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DIPLOMARBEIT

Titel der Diplomarbeit

Impact of wnt-Signalling Blockade on Survival Pathways in Colorectal Tumour Cells

Verfasser

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Contents:

1. Abstract

Colorectal cancer is the third most common cancer worldwide. The APC mutation is the first crucial step initiating tumor development in the colon. The consequence of this mutation is the progressive up-regulation of the wnt pathway. Consequently, this pathway is a prominent target of therapy. Sulindac is a drug inhibiting wnt-signalling that is already used in prevention therapy. In the human colon adenoma cell line LT97 a CD44+ subpopulation was identified that express high FGF18 and survivin and down-regulate the IGF-binding protein 3 (IGFBP3). Whether these events are causally related to wnt-signalling activity is not yet known, however.

In my diploma thesis I studied the impact of sulindac treatment on the RNA and protein expression levels of survivin, FGF18 and IGFBP3 in LT97 adenoma cells and in the colorectal cancer cell line HT29.
Sulindac caused a down-regulation of the wnt target gene survivin to 28% and 61% of the control in HT29 and LT97 cells respectively. The drug did not change IGFBP3 RNA levels in LT97 cells. In HT29 cells sulindac caused a slight elevation of IGFBP3 RNA levels.

In order to obtain a specific positive control for wnt blockade a dominant-negative Tcf4 mutant was cloned into an adenoviral construct. The resulting virus facilitates the transduction of all human cell lines.
HT29 and LT97 cells were transduced with this adenovirus. Tcf4 was expressed 4.7-fold and 1.7-fold in LT97 and HT29 cells respectively. No other significant effects on survivin, FGF18 and IGFBP3 could be detected.
The sulindac results showed that survivin but not FGF18 and IGFBP3 is regulated in a wnt-dependent manner. Mechanistic experiments with the newly produced Tcf4 virus will be necessary to actually demonstrate the wnt dependent reaction.

2. Introduction

2.1 Definition of cancer

Cancer is defined as a disease of abnormal deregulated cell division and subsequent invasion of other tissues. The malignant cells can spread to other body parts through the blood and lymph systems. Normally, cells grow and divide in a controlled manner. In cancer cells genetic mutations accumulate and increasingly prevent cell death when necessary and cause cell production without stimulatory signals (see figure 2.1.1). Cancer cells and their microenvironment form a tissue termed tumor which can be benign or malignant. Benign tumors can be curatively removed and do not spread to other body parts. Malignant tumors are cancerous and invade other tissues, a process which is called metastasis. Carcinomas are cancers that arise in the skin or epithelial tissues which internal organs (National Cancer Institute – Cancer definition).

