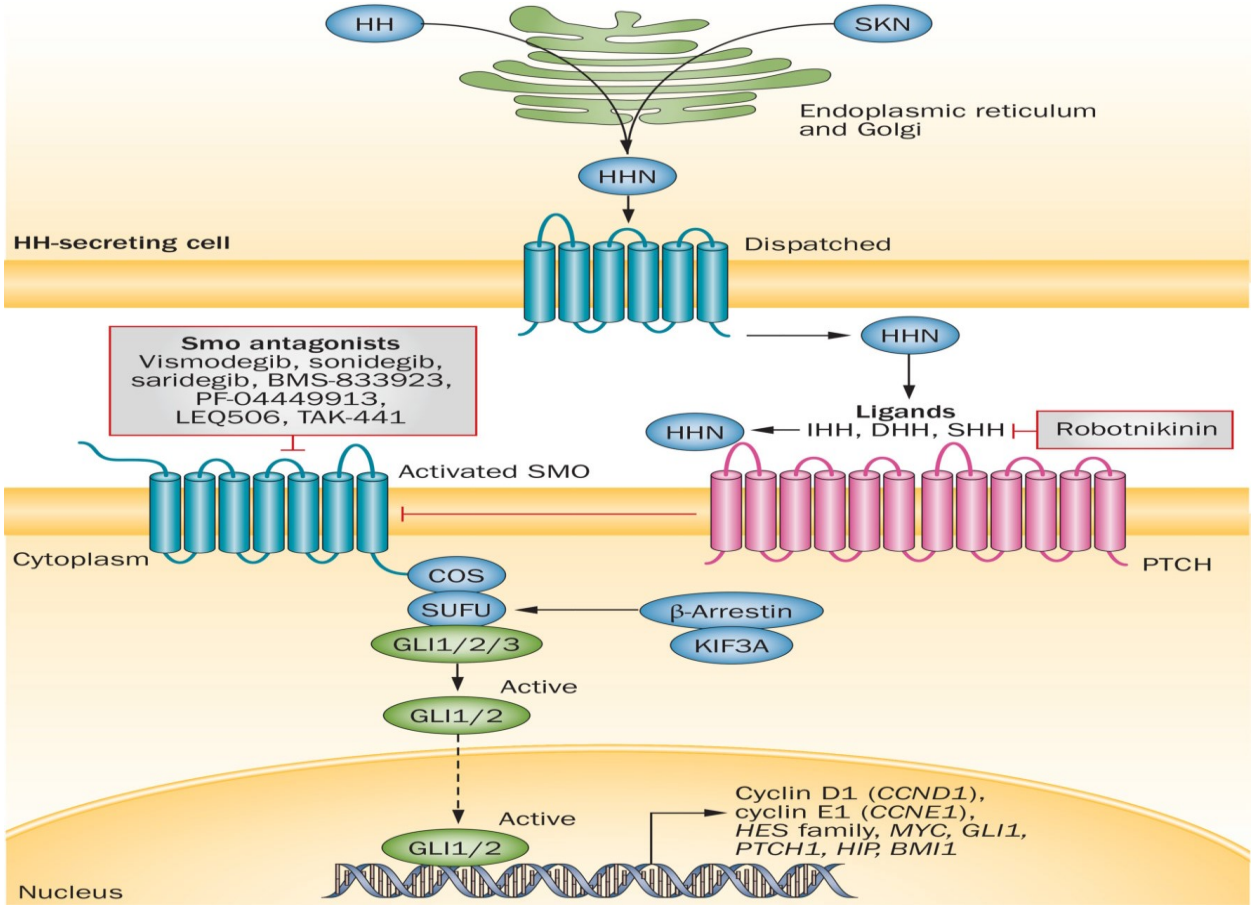

Figure 5. Hedgehog (HH) signaling pathway. The HH-secreting cell releases a ligand (shh, dhh, ihh) which binds to a transmembrane protein on the signal receiving cell, called PTCH (patched receptor). In the absence of a ligand, PTCH suppresses the activity of SMO, another transmembrane protein, and works therefore as a tumor suppressor. After binding of an HH-ligand to PTCH, SMO is activated and triggers a downstream signal cascade which leads to the activation and nuclear localization of GLI transcription factors. As a result expression of HH target genes is driven (figure from Takebe et al. 2015).

Wnt

In mammals, up to 20 secreted Wnt proteins exist (Clevers 2006). Wnt receptor activation plays a role in three different pathways: the canonical, the non-canonical and the Wnt/Ca^{2+} pathway (Clevers 2006). We focus on the canonical signal transduction (Fig. 6), as it is implicated in tumorigenesis and also the best understood of the three (Takebe et al. 2015). Wnt signaling is involved in embryonic development, homeostasis of adult tissues, and is also associated with cancer development when it is deregulated (Clevers 2006). The role of the pathway in carcinogenesis has been known for a long time. Rubinfeld, Bonnee, et al. (1993) showed that the cytoplasmic APC Protein (adenomatous polyposis coli), which triggers colon cancer progression when it is mutated, shows interaction with β-catenin, a main element of the Wnt signal transduction cascade. Malanchi et al. (2008) showed that increased β-catenin signaling in human squamous cell carcinomas plays an important role for the preservation of cells with CSC phenotype.

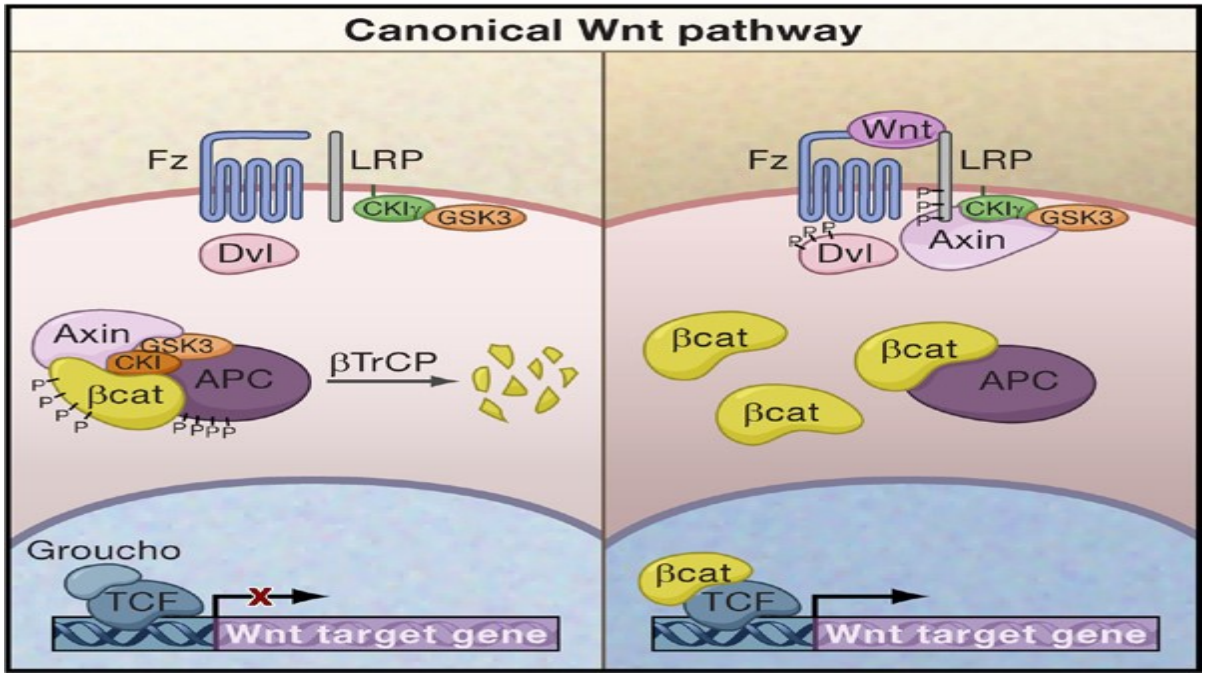

Figure 6. Left: In the absence of a Wnt ligand, a multiprotein destruction complex consisting of Axin, adenomatous polyposis coli (APC), and glycogen synthase kinase 3β (GSK3β) targets β-catenin for ubiquitination and proteasomal degradation. Right: A Wnt ligand binds to a receptor complex, consisting of „Fz" (Frizzled, G-protein-coupled receptor) and a co-receptor „LRP5/6" (low-density lipoprotein receptor-related protein). Ligand binding leads to the activation of the cytoplasmic phosphoprotein „Dv1". Activated Dv1 blocks Axin-mediated β-catenin phosphorylation and therefore ubiquitination and proteasomal degradation, resulting in accumulation of cytoplasmic β-catenin. Consequently, β-catenin can now translocate to the nucleus and interact with TCF–LEF transcription factors (Clevers 2006).

In conclusion, these three pathways affect physiologic and tumorigenic processes (Katoh 2007). Each pathway should not be regarded isolated, but rather as a component of a complex cellular signaling network (Katoh 2007). Substances which interfere with the signal transduction of Notch, Hedgehog and Wnt represent a novel approach to treat cancer (Takebe et al. 2011). Nevertheless, rational drug design turns out to be complicated because of the complex interplay between these three and other cellular signaling pathways (Takebe et al. 2011).

As such, a reporter construct to dynamically assess the activity of Notch, Hedgehog and Wnt in cancer, especially regarding their interplays, would be highly valuable for gaining more insight into cancer development and to find new approaches regarding therapies.

1.3. Components of our reporter construct

Reporter constructs can be used to visualize promoter activity of genes of interest (Hakkila et al. 2002). Nowadays, bioluminescent and fluorescent assays find broad application in molecular imaging (Troy et al. 2004). For our project, fluorescence imaging was chosen, as the development of various series of fluorophores allow to perform multi-label experiments (Ozawa et al. 2013).

We have built reporter plasmids to visualize signal transduction of developmental pathways in cancer. Each of these so-called entry vectors consists of a pathway-specific promoter, a specific fluorophore and a MuLE backbone (Fig. 7). Furthermore, mutated vectors containing functionless promoters and fluorophores with a different color compared to the unmutated version were built.

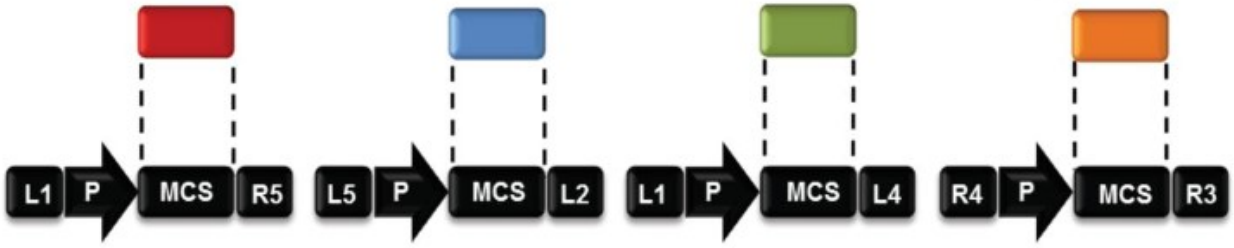

Figure 7. Entry vectors. A pathway specific promoter (P) and a fluorophore (colored blocks) are flanked by so called attL and attR sites from MuLE backbone vectors. The MuLE system will be described in the next chapter. A so-called MCS (multiple cloning site) allows to clone desired genetic inserts into the vector. Transcription factors from activated pathways will then interact with the corresponding promoter, leading to a measurable expression of the downstream fluorophore. In the mutated versions, the promoter region is altered and shows therefore no significant interaction with pathway specific transcription factors (figure from Albers et al. 2015).

The fluorophores

The use of fluorescent proteins in molecular imaging is a technique to noninvasively monitor biological processes like gene expression, tumor growth and metastasis (Lecoq und Schnitzer 2011). Generally, there are some requirements a fluorophore should have for a successful use (Shaner et al. 2005). We focused on:

1) High expression efficiency without toxicity.
2) A bright and sufficient signal above autofluorescence for significant detection.
3) Satisfying photostability to be imaged during the experiment.
4) Minimal crosstalk regarding excitation and emission wavelengths when used in multi-label experiments, such as in our approach.

- ***iRFP713:*** The infrared fluorescent protein is based on a bacteriophytochrome, incorporating biliverdin as the chromophore (Shu et al. 2009). It shows high expression in mammalian cells, as biliverdin is the initial intermediate in heme catabolism and therefore intracellularly ubiquitous, and excitation and emission maxima (684 and 708nm) penetrate tissues well (Shu et al. 2009).

- ***Tdtomato:*** The red tandem dimer Tdtomato is a derivate from DsRed, a red fluorescent protein discovered in the coral *Discosoma sp.* (Graewe, Stefanie, et al.

2009). The fluorophore shows excitation maximum at 554nm and emission maximum at 581nm (Shaner et al. 2004) and a very high photostability (Shaner et al. 2005).

- ***mTurq2:*** mTurquoise2 (excitation maximum: 434nm;emission maximum: 474nm) represents a subsequent development of the enhanced cyan fluorescent protein (ECFP) (Goedhart et al. 2012). It is characterized by a very high brightness, good photostability, fast maturation and the highest quantum yield measured for a monomeric fluorescent protein (Goedhart et al. 2012). Goedhart et al. (2012) showed via mutagenesis in their rational design of mTurq2 that the amino acids which surround the chromophore, a tryptophan, are essential for the lifetime and intensity of the fluorescence.

- ***EGFP:*** The enhanced green fluorescent protein is an optimized derivate of the green fluorescent protein, which was discovered in the *Aequorea* jellyfish (Tsien 1998). It shows excitation maximum at 488nm and emission maximum at 507nm (Shaner et al. 2005).

The Promoters

- ***Wnt pathway:*** TopFlash and FopFlash were used. TopFlash is a β -catenin-TCF (T cell factor)-dependent promoter, which contains a repetitive TCF-binding site (Chtarbova et al. 2002). In contrast, FopFlash has a mutated binding site for the transcription factor and shows therefore much less transcriptional activity (Korinek 1997).

  The Wnt promoter CTP4 was also used. It represents an optimized synthetic β-catenin-dependent promoter, containing a high number of TCF binding sites (Lipinski et al. 2004). There is no mutated CTP4 version available.

- ***Hedgehog pathway:*** hPTCH1 and hPTCH1mut were used. The hPTCH1 promoter contains a binding site for GLI transcription factors, mediators of the Hedgehog signaling pathway (Winklmayr et al. 2010). The available mutated version has an altered binding site, leading therefore to a lower transcriptional activity.

- ***Notch pathway:*** CBF and CBFmut were used. The CBF promoter contains a binding site for the transcription factor CBF1, which is a central component of the Notch signaling pathway, as it interacts with NICD (Notch Intracellular Domain) and afterwards with the DNA (Nowotschin et al. 2013). The mutated version features mutated NICD binding sites.

To conclude, seven vectors were built for three pathways, whereby the CTP4 construct was not used for further work. In each case one normal and one mutated version was necessary, to have a setup for the later testing of promoter pathway-specificity.

The final reporter construct contains the three entry vectors with non-mutated promoters (TopFlash, hPTCH, CBF) to visualize Wnt, Hedgehog and Notch signaling in cancer cells. Furthermore, the reporter construct includes an additional component. This fourth entry vector consists of the constitutive active cytomegalovirus (CMV) promoter (Wilkinson und Akrigg 1992) and the fluorophore EGFP, with the aim to have one color which is always expressed. These four vectors were recombined into a so-called destination vector via the MuLE system and afterwards packaged into a lentivirus to form the final reporter construct.

The mutated vectors differ from their non-mutated counterparts by the fluorophores used downstream of their altered promoters. The promoter specificity setup for each pathway consists of a MuLE based recombination reaction of the normal and mutated vector into a destination vector. After construction of the viruses, these systems will be used to transduce cancer cells. Afterwards, pathway specific activators will be added. Pathway-specificity is present when only the non-mutated promoter subsequently becomes activated, leading to a measurable expression of the downstream fluorophore, which is detectable e.g. by flow cytometry.

1.4. Introduction of the MuLE cloning system

The “Multiple Lentiviral Expression” (MuLE) system, based on MultiSite Gateway cloning, enables the generation of policistronic, replication-incompetent lentiviruses for the transduction of mammalian cells (Albers et al. 2015). Lentiviral vectors are modified versions of HIV-1 (human immunodeficiency virus) (Cockrell und Kafri 2007). In contrast to previously used simple retroviruses, lentiviruses have the ability to transduce dividing as well as non-dividing cells, which enables them to target various tissues and cell types (Cockrell und Kafri 2007). Furthermore, lentiviral vectors show qualities of a high transduction efficiency and stable, long-term transgene expression (Blömer, Ulrike, et al. 1997).

MultiSite Gateway cloning is a technique which enables the user to easily transfer genes between entry plasmid vectors into a large number of different destination plasmid vectors (Katzen 2007). The cloning method is based on a site-specific recombination reaction normally used by the lambda phage (Katzen 2007). It is characterized by specific attachment sequences found on the phage DNA, called attP(hage), and on the chromosome of the bacteria, called attB(acteria) (Katzen 2007). These recombination sites are called attL and attR after integration of the phage DNA into the bacterial chromosome (Katzen 2007). The Gateway system is using these attL and attR sites (Fig. 8). This technique represents a high-throughput approach, that can be used to transfer a variety of DNA segments highly specific and simultaneously into a vector background (Hartley 2000). As the reaction occurs site-specific, it will generate clones which carry the inserts of interest always in the right orientation (Hartley 2000).

To summarize, the MuLE system consists of a cloning method which enables the user to build expression clones containing different genes. Afterwards, the resulting so-called destination vector is used for generating the lentivirus for the transduction of mammalian cells. The MuLE developers generated a toolbox of building-blocks, containing a series of 104 Multisite Gateway compatible entry vectors (Albers et al. 2015). These can be used for an overnight attL-attR recombination reaction, utilizing up to four entry vectors getting recombined into a destination vector (Albers et al. 2015). We used pMuLE entry vectors which contained a so-called MCS (multiple cloning site) flanked by attL-attR sites,

and an antibiotic resistance (Kanamycin) for bacterial selection after transformation (Albers et al. 2015).

In our project, the MuLE system was used to recombine the four above mentioned entry vectors (chapter 1.3.) into a destination vector called Lenti Neo Dest. This vector contains the sequence information necessary for the generation of the virus, a different antibiotic resistance (Ampicillin) and a ccdb toxin gene for easier selection of the proper plasmid after the attL-attR recombination reaction.

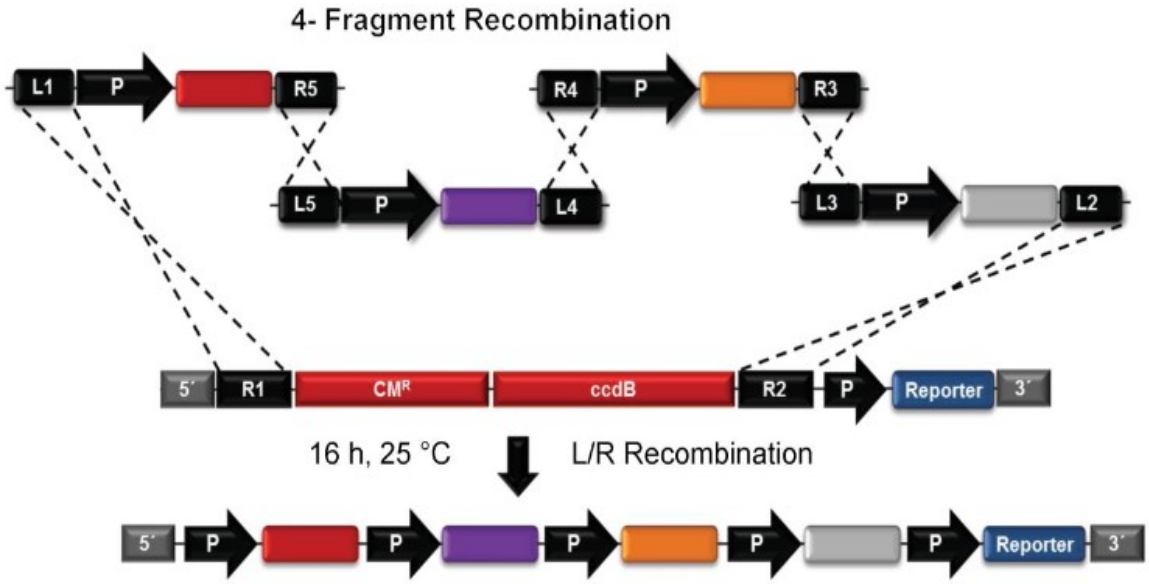

Figure 8. Schematic overview of the MultiSite Gateway–based recombination cloning of four MuLE entry vectors into a lentiviral destination vector to generate a multicistronic lentiviral expression vector. Site-specific recombination reactions between the attL and attR sites mediate the correct orientation of each insert. In this example, the destination vector contains a chloramphenicol resistance gene (CM^R) and a ccdB toxin gene for easier selection after transformation. Aside from the four-fragment recombination reaction for the reporter construct, two-fragment recombination reactions, using non-mutated and mutated vectors, were performed for the promoter specificity setup. (figure from Albers et al. 2015).

2. Aim of the thesis

The idea behind this project was to generate a system which enables us to dynamically visualize the activity of developmental pathways in cancer. These signal transduction cascades are likely deregulated in a tumor and lead to a poorer prognosis for patients, as they contribute to cancer proliferation, survival and angiogenesis.

Our reporter construct consists of pathway specific promoters and downstream fluorophores to visualize the signal transduction of the Wnt, Hedgehog and Notch pathway. Therefore, so-called entry vectors for each pathway were built. They were recombined into a destination vector, which was further packaged into a lentivirus. The finished virus then enables the transduction of e.g. cancer cells.

The aim of this thesis was to design and perform workflows regarding the building of these entry vectors. Afterwards an analytical setup, consisting of various steps, was used to detect if the right construct was generated.

We expect that this reporter system could be used to get a deeper insight into the activity of these three pathways in malignant cells. Possible applications could be for example cell sorting, CSC identification, visualization of pathway interplays and supporting drug development.

3. Material and Methods

3.1. Technical equipment

Table 1. List of technical equipment.

Name	Product number, supplier, location
Arium® pro ultrapure water systems	Sartorius®, Göttingen, GER
Biorad PowerPac 200 Electrophoresis Power Supply	4100-101-19, BIO-RAD®, Hercules, CA, USA
ChemiDoc™ XRS+ System	1708265, BIO-RAD®, Hercules, CA, USA
C1000 Touch™ Thermal Cycler	1851148, BIO-RAD®, Hercules, CA, USA
Drying chamber	940557, WTB BINDER®, Tuttlingen, GER
Eppendorf ThermoMixer® C	460-0222, VWR®, Radnor, PA, USA
Heraeus Megafuge 16R Refrigerated Centrifuge 230V	75004270, ThermoFisher Scientific®, Waltham, MA, USA
Herasafe™ KS, Class II Biological Safety Cabinet	51022484, ThermoFisher Scientific®, Waltham, MA, USA
MaxQ Mini 4450 Shaker	SHKA4450-1CE, ThermoFisher Scientific®, Waltham, MA, USA
Microcentrifuge, refrigerated, Micro Star 17R	VWRI521-1647, VWR®, Radnor, PA, USA
Precision balance	Sartorius®, Göttingen, GER
Spectrophotometer, GeneQuant™ 100	SCLI80-2130-00, VWR®, Radnor, PA, USA
Spectrophotometer, NanoVue™ Plus	28-9560-57, VWR®, Radnor, PA, USA
Sub-Cell® GT Horizontal Electrophoresis System	1704401, BIO-RAD®, Hercules, CA, USA
TC-412 Thermal Cycler	FTC41H2D, Techne®, Minneapolis, MN, USA

Ultrasonic Cleaner (waterbath)	470105-414, VWR®, Radnor, PA, USA
Wide Mini-Sub® Cell GT Horizontal Electro-phoresis System	1704405, BIO-RAD®, Hercules, CA, USA

3.2. Kits

If not mentioned otherwise all kits were used as per the manual. Only "ThermoFisher Scientific®" (Waltham, MA, USA) kits were used.

Table 2. List of kits.