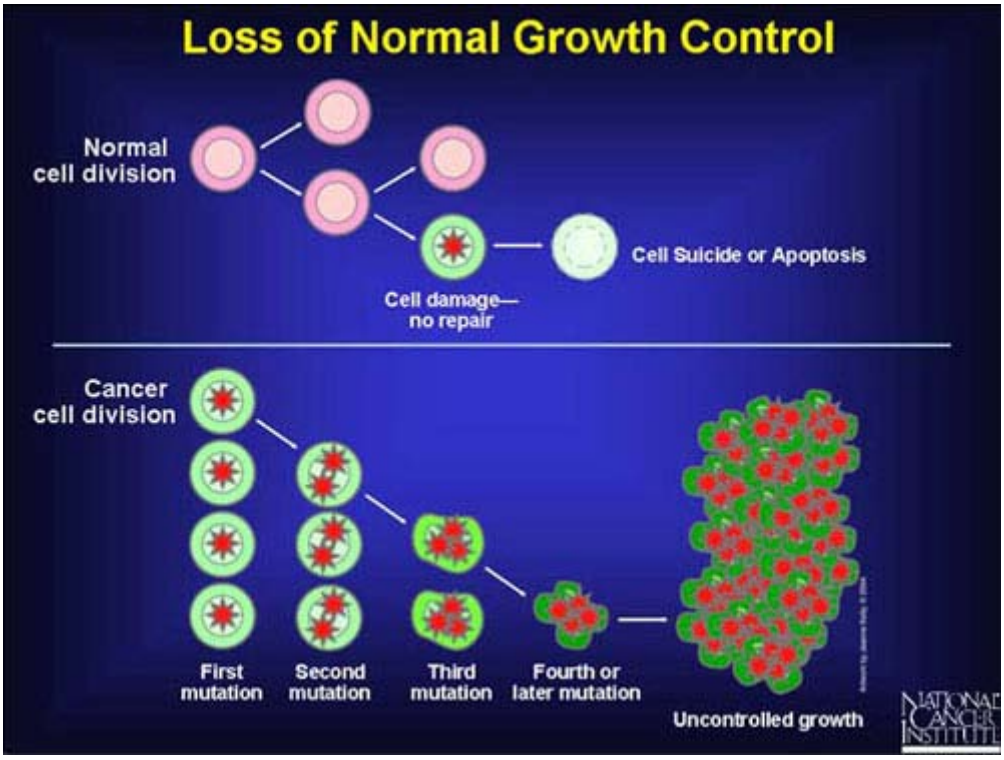


Figure 2.1.1 Loss of normal growth control in cancer cells
(http://www.cancer.gov/cancertopics/cancerlibrary/what-is-cancer)

2.2 Epidemiology of cancer

2.2.1 General cancer statistics

In 2008 12.7 millions new cancer cases and 7.6 millions cancer cases are counted worldwide. The numbers are age-standardized rates (ASR) in order to compare between countries because age has a strong influence on the incidence of cancer. In the incidence of cancer is extremely high in Australia/New Zealand (ASR 356.8 per 100000), closely followed by Northern America (ASR 334) and Western and Northern Europe (ASR 288.9 and 335.3). The high rates are the consequence of elevated incidence of prostate cancer in males (ASR higher than 80) and breast cancer in women (ASR higher than 75) in the concerning regions. The lowest incidence rates are recorded in Northern Africa for women and Middle and Western Africa and in South-Central Asia for men.

Interestingly, the incidence of cancer is much higher in developed regions than developing countries but the mortality is similar. These relationships are depicted in figures 2.2.1.1, 2.2.1.2 and 2.2.1.3. (Globocan for all cancers 2008).

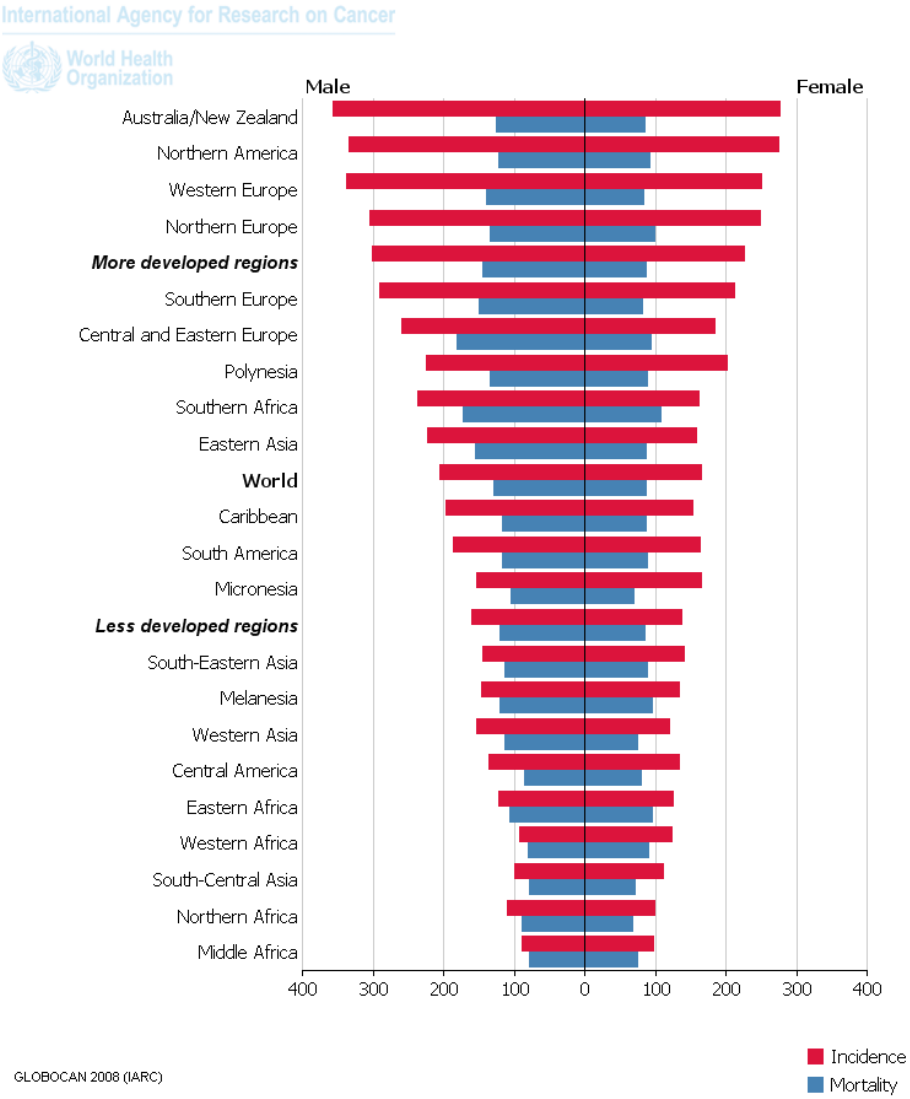


Figure 2.2.1.1 Cancer incidences in women and men in different world regions: estimated age-standardized rates per 100000 (Globocan for all cancers 2008)