Name	Specifications
GeneJET Gel Extraction and DNA Cleanup Micro Kit	#K0831 Lot: 00362185
GeneJET Plasmid Miniprep Kit	#K0503 Lot: 00191089
GeneJET Plasmid Maxiprep Kit	#K0492 Lot: 00368794 and 00398546

For higher elution yields, the elution step was split as follows:
The total amount of elution buffer was divided into two equal portions. Afterwards, the sample was eluted twice following the procedure of the manual. This led to DNA yields up to two times as much compared to samples which were just eluted once.

3.3. Gel materials

Table 3. Gel materials.

Name	Ingredients, product number supplier, location
SB-Buffer (sodium borate), self-made (20x)	○ 8g NaOH: A6829,1000, PanReac AppliChem®, Maryland Heights, MO, USA ○ 48g Boric acid: B6768, Sigma-Aldrich®, St. Louis, MO, USA ○ 1000ml ddH_2O: from arium® pro ultrapure water systems ○ 6M HCL: hydrochloric acid 30721-M, Sigma-Aldrich®, St. Louis, MO, USA
Agarose SERVA for DNA Electrophoresis	11404.04, Serva®, Heidelberg, GER
2-Log DNA Ladder (0.1-10.0 kb)	N3200L, New England BioLabs®, Ipswich, Massachusetts, USA

Instruction to make SB-buffer: NaOH and Boric acid were weighed in and dissolved in 800ml ddH_2O (magnetic stirrer for faster dissolution). pH was adjusted to 8,0 with 6M HCL and the mixture filled up to 1000ml with ddH_2O. Finally, the buffer was autoclaved.

3.4. Media and Agar

Table 4. Media and Agar.

Name	Ingredients, product number supplier, location
LB-Media (lysogeny broth)	○ Bouillon LB, X964.3, Lennox®, Richardson, TX, USA ○ Agar LB, X965.3, Lennox®, Richardson, TX, USA
SOC-Media (Super Optimal broth with Catabolite repression), self-made	○ 980ml ddH_2O: from arium® pro ultrapure water systems ○ 5g 0,5% yeast extract: 48503.01, Serva®, Heidelberg, GER ○ 20g 2% Tryptone: 95039-1KG-F, Sigma-Aldrich®, St. Louis, MO, USA ○ 10mM Nacl (58,44g/mol): A2942,5000, PanReac AppliChem®, Maryland Heights, MO, USA ○ 2,5mM KCL (74,55g/mol): A2939,1000, PanReac AppliChem®, Maryland Heights, MO, USA ○ 20mM $MgSO_4$ (120,37g/mol): M2643, Sigma-Aldrich®, St. Louis, MO, USA ○ 20mM Glucose (180,16g/mol): 3,6g in 20 ml of ddH_2O, 1.08337.1000, Merck®, Darmstadt, GER

3.5. Enzymes and reagents

Table 5. Fast digest Restriction Enzymes.

Name	#	*Lot*
BamHI	FD0054	00269078
BglII	FD0083	00269800
BcuI (=SpeI)	FD1254	00359558
Eco32I	FD0303	00380232
EcoRI	FD0274	00274229
HindIII	FD0504	00250958
KpnI	FD0542	00360782
NotI	FD0594	00363432
PvuII	FD0634	00418979
SacI	FD1133	00346553
SalI	FD0644	00202111
ScaI	FD0434	00178483
XbaI	FD0684	00269798

All enzymes ordered from ThermoFisher Scientific (Waltham, MA, USA). Additionally used for restriction digests: FD Green buffer (10x) from ThermoFisher Scientific (#B72; Lot:00198331).

Table 6. PCR and molecular biology reagents.

Name	Lot	Product number, supplier, location
Q5® High-Fidelity 2X Master Mix	0111512	M0492S, New England BioLabs®, Ipswich, Massachusetts, USA
DreamTaq Green PCR Master Mix (2X)	00389423	K1081, ThermoFisher Scientific®, Waltham, MA, USA
Blunt/TA Ligase Master Mix	0231602	M0367L, New England BioLabs®, Ipswich, Massachusetts, USA
T4 DNA Ligase kit	00029985	M0202S, New England BioLabs®, Ipswich, Massachusetts, USA
10x T4 DNA Ligase Reaction Buffer	00027707	B0202S, New England BioLabs®, Ipswich, Massachusetts, USA
Shrimp Alkaline Phosphatase (rSAP)	00027802	M0371S, New England BioLabs®, Ipswich, Massachusetts, USA
PEG 4000	00029741	Fermentas, Burlington, Ontario, CAN
ATP 100mM	00034391	R0441, Fermentas, Burlington, Ontario, CAN

3.6. DNA material

- MuLE Vectors , as well as plasmids, were all ordered from AddGene (Cambridge, MA, USA) except for hPTCH1-luc, which was a kind gift from Fritz Aberger, University of Salzburg.
- Primers, designed by Julia Maier, were all ordered from MicroSynth (Balgach, Switzerland).

Table 7. Primers.

Primer	Sequence
KpnI-TOPF_F	CGCACGCACTAGGTAC-CGAGCTCTTACGC
TOPF-PvuII_R	GCCCAGCTGATGGTGGCTTTAC-CAACAGT
PvuII-iRFP_F	ATAACAGCTGCACCATGGCG-GAAGGAT
iRFP-SpeI_R_TOPF	TGGAACTAGTGACTCAC-TCTTCCATCACGC
KpnI-CTP4_F	CGTGGTACCCGATAA-GCTCTAGCCCCTAGC
CTP4-PvuII_R	GCCCAGCTGATGGTGGCTTTAC-CAACAGT
iRFP-XbaI_R_CTP4	CGGATCTAGAGACTCAC-TCTTCCATCACGC
HindIII-iRFP_F	TATTAAGCTTGATCTGCCGCCACCAT
KpnI-CBFREmut_F	GTACGTAGGGAGGTACCGAGCTCTT
CBFREmut-HindIII_R	TATAAGCTTGTGGATCCCCCAGCTTTT
HindIII-FOP_F	GCCCGAAGCTTTCGATAGGTAC-CTTACG

3.7. Methods

3.7.1. Bacterial Work

Used bacteria strain for all experiments: competent DH5alpha *Escherichia coli* (*E. coli*). Just in one case methylation insufficient *dam*- and *dcm*- negative bacteria were necessary: *E. coli* strain K12_ER2925. All steps, where contamination of the samples could occur, were done using a Herasafe™ KS, Class II Biological Safety Cabinet. Filter tips were used for DNA pipetting, and the samples were handled at 4°C.

- **Colony evaluation and picking**

Colonies growing on LB agar were examined after incubation at 37°C for 18-24 hours. Important characteristics are the shape, the size and the color of the colonies and how their bacterial neighborhood looks like. A colony which was big enough, similar shaped and colored as the surrounding colonies and single enough to be picked was chosen. Picking was done by using a pipette tip. The colony was carefully scrapped from the agar and the whole pipette tip was put into a 50ml falcon tube filled with media for further processing (see “pre-culture”). Careful picking is essential, as only one colony should be further processed and not a mixture of several.

- **Pre-culture**

Picked colonies from agar LB were incubated on a MaxQ Mini 4450 Shaker in 50ml falcon tubes filled with media at 37°C and shaking for 6 to 8 hours for optimal growth. A turbid suspension is the goal. 5 to 10ml of bouillon LB were used per falcon tube. For checking the viability of the bacteria, no antibiotics were added. For selective growth, antibiotics were added, dependent on the resistance gene. Per ml of bouillon LB 4µl of antibiotic were added. The pre-culture was then used for DNA yielding via Miniprep or for further growth steps (see “overnight-culture”).

➢ Overnight-culture

At least a quarter of an overnight flask (generally 1000ml total flask volume) was filled with bouillon LB. Additionally 4µl of antibiotics per ml LB were added for selective growth. Afterwards, 20µl from the pre-culture suspension was transferred into the culture flask. The flask was then incubated on a MaxQ Mini 4450 Shaker at 37°C and shaking overnight 16 to 18 hours for optimal growth. A turbid suspension is the goal.

On the next day, the bacterial suspension was directly used for doing a Maxiprep or further processed, to be stored at the 4°C, after a step of centrifugation of the bacteria into a pellet, and decanting of the supernatant.

➢ Transformation

All transformations were performed by using the so called “heat shock technique”, which is based on changes in temperature to make bacteria incorporate plasmid DNA. Additionally, the used bacteria were made chemically competent beforehand by Haider Sami.

DNA for the transformation of the bacteria was taken out of the fridge and put into an ice box where it thawed. SOC-Media (self-made, described in chapter 3.4) was taken out of the fridge and thawed in a water bath (Ultrasonic Cleaner) at 37°C for 0,5 hours or in a drying chamber for 3,0 hours. It is later needed as growing medium for the bacteria after the stressful heat shock. Enough 1,5ml Eppendorfs were prepared for the pipetting work afterwards and cooled in the fridge until they were needed. This improves transformation efficiency as the bacteria lose their chemical competence when they are exposed to above 4°C. Eppendorf ThermoMixer® C was set to 37°C and 700rpm. Agar LB plates with and without antibiotics were prepared and pre-heated in a drying chamber at 37°C, and are needed for the streaking of the transformed bacteria. When everything was prepared, the competent bacteria cells were taken out of the -80°C fridge and immediately put on ice were they thawed slowly. The following steps should be performed in a speedy fashion as to not let the bacteria warm up. In every prepared Eppendorf at least 50µl of competent bacteria and 2µl of plasmid DNA was pipetted. Beside our samples for the project, positive controls, negative controls and viability testing were always done. Fur-

thermore, transformations with bigger amount of material using 100µl of competent bacteria and 4µl of plasmid DNA were additionally done for all transformations. The idea behind this was to be on the safe side if the approaches with lesser material fail. After pipetting everything together, the samples were put on ice for 30 minutes. Afterwards, heat shock was done in a water bath at 42°C for 45 seconds. The last step of the technique was to incubate the samples for 2 minutes on ice. Then, 450µl of warm SOC-Media was added per Eppendorf and incubated on ThermoMixer® C for 45 minutes at 700rpm and 37°C. In the end the transformed bacteria were plated on the labeled and pre-heated LB agar plates. Plates were then incubated overnight in the drying chamber at 37°C for max. 24 hours. Colonies were examined, numbered and the plates were stored at 4°C. Additionally, marked colonies were picked for making a master plate, which contained all positive colonies, for further experiments.

➢ Master plate

A master plate is also an agar-antibiotic-plate, with the advantage that it contains all the colonies which were presumably successfully transformed during the experiment.
For this, a number grid was fixed on the agar-plate. Numbered colonies were picked from the transformation plates by using a fresh pipette tip for each colony (described in chapter "colony evaluation and picking") and transferred into the grid area with the right number. Afterwards the master plate was incubated overnight for max. 24h at 37°C and stored the next day at 4°C.

➢ Glycerol stocks

As a backup, up to 3 glycerol stocks per colony were done. After an overnight culture 500µl of bacterial suspension and 500µl of 50% glycerol in ddH_2O were mixed together in barcoded tubes. This was then stored at -80°C.

3.7.2. Plasmid work

DNA material and enzymes were always stored in an ice box during work. All steps, where contamination of the samples could occur, were done using a Herasafe™ KS, Class II Biological Safety Cabinet. Working with DNA was always done by using filter tips.

➢ PCR

Primer design was done by Julia Maier. Components for doing the PCR were pipetted together in PCR strip tubes. Less than 1,0ng of the DNA which had to be amplified was used for each PCR sample. Therefore, higher concentrated starting materials had to be diluted beforehand, using nuclease free water. Afterwards, 2,5µl of 10µM forward primer, 2,5µl of 10µM reverse primer and 25µl of Q5® High-Fidelity 2XMaster Mix (1000bp/30sec) were added. The whole mixture was then diluted up to 50µl using nuclease free water. TC-412 Thermal Cycler or C1000 Touch™ Thermal Cycler were used for performing the PCR.

For the calculation of PCR settings, the tmcalculator from New England BioLabs was used: http://tmcalculator.neb.com/#!/ (13.08.2017). Initial denaturation, different cycles and final extension were performed. Two different cycles were always programmed as we used primers with non-complementary, overhanging sequences. Such primers are normally used to add restriction sites to the amplified sequences. The first cycle of ten rounds was adjusted for binding of the complementary part of the primer without overhanging ends. The second cycle of 25 rounds enabled binding of the whole primer and DNA amplification. Times and temperatures during the cycles are dependent on the size of the insert which has to be amplified, primer sequences and polymerase efficiency. After PCR, samples were stored at 4°C.

➢ Colony PCR

This technique enables screening a lot of presumably positive colonies from a transformation experiment (described in chapter 3.6.1). Primers with complementary sequences to parts of bacterial plasmids are used to search for the right insert. Compared

to culturing every colony, miniprepping them and digestion the yielded DNA, this technique is much faster. 1,5 ml Eppendorfs were labelled and filled with 75µl nuclease free water each. A master plate from a transformation (described in chapter 3.6.1) was taken out of the fridge. By dibbing a pipette tip gently on the according spot of the master plate, the colonies were picked. Afterwards they were transferred into nuclease free water by putting the tip into the 1.5ml Eppendorf with the right number, squishing it around and pipetting up and down. To release the bacterial DNA, the Eppendorfs were then heated up in an Eppendorf ThermoMixer® C for 10 minutes at 95°C. The next step was to perform the PCR. Components for doing the PCR were pipetted together in PCR strip tubes. A master mix containing 10µl DreamTaq Green PCR Master Mix 2X (1kb/20-30seconds) + 0,5µl of 10µM forward primer + 0,5µl of 10µM reverse primer was made. In each tube of a strip, 9µl of cooked bacteria and 11µl of master mix were added. As positive control a parallel approach with the plasmid which contained the insert that we were looking for in the positive transformants was always done. This positive control consisted of 11µl of master mix, varying amounts of a dilution (<1ng) of the plasmid DNA and nuclease free water up to 20µl. PCR was then performed as described in the chapter “PCR”. After PCR, the samples were stored at 4°C or directly used for a gel electrophoresis (described later).

- **Restriction digest (RD) and Diagnostic restriction digest (DRD)**

In both cases, RD and DRD, a gel was the next step. In case of a restriction digest, gaining building blocks and thus a high DNA yiel was normally the purpose, e.g. for promoters, fluorophores and backbones. Additionally, RDs were used to prepare DNA elements for ligation reactions. Through the process of digestion, restriction sites were made sticky, enabling them to interact with their corresponding structures. In case of a DRD the gel was just examined and evaluated using ChemiDoc™ XRS+ System, as an analytical method at the end of every vector generation. In RDs higher amounts of plasmid DNA (0,5µg to 1µg) were used as we wanted to obtain the products afterwards via gel purification and elution. In DRDs 200ng of Plasmid per sample were utilized. Plasmid sequences were examined and the right restriction enzymes chosen. The plasmid DNA which should be digested and the restriction enzyme were pipetted together. 1µl of each

restriction enzyme (see table 5.), 2µl of FD Green buffer (10x), plasmid sample and nuclease free water to 20µl were mixed in PCR strip tubes. After pipetting everything together, the samples were put in a TC-412 Thermal Cycler for digestion. 120min at 37°C were followed by a heat inactivation for 10min at 80°C. The samples were then stored at 4°C or further processed.

➢ Ligation

Two and three fragment ligations were performed. Components for the reaction were pipetted together in a 1,5ml Eppendorf. 40ng of vector backbone was always used. Afterwards the amount of insert was calculated using this formula from http://nebiocalculator.neb.com/#!/ligation (13.08.2017):

$$\text{Insert Mass in ng} = 3 \times \left[\frac{\text{Insert Length in bp}}{\text{Vector Length in bp}} \right] \times \text{Vector Mass in ng}$$

Vector and inserts were pipetted together, 5µl of Blunt/TA Ligase Master Mix added and the whole mixture diluted with nuclease free water to 10µl. Additionally a recirculation control was always done, using 20ng of vector only, adding 2,5µl of Blunt/TA Ligase Master Mix and diluting with nuclease free water to 5µl. The purpose of doing this, was to see how strong the linearized backbone is able to recircularize without the proper inserts. All of the ligation reactions were later used for transformations. The recircularization control was done for estimating how many of the positive colonies are false positives, as the backbone carries the antibiotic resistance.

The samples were incubated at room temperature (25°C) for 120 minutes in an Eppendorf ThermoMixer®. Afterwards they were directly used for transformations or stored at 4°C. In one case the protocol was changed by using a Shrimp Alkaline Phosphatase (rSAP) treatment and different adjustments during the reaction. This treatment is done by using 1µg of plasmid DNA + rSAP + restriction enzymes. This procedure was necessary as a linearized backbone vector was used which had two identical restriction sites on both ends. As same restriction sites can pair with each other, the risk of recircularization was very high. The rSAP treatment should alleviate this problem.

➢ Gels

Ethidium bromide agarose gels between 0,5%w/v and 2%w/v, dependent on the size of the DNA fragments, were produced. An electrophoresis comb was fixed in the electrophoresis mold. Agarose SERVA for DNA Electrophoresis was weighed in a laboratory flask on a precision balance. 100ml of SD-buffer were added. After pivoting the flask a little bit, the mixture was heated up in a microwave until the agarose was dissolved. 4,2µl Ethidiumbromide were added and the liquid rapidly filled in the electrophoresis mold. After 30 to 45 minutes the comb was removed to reveal the pouches. The mold with the polymerized gel was transferred into an electrophoresis system. After filling the system with SB buffer, 10- 20µl of each sample and 1µl of 2-Log DNA ladder (0.1-10.0 kb) were pipetted into the pouches. Running conditions were set to 80 Volt for 1-2 hours. Imaging was done every 30 minutes using ChemiDoc™ XRS+ System. Pictures of the gel were made and saved for further evaluation, or a gel cutting was performed.

➢ Gel cutting and elution

If post-processing of digested DNA fragments was desired, a gel cutting was done. This technique was performed by visualizing the separated nucleic acid bands in the gel via UV light of the ChemiDoc™ XRS+ System. Then, the bands of interest were cut out by using a scalpel. The gel fragments were then stored in a 1,5ml Eppendorf at 4°C or directly eluted. Gel elution was performed using a GeneJET Gel Extraction and DNA Cleanup Micro Kit.

➢ DNA measurement for Mini/Maxiprep and gel elution

Measuring DNA concentrations was done by using a NanoVue™ Plus spectrophotometer. 2µl of sample was pipetted onto the according area of the photometer and measured against elution buffer as the blank. Measurements were repeated 2-3 times and mean value was calculated.

4. Results

Table 8. Overview of all relevant constructs.

Pathway	Name	Mu-tant	Origin	Reporter construct
Wnt	TOPFlash_iRFP_L5-L4	☒	Self-made	☑
Wnt	CTP4_iRFP_L5-L4	☒	Self-made	☒
Wnt	FOPflash_mTurquoise_R4-R3	☑	Self-made	☒
Notch	CBF_tdtomato_L3-L2	☒	Julia Maier	☑
Notch	CBFmut_iRFP_L1-L4	☑	Self-made	☒
Hedgehog	hPTCH1_mTurquoise2_R4-R3	☒	Julia Maier	☑
Hedgehog	hPTCH1mut_iRFP_L1-L4	☑	Self-made	☒
Constitutively active	CMV_EGFP_L3-L2	☒	Addgene	☑

4.1. pMuLE_ENTR_TOPflash_iRFP_L5-L4

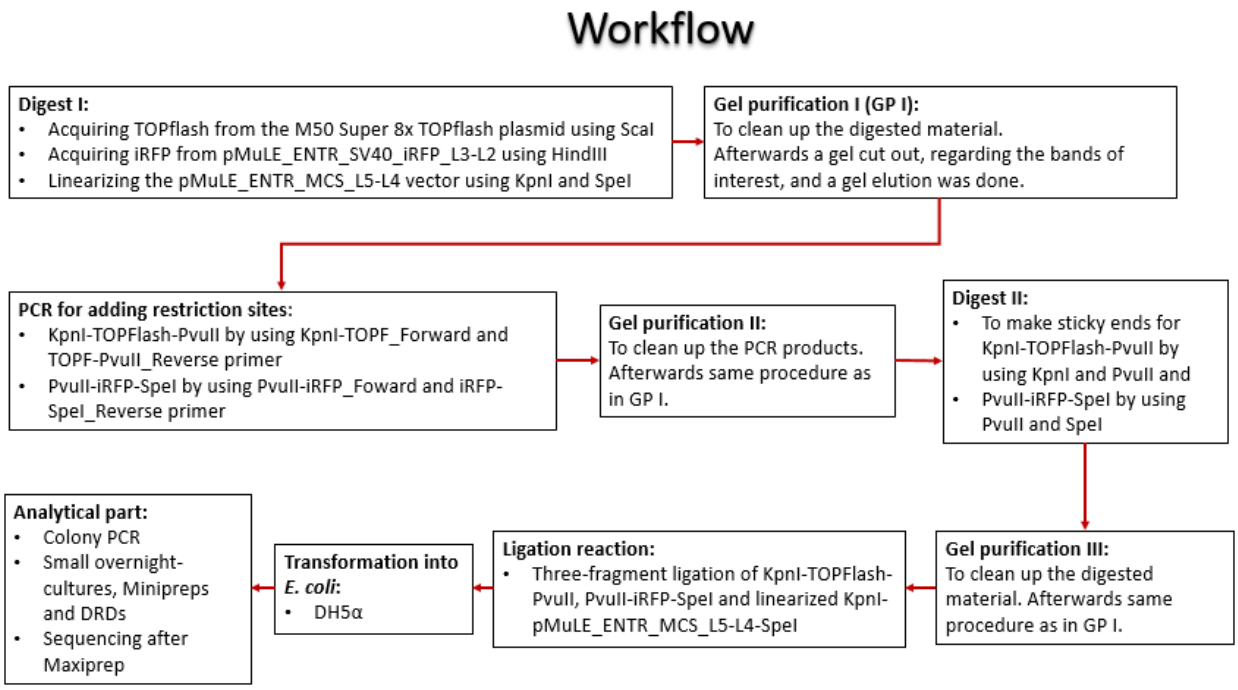

Figure 9. Overview of the steps to build the TOPflash_iRFP_L5-L4 construct.

TOPflash_iRFP_L5-L4 is a Wnt dependent entry vector used for the reporter construct. It consists of a pMuLE backbone vector with an attL5 and attL4 side, the TCF dependent promoter TOPflash and the downstream fluorophore iRFP.

Digest I: Procedure done as in Fig. 9.

Gel purification I: Promoter and fluorophore were purified in the same gel, linearized ENTR_MCS in a different one. To easier differentiate between ENTR_MCS uncut (2665bp) and the cut version (2651bp), both were sent through the same gel in neighboring lanes.

- *ScaI-TOPflash-ScaI: 512bp (0,7% agarose gel,1h): 10,1ng/µl*
- *HindIII-iRFP-HindIII: 1006bp (0,7% agarose gel, 1h): 15,6ng/µl*
- *KpnI-ENTR_MCS-SpeI: 2651bp (0,7% agarose gel, 40 mins): 79,1ng/µl*

PCR for adding restriction sites: The aim of this step is to equip the building elements with the correct restriction sites for the later performed ligation reaction. The backbone vector was linearized using KpnI and SpeI. As TOPflash and iRFP were cut out from their origin plasmids with different restrictions enzymes, their sequences had to be modified using the right primers.

Gel purification II: To get rid of primers and unwanted bands, caused by primer unspecificity, the PCR products were cleaned up using an agarose gel. Afterwards a gel cut out, gel elution and DNA value measurement were performed. Values after gel elution were at:

- *KpnI-TOPFlash-PvuII: 275bp (1,0% agarose gel,1h): 48,5ng/µl*
- *PvuII-iRFP-SpeI: 978bp (1,0% agarose gel,1h): 91,5ng/µl*

Digest II: This step is necessary to get sticky ends. By PCR added restriction sites are digested, which enables them to pair afterwards with their corresponding structures during the ligation reaction.