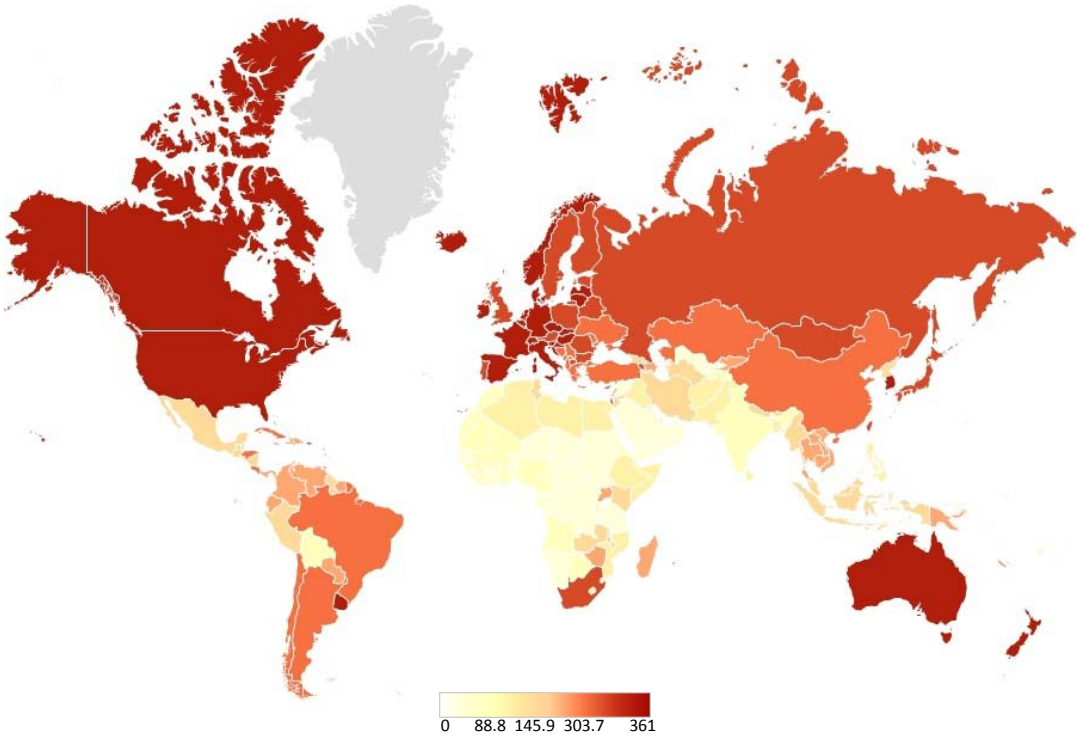


Figure 2.2.1.2 Cancer incidences in men worldwide in 2008
Age-standardized incidence rates per 100000 (adapted figure Globocan for all cancers 2008)