Gel purification III: This last purification step was done to clean up the products from "Digest II". The sticky-ended building elements were sent through a gel, cut out and eluted. Results from the DNA measurement were as followed:

- *KpnI-TOPFlash-PvuII: 253bp (1,0% agarose gel, 40mins): 28,5ng/µl*
- *PvuII-iRFP-SpeI: 954bp (0,7% agarose gel, 40mins): 63,0ng/µl*

Ligation reaction: Procedure done as in Fig. 9. Furthermore, the cut backbone KpnI-pMuLe_ENTR_MCS_L5-L4-SpeI and ligase were mixed to have a recirc. control.

Transformation: All transformations were performed using competent DH5alpha *E. coli*. As positive controls 2µl of pGluc and 2µl of uncut pMuLE_ENTR_MCS_L5-L4 were used. pGluc represents a plasmid that contains an Ampicillin resistance gene and shows a very high transformation efficiency. It was used as positive control for transformation. The uncut backbone is a plasmid which carries the antibiotic resistance for Kanamycin. As bacteria can easier take up cyclized DNA, transformations should work, and growth on agar-

antibiotic-plates should be detectable. To estimate how many positive colonies were false positives, 2µl from the ligated recirc. control were also used in the transformation approach. Furthermore, just competent cells without any added plasmid were used to have a testing for viability and for random resistance. Four approaches with the three-fragment ligation TOPflash_iRFP_L5-L4, using two times 2µl and two times 4µl of plasmid DNA, were done. Afterwards all transformations were plated on the according LB plates. After incubation, the plates were examined. Transformation success was satisfactory, with over 110 positive-colonies on the ligation plates and only three colonies on the recirc. control plate. In the end, positive colonies were transferred on master plates.

Analytical part: As there were so many hits a colony PCR was done to rapidly screen the positive colonies, using primers for the iRFP insert: PvuII-iRFP_Forward and iRFP-SpeI_Reverse. Afterwards a gel was done (0,7% agarose gel, 1,5h) searching for the iRFP band at 978bp. 14 positive colonies were detected: colony 7, 8, 12, 13, 15, 17, 19, 22, 23, 25, 34, 35, 43, 14. Minipreps were done for these colonies. The next step was a DRD, using BglII for once cut and PvuII and EcoRI for multi cut. Gel conditions: 1,0%, 1h

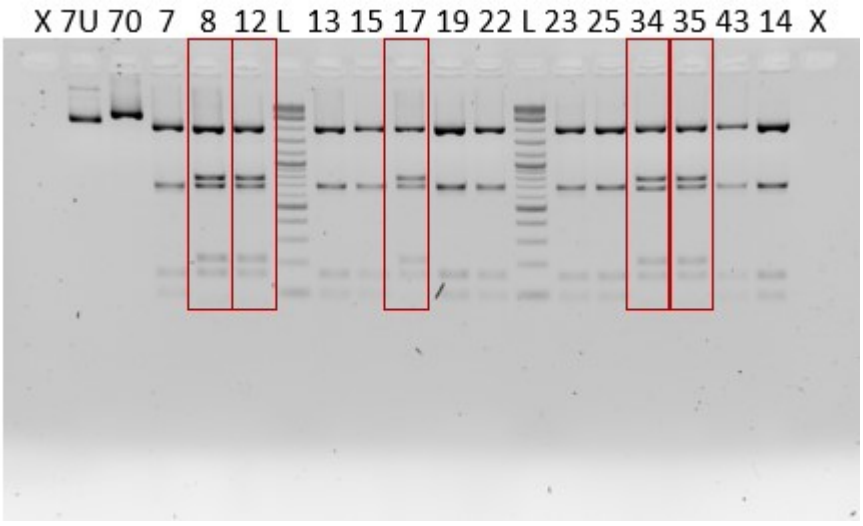

Figure 10. DRD of positive colonies from transformation with TOPflash_iRFP_L5-L4. Expected bands for the multi cut construct: 1955bp, 770bp, 689bp, 211bp, 161bp, 74bp; Therefore colonies 8,12,17, 34 and 35 contain the right plasmid. U = uncut, O = once cut, all other samples were multi cut.

Colony 8 was chosen for subsequent experiments, and sequenced. Sequencing showed fidelity of the sequence (data not shown).

4.2. pMuLE_ENTR_CTP4_iRFP_L5-L4

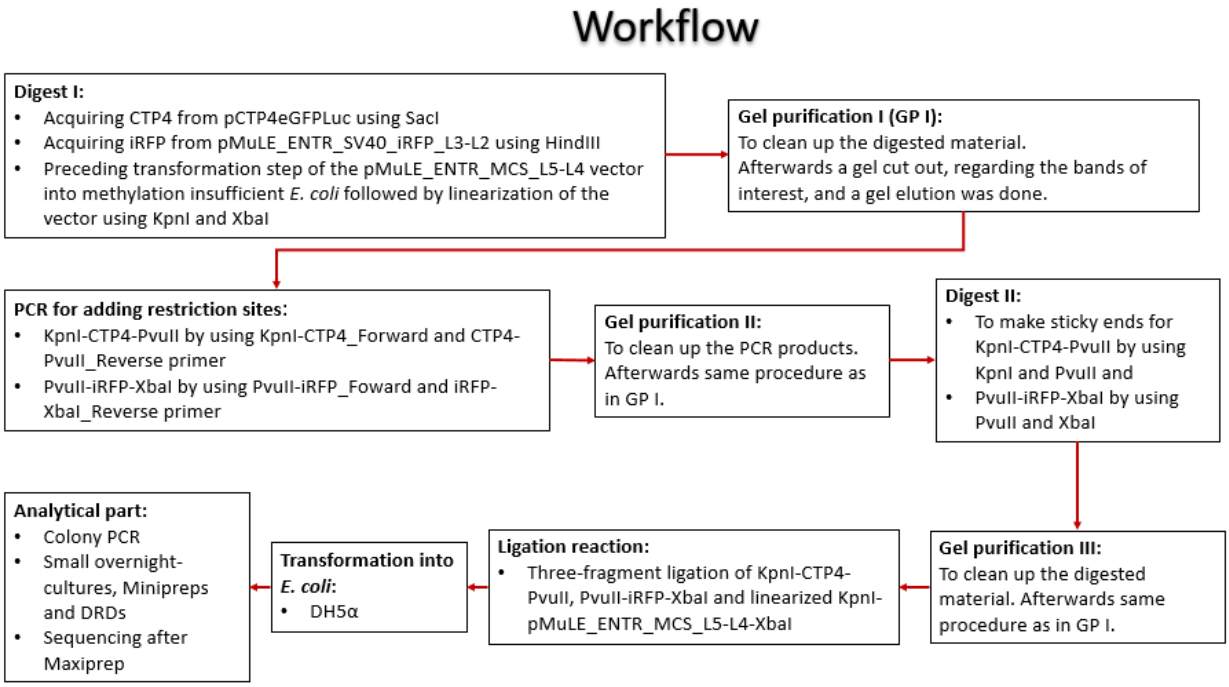

Figure 11. Overview of the steps to build the CTP4_iRFP_L5-L4 construct.

CTP4_iRFP_L5-L4 is the second construct which represents a Wnt dependent entry vector. It consists of a pMuLE backbone vector with an attL5 and attL4 side, flanking the optimized synthetic β-catenin-dependent promoter CTP4 and the downstream fluorophore iRFP. This vector was not used for the reporter construct, as there is no mutated version of CTP4 available, making pathway-specificity tests impossible. The building of this construct was more complex and therefore shows how to overcome different obstacles which can occur during vector construction.

Digest I: Procedure done as in Fig. 11. For the TOPflash project the MCS_L5-L4 backbone was linearized using KpnI and SpeI. As the CTP4 promoter has a SpeI restriction site in its sequence, the same approach could not be used. If the promoter needs to be cut out of the vector backbone one day, SpeI would destroy CTP4. To circumvent this situation, a different restriction enzyme was chosen to linearize the backbone, called XbaI. This lead to a novel problem, as the MCS_L5-L4 plasmid was beforehand amplified

in methylation sufficient bacteria. XbaI can only cut at unmethylated XbaI sites and could therefore not digest the backbone.

The new approach was to transform the pMuLE_ENTR_MCS_L5-L4 vector into so-called methylation insufficient, *dam-* and *dcm-* negative K12_ER2925 *E. coli*. Afterwards the unmethylated plasmid was gathered using a Maxiprep and afterwards linearized with KpnI and XbaI.

Gel purification I: Building elements were purified in an agarose gel, cut out and eluted. Results from the DNA measurement were as followed:

- *SacI-CTP4-SacI: 1163bp (0,7% agarose gel, 1h): 11,1ng/µl*
- *HindIII-iRFP-HindIII: 1006bp (0,7% agarose gel, 1h): 15,6ng/µl (from 4.1.)*
- *KpnI-MCS_L5-L4-XbaI: 2581bp (1,0% agarose gel, 1h) 22,5ng/µl*

PCR for adding restriction sites: The backbone vector was linearized using KpnI and XbaI. As TOPflash and iRFP were cut out from their origin plasmids with different restrictions enzymes, their sequences had to be modified using the right primers.

Gel purification II: PCR products were cleaned up using an agarose gel. Afterwards a gel cut out and elution was done. Values after gel elution were at:

- *KpnI-CTP4-PvuII: 408bp (1,0% agarose gel, 1h): 52,2ng/µl*
- *PvuII-iRFP-XbaI: 978bp (1,0% agarose gel, 1h): 64,0ng/µl*

Digest II: Procedure done as in Fig. 11.

Gel purification III: This last purification step was done to clean up the products from "Digest II". The sticky-ended building elements were sent through a gel, cut out and eluted. Results from the DNA measurement were as followed:

- *KpnI-CTP4-PvuII: 394bp (1,0% agarose gel, 40mins): 38,5ng/µl*
- *PvuII-iRFP-XbaI: 962bp (1,0% agarose gel, 40mins): 49,0ng/µl*

Ligation reaction: Procedure done as in Fig. 11. Furthermore, the cut backbone KpnI-pMuLe_ENTR_MCS_L5-L4-XbaI and ligase were mixed to have a recirc. control.

Transformation: All transformations were performed using competent DH5alpha *E. coli*. Same setup of controls as in 4.1. was used. Four approaches with the three-fragment ligation CTP4_iRFP_L5-L4, using two times 2µl and two times 4µl of plasmid DNA, were done. Afterwards all transformations were plated on the according LB plates. After incubation, the plates were examined. There were over 150 hits on the three-fragment ligation plates but also over 70 colonies on the recirc. control plates. In the end, positive colonies were transferred on master plates.

Analytical part: As there were so many presumably positive colonies, a colony PCR was done for rapid screening, using primers for the iRFP insert (see 4.1.). 41 positive colonies were used. Afterwards a gel was done (0,7% agarose gel, 30mins) searching for the iRFP band at 978bp. 5 positive colonies were detected: colony 1, 8, 9, 18, 23. Minipreps were performed for these colonies. The next step was a DRD, using BamHI for once cut and Eco32I and SpeI for multi cut. Gel conditions: 1,0%, 1h

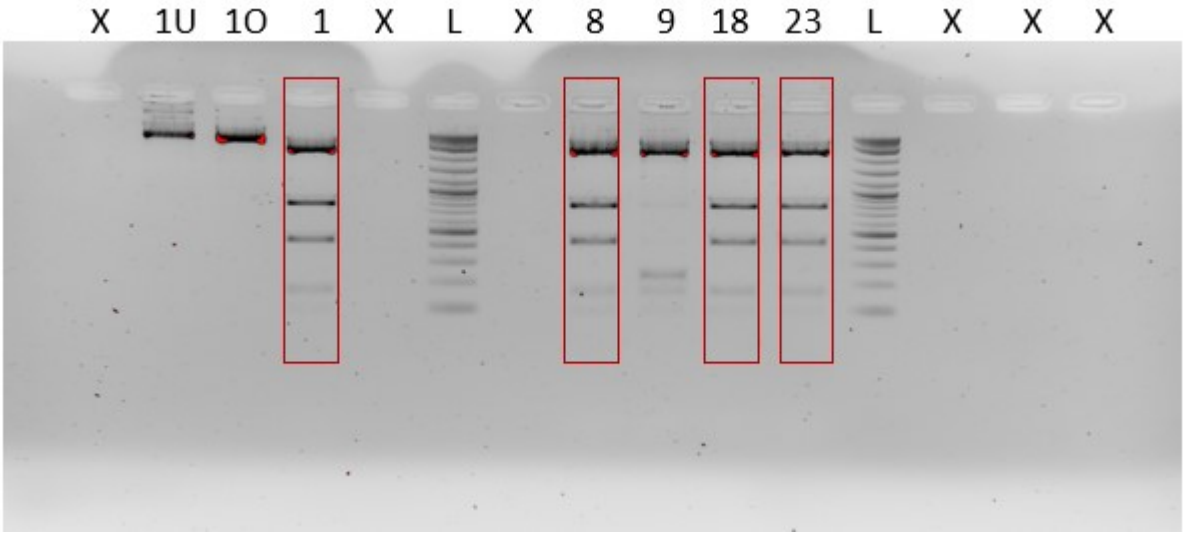

Figure 12. DRD of positive colonies from transformation with CTP4-iRFP-L5-L4. Expected bands for once cut: 3937bp and for multi cut: 2476bp, 770bp, 425bp, 166bp, 99bp; Therefore colonies 1,8,18,23 contain the right plasmid. U = uncut, O = once cut, all other samples were multi cut

Colony 1 was chosen for subsequent experiments, and sequenced. Sequencing showed fidelity of the sequence (data not shown).