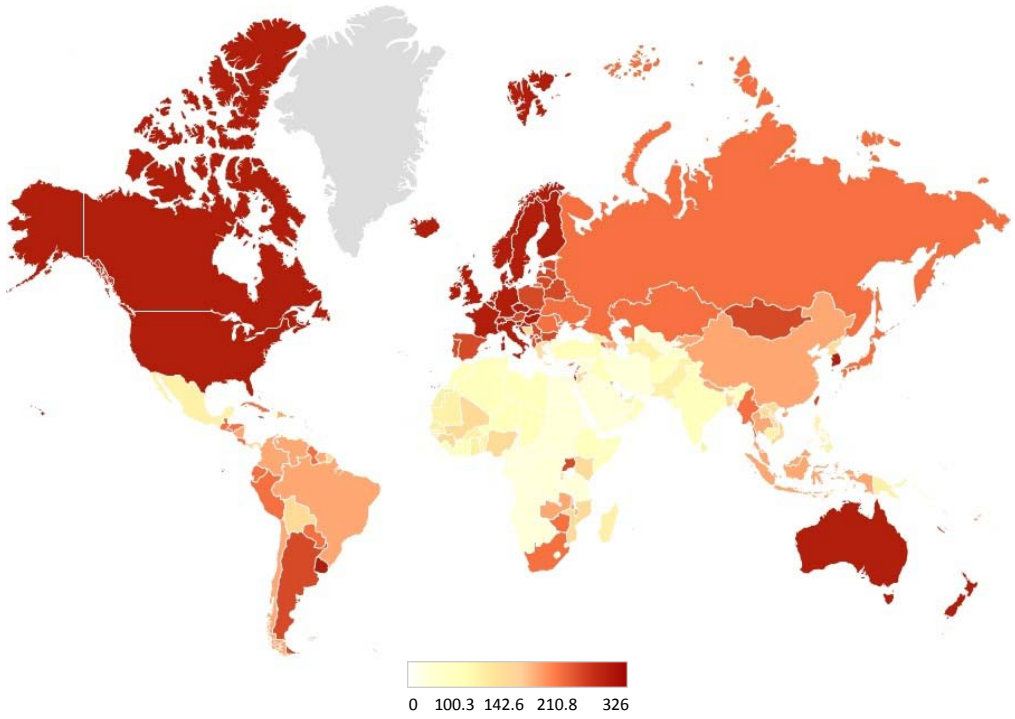


Figure 2.2.1.3 Cancer incidences in women worldwide in 2008
Age-standardized incidence rates per 100000 (adapted figure Globocan for all cancers 2008)

Worldwide the most frequent cancers in men are lung, prostate, colorectum, stomach and liver cancer. In women the list is headed by cancers of the breast, colorectum, cervix uteri, lung and stomach cancer worldwide. These facts are depicted in figures 2.2.1.4 and 2.2.1.5 (Globocan Fast Stats world 2008).

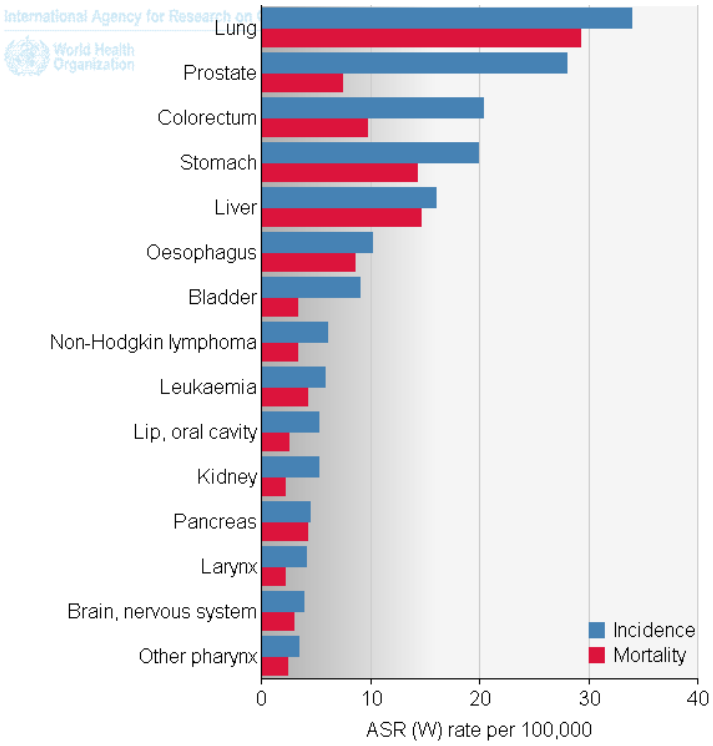


Figure 2.2.1.4 The most frequent cancers in men worldwide
(Globocan Fast Stats world 2008)

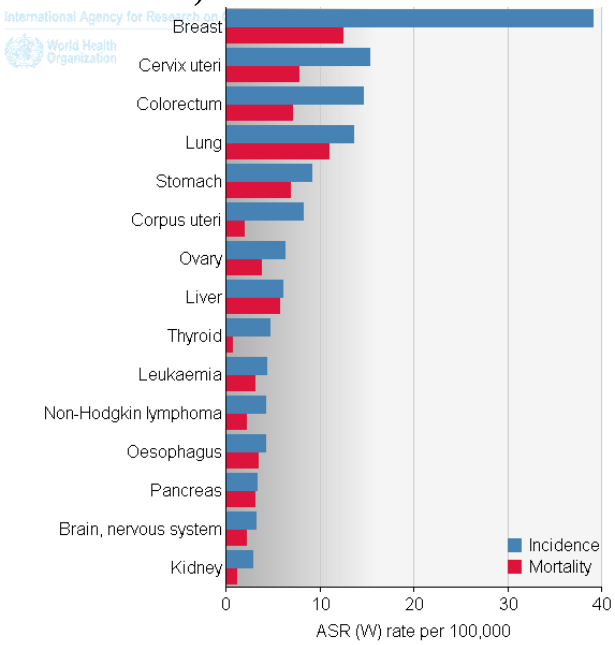


Figure 2.2.1.5 The most frequent cancers in women worldwide
(Globocan Fast Stats world 2008)