4.3. pMuLE_ENTR_hPTCH1mut_iRFP_L1-L4

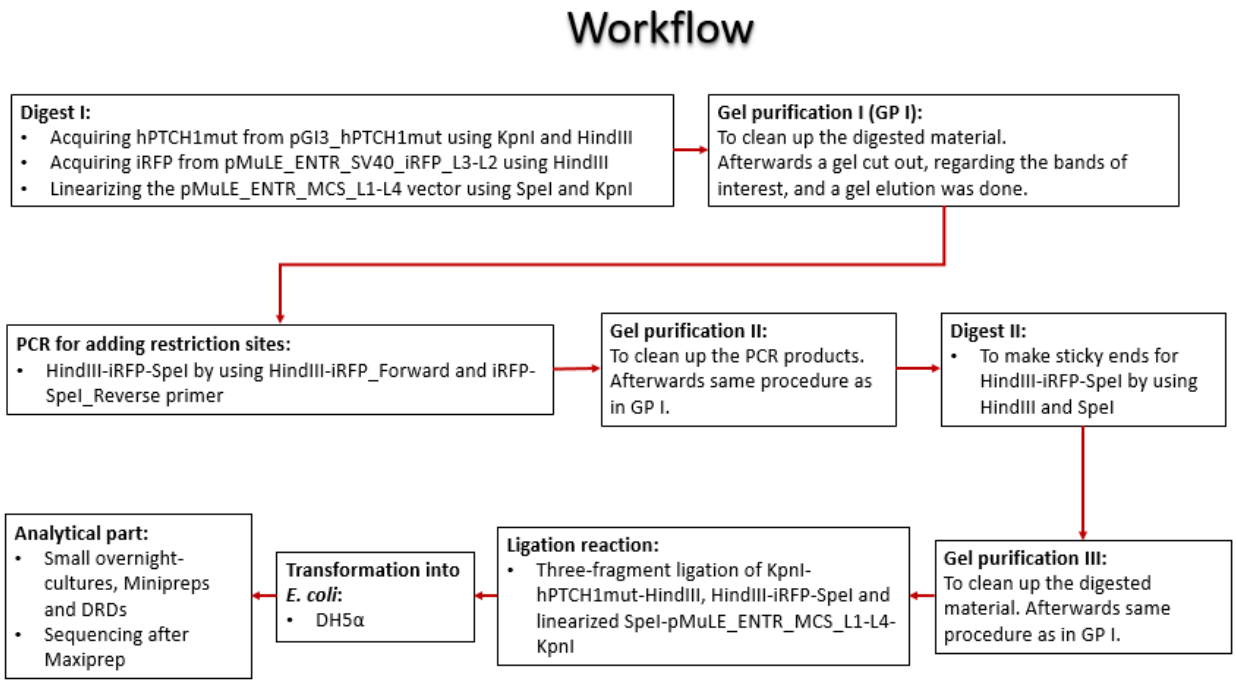

Figure 13. Overview of the steps to build the hPTCH1mut_iRFP_L1-L4 construct.

This plasmid represents the first mutated vector. It will be used in further experiments to test the Hedgehog pathway specificity of the hPtCH1 promoter from the pMuLE_ENTR_hPTCH1_mturq2_R4-R3 entry vector. The mutated plasmid consists of the altered hPTCH1mut promoter, the downstream fluorophore iRFP and the backbone vector pMuLE_ENTR_MCS_L1-L4.

Digest I: Procedure done as in Fig. 13.

Gel purification I: Building elements were purified in an agarose gel, cut out and eluted. Results from the DNA measurement were as followed:

- *KpnI-hPTCH1mut-HindIII: 1375bp (1,0% agarose gel, 1h): 10,0ng/µl*
- *HindIII-iRFP-HindIII: 1006bp (1,0% agarose gel, 1h): 11,2ng/µl*
- *SpeI-MCS_L1-L4-KpnI: 2654bp (1,0% agarose gel, 1h): 22,5ng/µl*

PCR for adding restriction sites: Only the fluorophore had to be equipped with new restriction sites for the later performed ligation reaction.

Gel purification II: PCR products were cleaned up using an agarose gel. Afterwards a gel cut out and elution was done. Value after gel elution:

- *HindIII-iRFP-SpeI: 987bp (1,0% agarose gel, 1h): 60,5ng/µl.*

Digest II: Procedure done as in Fig. 13.

Gel purification III: This last purification step was done to clean up the products from "Digest II". The sticky-ended building element was sent through a gel, cut out and eluted. Result from the DNA measurement:

- *HindIII-iRFP-SpeI: 974bp (1,0% agarose gel, 1h): 35,0ng/µl*

Ligation reaction Procedure done as in Fig. 13. Furthermore, the cut SpeI-pMuLe_ENTR_MCS_L1-L4-KpnI and ligase were mixed to have a recirc. control.

Transformation: All transformations were performed using competent DH5alpha *E. coli*. Same setup of controls as in the already mentioned transformations was used. Four approaches with the three-fragment ligation hPTCH1mut_iRFP_L1-L4, using two times 2µl and two times 4µl of plasmid DNA, were done. Afterwards all transformations were plated on the according LB plates. After incubation, the plates were examined. This transformation approach showed a very low efficiency, as only five positive colonies on the ligation plates were detected. In the end, they were transferred on a master plate.

Analytical part: As there were so few hits, colony PCR was skipped and directly small overnight cultures and Minipreps done. The next step was a DRD, using BamHI for once cut and PvuII, EcorI and KpnI for multi cut.

Colonies 1 to 5 represent positive colonies from the ligation plates (Fig. 14). Colony 6 is a false positive from the recirc. control plate, which should therefore only contain the MCS_L1-L4 backbone. The MCS_L1-L4 lane represents the multi cut backbone from our stock. The idea behind this was to have an additional control for a better differentiation between “just backbone” and “backbone plus insert”. Gel conditions: 1,0%, 1h

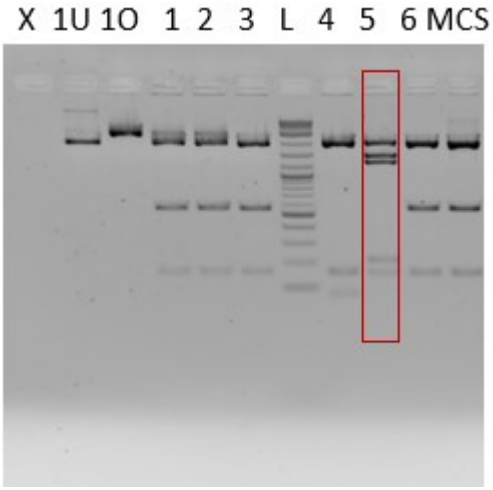

Figure 14. DRD of positive colonies from transformation with hPTCH1mut_iRFP-L1-L4. Expected bands for once cut: 5003bp and for multi cut: 1955bp, 1427bp, 1249bp, 211bp and 161bp; Therefore, just colony 5 contains the right plasmid. U = uncut, O = once cut, all other samples were multi cut

Only colony 5 contained the construct of interest. All other colonies show the pattern of the multi cut MCS_L1-L4 backbone. Therefore, colony 5 was chosen for subsequent experiments, and sequenced. Sequencing showed fidelity of the sequence (data not shown).

In contrast, the sequencing results of the non-mutated hPTCH1_mturq2_R4-R3 vector were not satisfying (see “5. Discussion”).

4.4. pMuLE_ENTR_CBFmut_iRFP_L1-L4

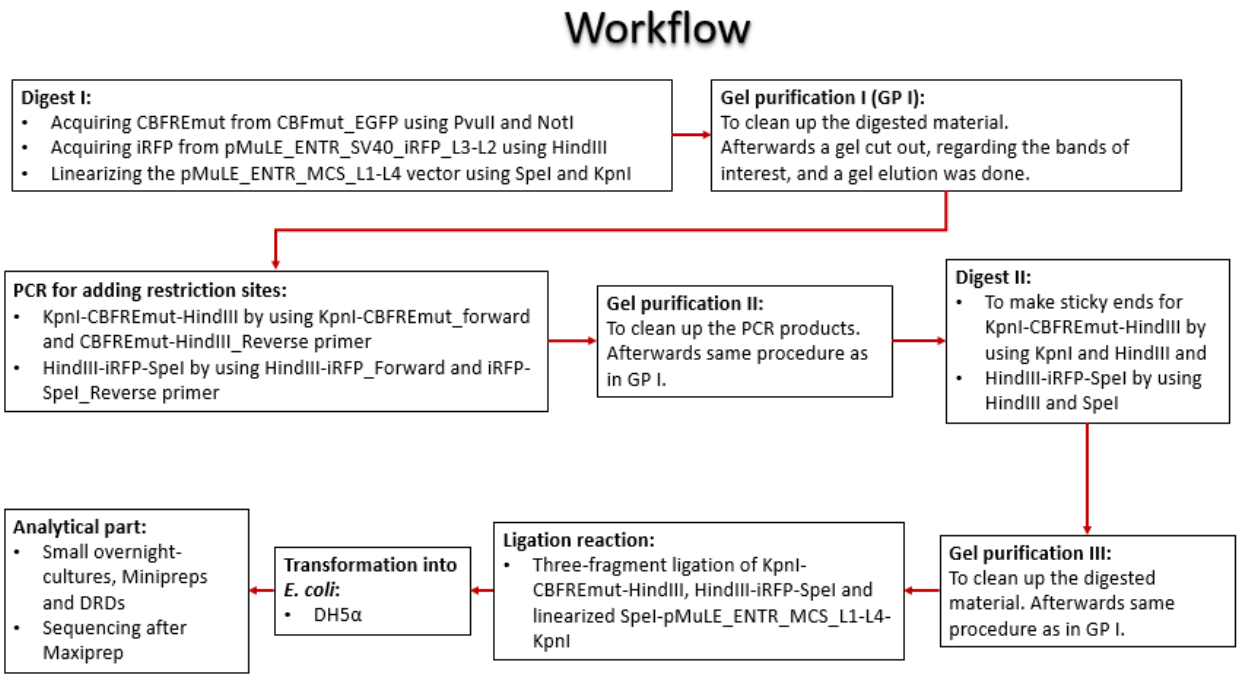

Figure 15. Overview of the steps to build the CBFmut_iRFP_L1-L4 construct.

This plasmid represents the second mutated vector. It will be used in further experiments to test the Notch pathway specificity of the CBF promoter from the pMuLE_ENTR_CBF_tdtomato_L3-L2 entry vector. The mutated plasmid consists of the altered CBFmut promoter, the downstream fluorophore iRFP and the backbone vector pMuLE_ENTR_MCS_L1-L4.

Digest I: Procedure done as in Fig. 15. The building element HindIII-iRFP-SpeI (974bp; 35,0ng/µl) was already generated in the pMuLE_ENTR_hPTCH1mut_iRFP_L1-L4 project. As we had stocks of it left, we only had to acquire the promoter and backbone.