The risk of developing cancer before age 75 is 21.2% for males and 16.5% for females worldwide. The risk of dying from cancer before age 75 is 13.4% for males and 9.1% for females (Globocan Fast Stats world 2008). The most frequent cancers in Austria are shown in figure 2.2.1.6 and 2.2.1.7.

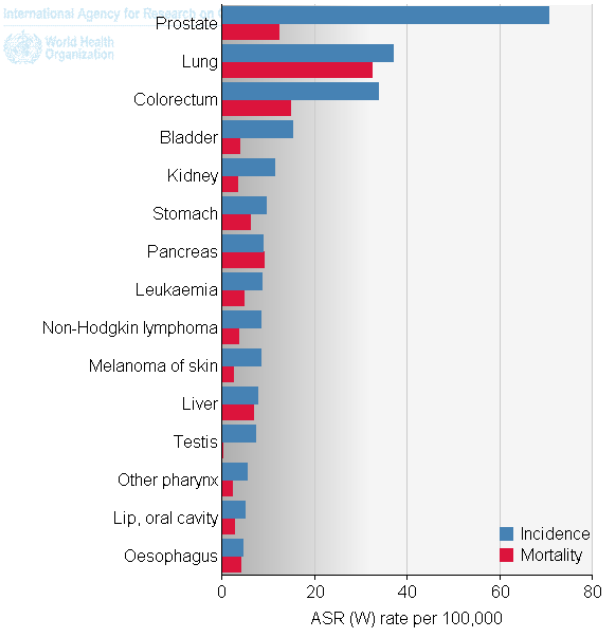


Figure 2.2.1.6 The most frequent cancers in men in Austria
(Globocan Fast Stats Austria 2008)

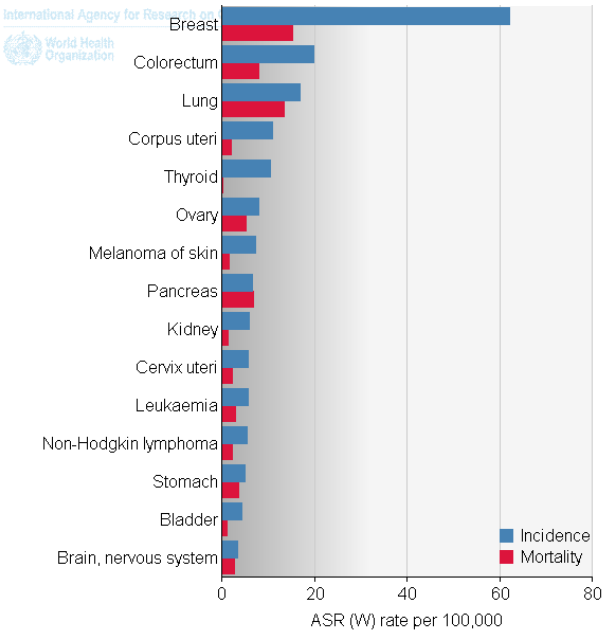


Figure 2.2.1.7 The most frequent cancers in woman in Austria
(Globocan Fast Stats Austria 2008)

In Austria the most frequent cancers in men are prostate, lung, colorectum, bladder and kidney cancer. For women they are breast, colorectum, lung, corpus uteri and pancreas cancer. The risk of developing cancer before age 75 is 27.8% for males and 19.7% for females in Austria. The risk of dying from cancer before age 75 is 13.3% for males and 8.6% for females (Globocan Fast Stats Austria 2008).

2.2.2 Colorectal cancer statistics

Worldwide colorectal carcinoma is the third most common cancer type in men (10%) and the second in women (9.4%). 60% of colorectal cancer cases occur in developed regions. The highest rates are observed in Australia/New Zealand and Western Europe. The lowest rates are in Africa and South and Central Asia. Colorectal cancer occurs more frequently in men than in woman. Colorectal cancer causes 8% of overall cancer deaths. The highest mortality rates are seen in Central and Eastern Europe. Worldwide incidents rates are depicted in figure 2.2.2.1 (Globocan colorectal cancer 2008).

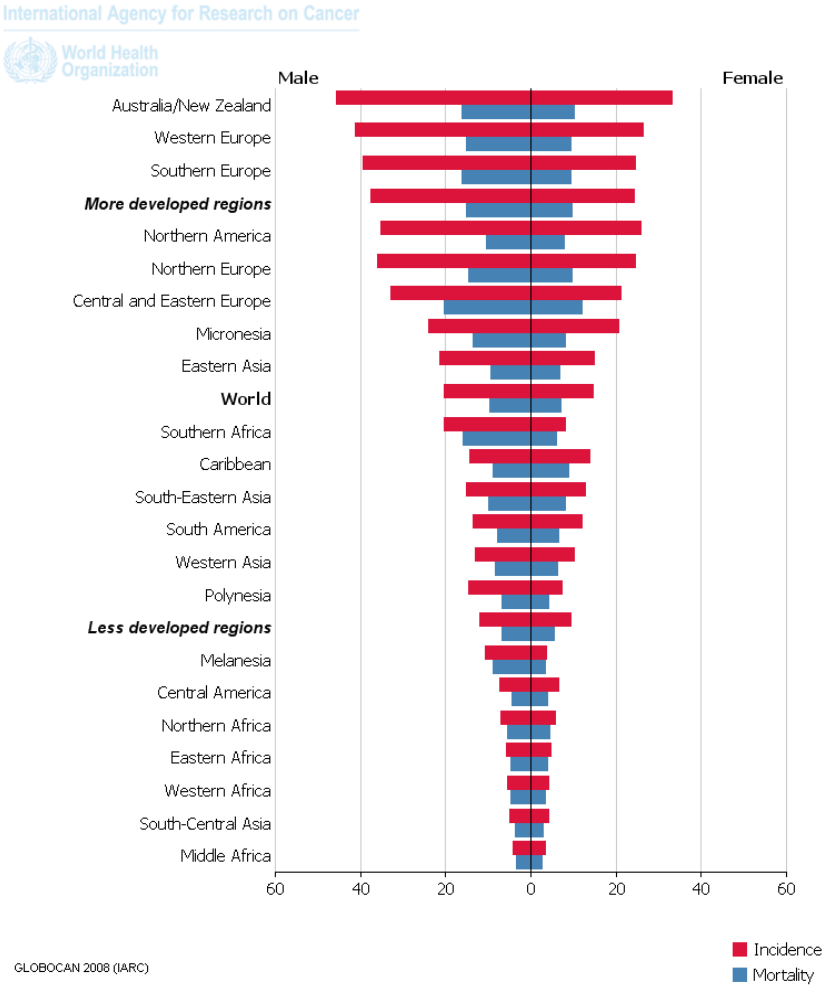


Figure 2.2.2.1Colorectal cancer incidences of colorectal cancer worldwide: estimated age-standardized rates per 100000 (Globocan colorectal cancer 2008)

In Austria between 4600 and 5000 malignant colorectal tumors are diagnosed in the colorectal region each year. This represents 13% of all new cases of malignant diseases. 1988 more women than men developed colorectal cancer in Austria but from 1994 on the gender ratio has reversed. So that currently, the risk of developing colorectal is higher in men than women. 4 of 100 men but only 2 of 100 women developed colorectal cancer till the age of 75 in the year 2008. The risk of dying from colorectal cancer before the age of 75 has been declining over the past 20 years (2% to 1.1%) as consequence of early diagnosis of adenomas.

The incidence and mortality rates for malignant neoplasms of the intestine in Austria are shown in figure 2.2.2.2 (Statistik Austria 2011).