Gel purification I: Building elements were purified in an agarose gel, cut out and eluted. Results from the DNA measurement were as follows:

- *PvuII-CBFREmut-NotI: 1276bp (1,0% agarose gel, 1h): 26,5ng/µl*
- *SpeI-MCS_L1-L4-KpnI: 2654bp (1,0% agarose gel, 1h): 46,0ng/µl*

PCR for adding restriction sites: Only the promoter had to be equipped with new restriction sites for the later performed ligation reaction.

Gel purification II: The promoter with newly added restriction sites was cleaned up using an agarose gel. Afterwards a gel cut out and elution was done. Value after gel elution:

- *KpnI-CBFREmut-HindIII: 411bp (1,0% agarose gel, 1h): 41,0ng/µl*

Digest II: Procedure done as in Fig. 15.

Gel purification III: This last purification step was done to clean up the products from "Digest II". The sticky-ended building element was sent through a gel, cut out and eluted. Result from the DNA measurement:

- *KpnI-CBFREmut-HindIII: 411bp (1,0% agarose gel, 1h): 16,2ng/µl*

Ligation reaction: Procedure done as in Fig. 15. Furthermore, the cut SpeI-pMuLe_ENTR_MCS_L1-L4-KpnI and ligase were mixed to have a recirc. control.

Transformation: All transformations were performed using competent DH5alpha *E. coli*. The same setup of controls as in the already mentioned transformations was utilized, except for 1µl pUC19 instead of the previously used 2µl pGluc as one of the positive controls. pUC19 represents a plasmid that contains an Ampicillin resistance gene and shows a very high transformation efficiency. Four approaches with the three-fragment ligation hPTCH1mut_iRFP_L1-L4, using two times 2µl and two times 4µl of plasmid DNA, were done. Afterwards all transformations were plated on the according LB plates. After incubation, the plates were examined. This transformation approach showed a very low efficiency. Only 1 positive colony was detected and no growth on the recirc. plate was apparent.

Analytical part: As there was just one hit, no colony PCR was needed. Thus, a small overnight culture of the colony, a Miniprep and afterwards a DRD was done.
For the DRD, BglII was used for the once cut and HindIII and Eco32I for multi cut.
Gel conditions: 1,0%, 1h

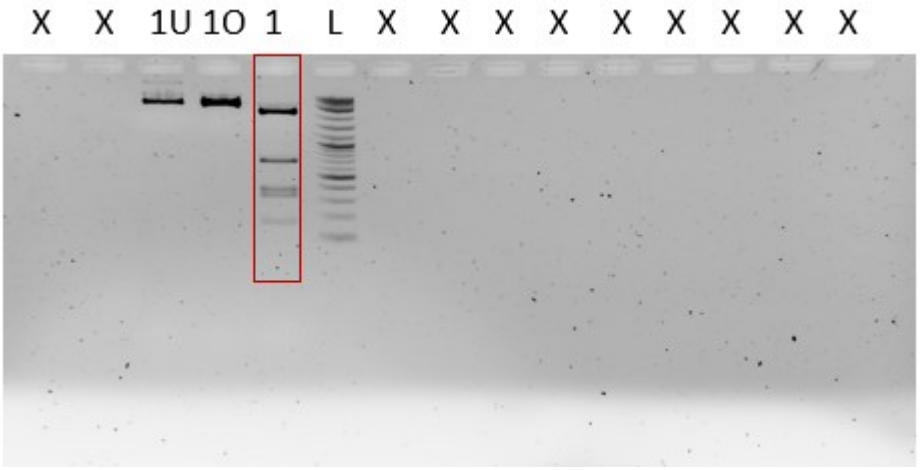

Figure 16. DRD of positive colony from the transformation with CBFmut_iRFP-L1-L4. Expected bands for once cut: 4015bp and for multi cut: 2440bp, 682bp, 397bp, 329bp and 167bp; Therefore, colony 1 contains the right plasmid. UC = uncut, OC = once cut

Colony 1 was chosen for subsequent experiments and sequenced. Sequencing results showed only deviations in areas which should not affect the functionality of the construct (data not shown).

4.5. pMULE_ENTR_FOPflash_mTurquoise2_R4-R3

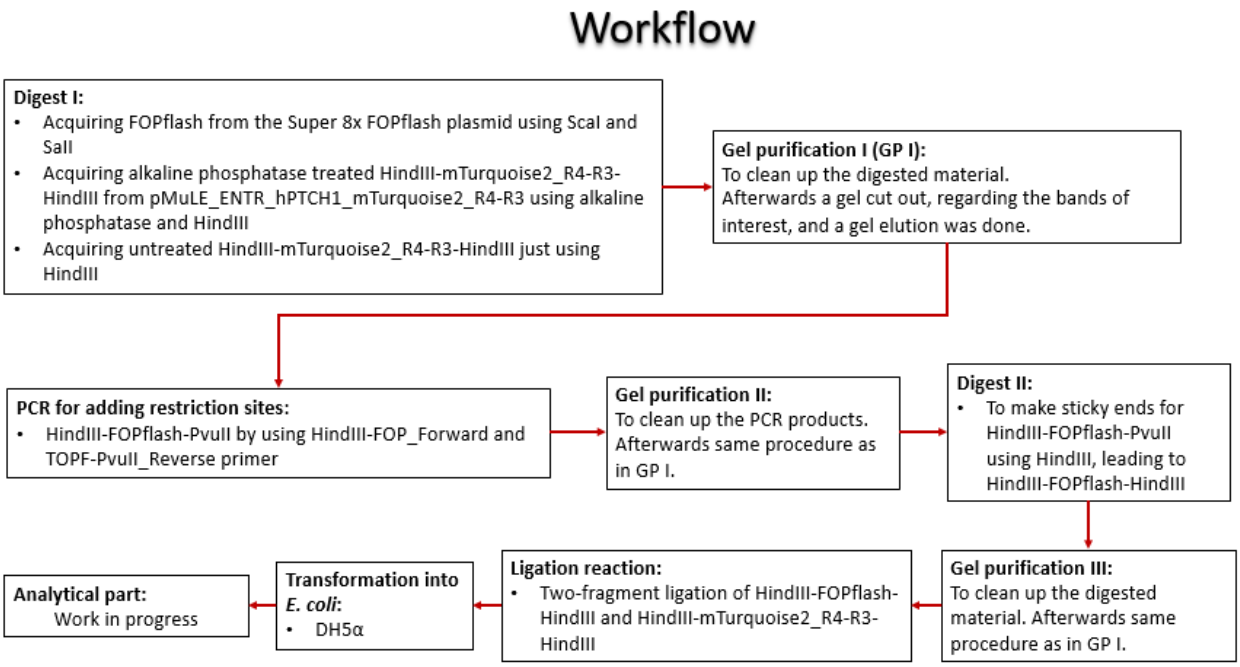

Figure 17. Overview of the steps to build the FOPflash_mTurquoise2_R4-R3 construct.

This plasmid represents the last mutated vector. It will be used in further experiments to test the Wnt pathway specificity of the TOPflash promoter from the pMuLE_ENTR_TOPflash_iRFP_L5-L4 entry vector. The mutant consists of the altered FOPflash promoter, the downstream fluorophore mTurquoise2 and the backbone vector pMuLE_ENTR_MCS_R4-R3.

Digest I: Procedure done as in Fig. 17. To get the backbone and fluorophore of interest, the self-made entry vector hPTCH1_mTurquoise2_R4-R3 was digested using HindIII. This lead to the building block HindIII-mTurquoise2_R4-R3-HindIII, which carries a Kanamycin resistance gene. As this linearized element had identical restriction sites on both ends, the risk of recapturing its circular form was very high. As such, we followed a normal and a modified approach for dealing with this challenge: two digests were performed to be able to circumvent this problem of recircularization, which can potentially lead to a lot of false positives during transformation. A cut out of HindIII-mTurquoise2_R4-R3-HindIII

was done by using HindIII only. Additionally, a Shrimp Alkaline Phosphatase (rSAP) treated digest with HindIII was performed. The latter leads to the loss of phosphate groups from the nucleotides at the 5´ and 3´ ends of the DNA element, with the aim to prevent recircularization.

Gel purification I: Building elements were purified in an agarose gel, cut out and eluted. Results from the DNA measurement were as followed:

- *ScaI-FOPflash-SalI: 510bp (1,0% agarose gel, 1h): 20,0ng/µl*
- *HindIII-mTurquoise2_R4-R3-HindIII: 3574bp (1,0% agarose gel, 1h): 22,0ng/µl*
- *rSAP treated HindIII-mTurquoise2_R4-R3-HindIII: 3574bp (1,0% agarose gel, 1h): 47,0ng/µl*

PCR for adding restriction sites: Only the promoter had to be modified. Therefore, we used a newly designed HindIII-FOP_Forward primer and the already existing TOPF-PvuII_Reverse primer from 4.1.

Gel purification II: The promoter with newly added restriction sites was cleaned up using an agarose gel. Afterwards a gel cut out and elution was done. Value after gel elution:

- *HindIII-FOPflash-PvuII: 270bp (1,0% agarose gel, 1h): 38,0ng/µl*

Digest II: Procedure done as in Fig. 17. In this case, we are targeting a newly added HindIII site and another HindIII site which is upstream to the added PvuII site, leading to the building element HindIII-FOPflash-HindIII.

Gel purification III: This last purification step was done to clean up the products from “Digest II”. The sticky-ended building element was sent through a gel, cut out and eluted. Result from the DNA measurement:

- *HindIII-FOPFlash-HindIII: 219bp (1,0% agarose gel, 1h): 15,4ng/µl*

Ligations and transformations: Multiple two fragment ligations were done. Furthermore, the cut backbone HindIII-mTurquoise2_R4-R3-HindIII and ligase were always mixed to have a recirc. control.

First, the rSAP treated backbone and HindIII-FOPflash-HindIII were ligated using our normal protocol of 120mins at 25°C with the TA/Blunt ligase master mix. As the transformation afterwards lead to no apparent growth, we decided to modify the ligation reaction. We used the T4-DNA-Ligase Master mix and a modified protocol for incubation, consisting of 891 cycles at 10°C for 30 secs and 30°C for 30 secs. As the transformation afterwards showed again no apparent growth, we decided to do parallel ligation reactions using the normal and changed protocol.

In these ligations, we used the untreated HindIII-mTurquoise2_R4-R3-HindIII backbone and HindIII-FOPflash-HindIII. This tactic worked, as the normal ligation protocol lead to six colonies on the recircularization plate and over 70 positive colonies on the ligation plates. In contrast, the optimized protocol delivered four colonies on the recircularization plate and only 40 colonies on the ligation plates. In the end, positive colonies were transferred on master plates. Ligation and transformation observed in this experiment will be further discussed.

Analytical part: My time in the lab ended before finishing this construct. After consultation with Julia Maier, the analytical part of the construct went well. A colony with the right plasmid was detected and sequenced. Sequencing showed fidelity of the sequence (data not shown).

5. Discussion

The whole project focused on the generation of a reporter construct, consisting of plasmids which have the ability to visualize deregulated developmental pathways in cancer. This chapter of the thesis will focus on problems which occurred during the construction process and furthermore will give a glimpse into the future of the finished reporter construct.

mTurquoise2 – a mutation prone sequence?

As mentioned in the results of this work, sequencing of the plasmids showed a high fidelity. Only the plasmid pMuLE_ENTR_hPTCH1_mTurquoise2_R4-R3 contained sequence deviations which could affect the functionality of the construct. A colony PCR during the construction work of this vector detected various positive colonies. Ten colonies were chosen, cultured, Minipreps performed and DRDs done. This lead to five potential candidates: colony 51, 59, 65, 67 and 78. All of them were sequenced by Julia Maier. Results of the sequencing are as followed:

- Colony 51 showed three different point mutations in the mTurquoise2 gene:
  - Silent: 2229C(Ala) -> A(Ala)
  - Silent: 2682C(Ile) -> A(Ile)
  - Non-silent: 2327G(Cys) -> A(Tyr)

  In the latter, a thiol-function is changed against a phenolic hydroxy-group. As thiols are capable to form disulfide bonds and therefore enhance protein stability (Hogg 2003), this colony was not chosen for further experiments.

- Colony 59 was sequenced twice. Results of the first sequencing are as followed:
  - Silent: point mutation in position 2211 leading to Gly30 -> Gly30
  - Non-silent: point mutation in position 2731 leading to Ser205 -> Ala205

Botch mutations occurred again in the mTurquoise2 gene. In the latter, a hydroxy group is changed against an aliphatic side chain, leading to loss of a hydrogen bond donor and polarity. Goedhart et al. (2012) showed that a serin at this position stabilizes the chromophore of mTurq2, a tryptophan, via hydrogen bonding. Loss of this interaction leads to a mis-positioned chromophore in the excited state and therefore to a lower fluorescence (Goedhart et al. 2012). This mutation could have a high negative impact on the function of the construct. Therefore, a second sequencing was done. In this repeat no mutations in the mTurquoise2 gene were found. We consider that something during the first sequencing approach went wrong. One of the most obvious sources of error in such a situation represent the used primers. Based on the second sequencing, colony 59 was chosen for further work. More colonies were sequenced to have a backup.

- Colony 65 and Colony 67 could not be used, as they showed a single base substitution in the mTurquoise2 gene, leading to a stop-codon.

- Colony 78 showed one silent mutation in the mTurquoise2 gene:
  - ATC against ATA -> both code for Ile128

Furthermore, there was one mutation in the non-coding area between mTurq2 and the recombination site att3. Colony 78 was chosen to be our backup if colony 59 was non-functional in later steps, as the silent mutation and the sequence deviation in the non-coding are should not change the function of the construct.

- As the mTurquoise2 gene showed so many mutations, Julia decided to sequence the origin plasmid mTurq2-NES, to see if it was mutated from the beginning. The sequencing results showed the same nucleotide sequence as specified by Addgene.

In conclusion, 5 plasmids were sequenced, whereby 4 of 5 showed mutations in the gene encoding for the fluorophore. As Colony 59 showed no mutations in critical regions, we decided to take this plasmid for our reporter construct.

mTurquoise2 seems to be an error prone sequence. We can only speculate why these mutations occurred. The PCR step is most likely for introducing mutations. The fluorophore underwent PCR to add the right restriction sites for the ligation reaction afterwards. Although we used the Q5® High-Fidelity DNA polymerase, which was chosen because of its advertised superior fidelity rate, mutations probably occurred in this step. In conclusion, we have chosen the correct plasmid by using the right tactic, meaning that in such a project it is better to sequence more often than too less.

Shrimp alkaline phosphatase ligation efficiency

During the building of FOPflash_mTurquoise2_R4-R,3 we worked with building elements which had identical restriction sites on both ends: HindIII-FOPFlash-HindIII and HindIII-mTurquoise2_R4-R3-HindIII. Same restriction sites pair, and ligate, with each other. In this case, the main problem is originating from the backbone, as it carries the antibiotic resistance. Theoretically, transformations with high amounts of recircularized backbones lead to a lot of false positive colonies and lower therefore transformation efficiency.

To circumvent this problem, a Shrimp alkaline phosphatase treatment was tried. This technique is used in cloning to prevent religation of linearized plasmid DNA, as rSAP (recombinant Shrimp alkaline phosphatase) nonspecifically catalyzes the dephosphorylation of 5´ and 3´ ends of DNA (description from https://www.neb.com/products/m0371-shrimp-alkaline-phosphatase-rsap (15.08.2017)). Theses phosphates are necessary for stable ligations, as they are needed to covalently connect the sugar backbones of interacting nucleotides. In theory, the phosphate groups will only get reintroduced during the ligation reaction by the enzyme Ligase, leading exclusively to products containing the ligated elements and no recirculated structures.

Our results show that the use of this treatment lowered our ligation and transformation efficiency instead of increasing them. All transformations where rSAP treated elements were used had no true positive colonies. Even modifications in the ligation protocol did not change the outcome. In contrast to this, switching to non-rSAP treated building blocks and ligating them led to true positives. Therefore, we conclude that the working procedure was not the source of error. Why the ligations failed remains unclear. Probably

the Ligases we used were not able to reintroduce the phosphate groups into the dephosphorylated nucleotides, making stable ligations impossible. In conclusion, the results from the last two transformations, where the untreated backbone was used for the ligations, showed that our fear of backbone religation was exaggerated.

Packaging limit of lentiviral vectors

The destination vector itself has a size of 9237bp, containing the important sequences for the recombination reaction and the lentivirus generation. The multiple gateway cloning reaction is used to recombine the entry vectors TOPFlash_iRFP_L5-L4 (3866bp), hPTCH1_mTurquoise2_R4-R3 (4959bp), CBF_tdtomato_L3-L2 (4509bp) and CMV_EGFP (4081bp) into Lenti Neo Dest, leading to a plasmid with a total size of 14.685bp. This construct is very big and has now to be packaged into a lentivirus.

Kumar et al. (2001) showed in their study that viral titers of HIV-based vectors decrease semi-logarithmically with increasing vector length. They also showed that measurable titers were even obtained when the DNA was bigger than 18.000bp, implying that there is no absolute packaging limit. The step of generating the lentiviral reporter construct should therefore not present a major challenge.

Interplay of the fluorophores

Four compatible fluorophores were chosen. For this, fluorophores should show minimal interplay regarding excitation and emission wavelengths when used in multi-label experiments (Shaner et al. 2005).

Excitation and emission maxima of iRFP, Tdtomato, mTurquoise2 and EGFP are mentioned in the introduction. Emission and excitation of a fluorophore cannot be regarded as a sharp line when wavelength is plotted against signal intensity. Rather, it represents a spectrum extending over a wavelength range (Fig.18). Emission-excitation-interplays can lead to misinterpretation of measured signals.

Therefore, the fluorophores have to be tested in combination, e.g. in an experimental setup. Cells which show developmental pathway activity should be transfected with our self-made entry vectors. First, each entry vector has to be tested alone, to see how strong the signal activity is, after addition of the right pathway activator. Afterwards, combinations

should be tested, to see if the emission of a fluorescent protein from one pathway is eventually exciting a fluorescent protein from another pathway. Furthermore, this setup can visualize if pathway activators lead to a specific activation of just one pathway or activate additionally the others.

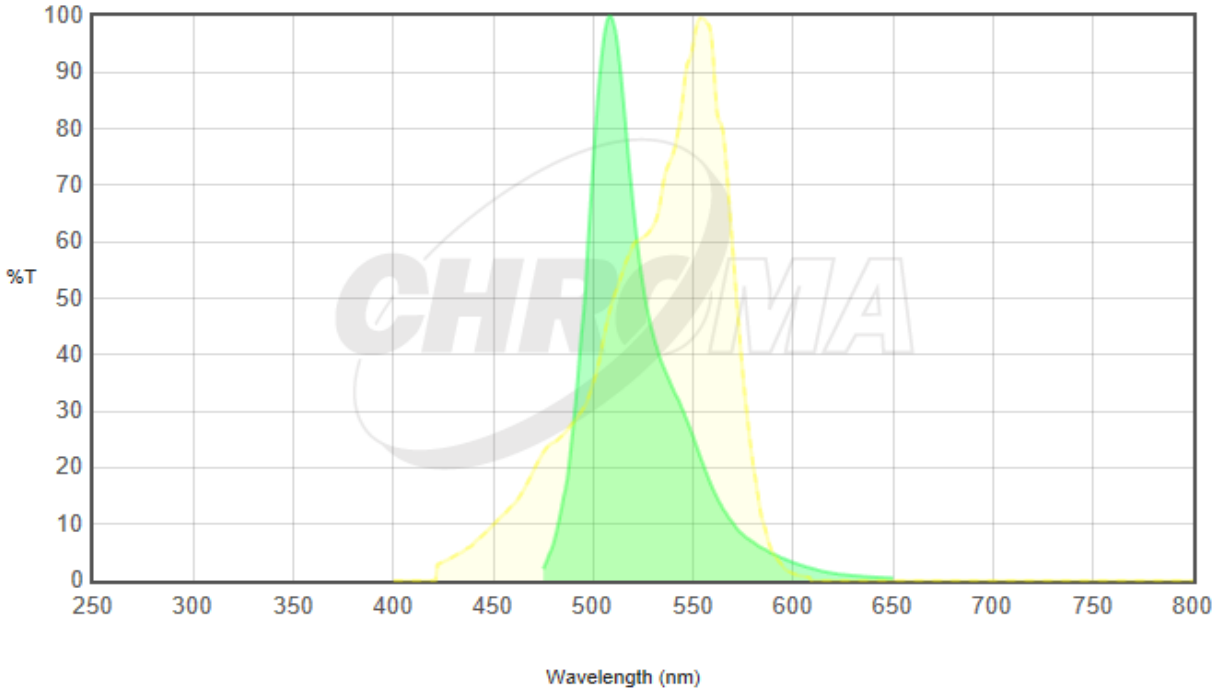

Figure 18. Example for emission-excitation-interplay. This plot was done using the Chroma Spectra Viewer from https://www.chroma.com/spectra-viewer (15.08.2017). It is showing the emission spectrum of EGFP and the excitation spectrum of Tdtomato. Wavelength (x) is plotted against signal intensity (y). The signal emitted from EGFP is overlapping with the excitation area of Tdtomato. The significance of this overlap must be further tested in *in vitro* assays.

6. Appendix

6.1. Abstract

Cancer is still one of the leading causes of death worldwide. Therefore, the pharmaceutical industry and independent research groups try to get a better understanding of this disease. Research has shown that pathways, which normally play an important role in tissue homeostasis and embryonic development, are deregulated in cancer. Aberrations in these signal transduction cascades are associated to tumor initiation, progression, therapy resistance and the controversially discussed tumor subpopulation of cancer stem cells.

Our project focused on the generation of a MuLE-system-based reporter construct. This construct has the ability to transduce malignant cells and dynamically visualize the activity of the developmental pathways Wnt, Hedgehog and Notch.

This thesis is an example for how to build such a reporter. It illustrates the stepwise building of each needed component. The results show how workflows can look like and how obstacles, which can occur during the construction of a such a system, can be prevented and circumvented.

This reporter can enable us to get a deeper understanding of the role of developmental pathway signaling in cancer. Various applications of the system are intended, e.g. cell sorting, CSC identification, measurement of pathway interplays and supporting drug development.

Altogether, the constructed, developmental pathway specific vectors will hopefully brighten our understanding of cancer and will therefore facilitate the development of novel therapies.

6.2. Zusammenfassung

Krebs stellt immer noch eine der Hauptursuchen für den Tod weltweit dar. Daher versuchen die pharmazeutische Industrie sowie unabhängige Forschungsgruppen diese Bedrohung besser zu verstehen. Forschungsergebnisse zeigen, dass Signaltransduktionswege, die normalerweise eine Rolle in der Gewebshomöostase und der Embryonalentwicklung spielen, in Krebs dereguliert sind. Anomalien in diesen Signalkaskaden werden in Verbindung gebracht mit der Entstehung von Tumoren, dem Fortschreiten der Erkrankung, Therapieresistenz und der umstrittenen Subpopulation von Krebsstammzellen.

Unser Projekt hat sich mit der Entwicklung eines MuLE-System-basierenden Reporterkonstrukts beschäftigt. Dieses Konstrukt besitzt die Fähigkeit Krebszellen zu transduzieren und anschließend die Aktivität der Signalwege Wnt, Hedgehog und Notch dynamisch zu visualisieren.

Diese Arbeit stellt ein Beispiel dafür dar wie man so einen Reporter bauen kann. Der schrittweise Aufbau jeder benötigten Komponente wird beschrieben. In den Resultaten sieht man wie Arbeitsabläufe ausschauen können und wie Probleme, die während des Bauprozesses von so einem System möglich sind, verhindert beziehungsweise umgangen werden können.

Dieser Reporter kann uns ein tieferes Verständnis dafür geben welche Rolle diese Signalwege in Krebs haben. Unterschiedliche Anwendungsgebiete für den Reporter sind vorgesehen, wie z.B. Zellen zu sortieren, Krebsstammzellen zu identifizieren, Wechselwirkungen zwischen den Signalwegen zu messen sowie die Arzneimittelentwicklung zu unterstützen.

Abschließend betrachtet hoffen wir, dass die gebauten, signalwegspezifischen Vektoren unser Verständnis bezüglich Krebs erweitern und somit die Entwicklung von neuen Therapien erleichtern.

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