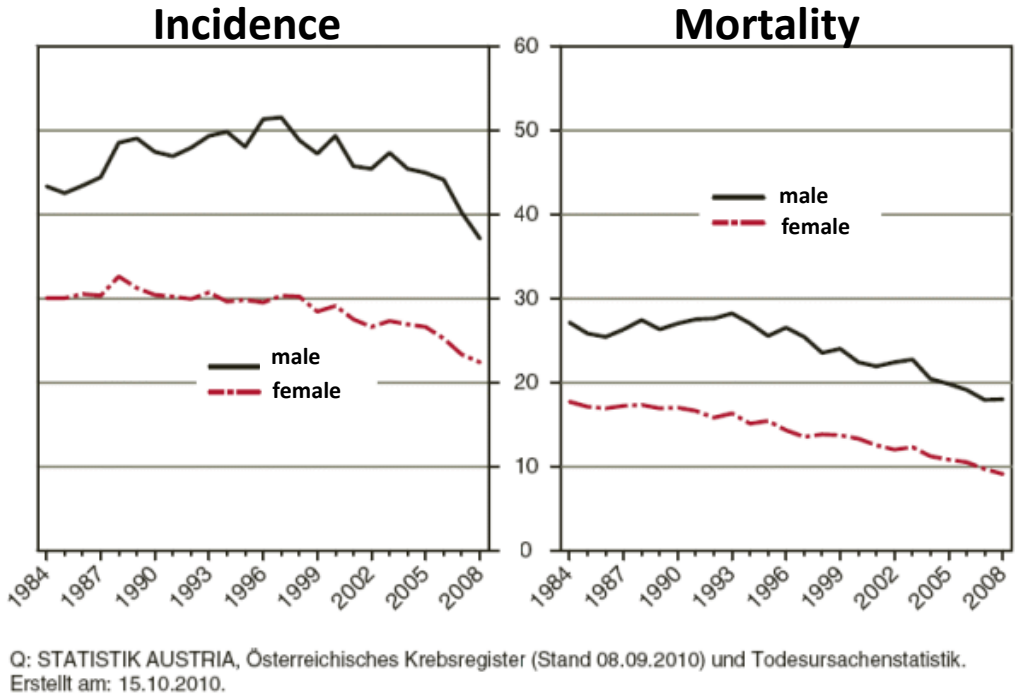


Figure 2.2.2.2 Incidence and mortality rates for malign neoplasms of the intestine in Austria: age-standardized rates per 100000
(adapted Statistik Austria 2011)

2.3 Tumor development

2.3.1 General features of tumor development

As mentioned above genetic alterations are important factors in the pathogenesis of cancer. The relevant mutations can be in oncogenes resulting in dominant gain of function as well as in tumor suppressor genes causing recessive loss of function (Hanahan and Weinberg 2000).

The gain of function mutation of proto-oncogenes turns them into oncogenes that enhance proliferation and survival of cells (Alberts 2008). Important examples are the RAS-oncogenes which is mutated in 50% of all colon carcinomas (Kinzler and Vogelstein 1996). Tumor suppressor

genes prevent cancer in their wild type version and their recessive loss supports cancer e.g. Rb gene (Alberts 2008).

The mutations can be point mutations as well as more severe changes in the chromosomes.

Thus cancer is a disease of dynamic changes in the genome. In humans tumorigenesis is a multistep process (also see figure 2.1.1). These steps are genetic alterations which cause the progressive transformation of normal human cells into highly malignant cancer cells. Hence the process of tumor development can be compared with the Darwinian evolution in which the succession of genetic changes leads to growth advantages and they cause the progressive transformation of normal human cells into cancer cells (Hanahan and Weinberg 2000). Thus the process is characterized by new cell phenotypes and selection of those populations that grow best (Schulte-Hermann et al. 1999).

Hanahan and Weinberg (2000) explained in their review “hallmarks of cancer” that cancer cells have six essential acquired capabilities that cause malignant growth. These hallmarks include self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis (programmed cell death), limitless replicative potential, tissue invasion, metastasis and sustained angiogenesis (see figure 2.3.1.1). The sequence of the acquisition of these capabilities and thus the pathway how the cells become malignant is very variable. Therefore the capabilities can occur at different stages of the transformation. One genetic event can cause the acquisition of more than one capability or only partially support a single capability (see figure 2.3.1.2).

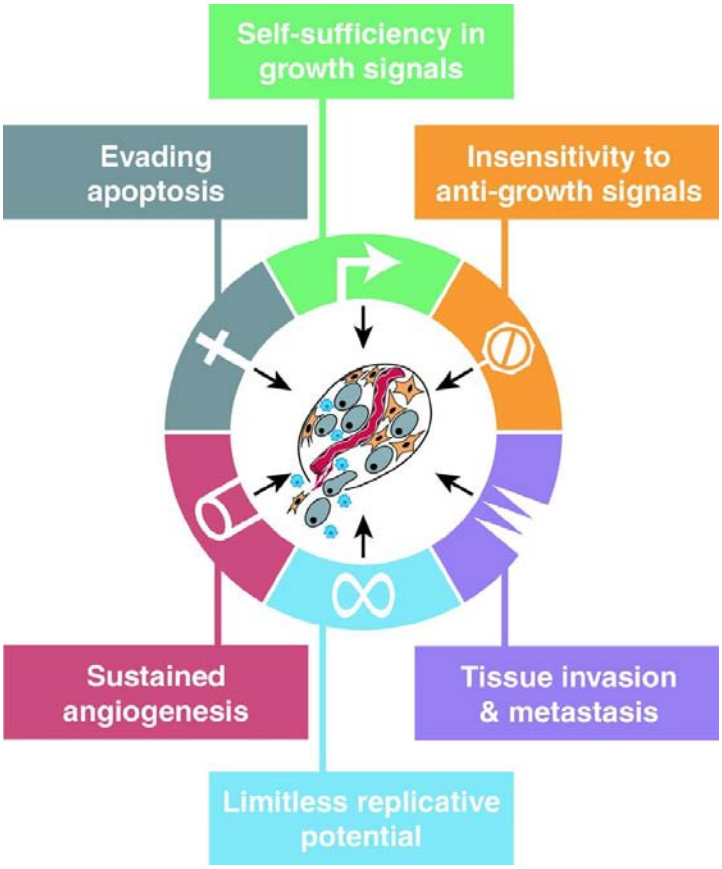


Figure 2.3.1.1 The 6 essential acquired capabilities of cancer cells
(Hanahan and Weinberg 2000)

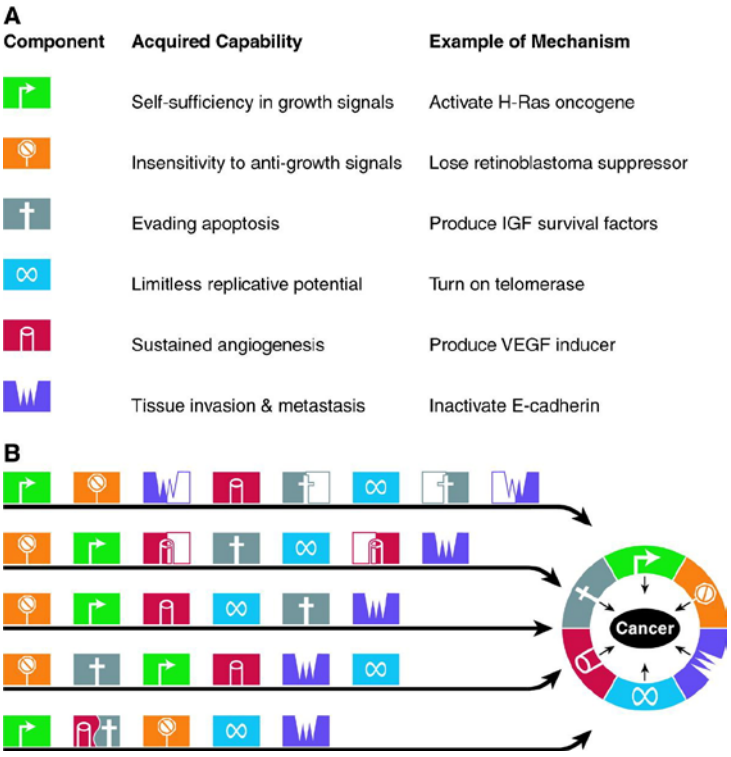


Figure 2.3.1.2 The very variable sequence of acquisition of the acquired capabilities
(Hanahan and Weinberg 2000)

Various anti-cancer defense mechanisms such as cell cycle checkpoints and DNA repair machinery prohibit these 6 acquired capabilities and thus cancer stays a rare event in human life. Therefore mutations in these defense mechanisms lead to increased mutability and genome instability. Under such conditions multiple mutations which are found in tumor cells become more likely. A very important example is the p53 tumor suppressor which responses to DNA damage either with cell cycle arrest or apoptosis (Hanahan and Weinberg 2000). Normal p53 function is lost in most human cancers (Levine 1997).

2.3.2 The initiation-promotion-progression model

The multistage concept shows that malignant cells develop stepwise in a sequence of intermediary cells which distinguish increasingly from the original cell. The population of intermediary cells originates from single cells ("clonal genesis") and they grow faster than the original tissue. This proliferative advantage is low in early stages and increases during cancerogenesis.

The development of cancer can be divided into 3 different stages: Initiation – Promotion – Progression (see figure 2.3.2.1)

Initiation: Initiation is caused by carcinogens and leads to the first mutation. Non-mutational causes of initiation are also possible e.g. epigenetic mechanisms. Initiation is a persistent transformation which is transmitted to daughter cells and causes a proliferation advantage for initiated cells. It is an infrequent event and does not lead to tumor formation alone.

Promotion: It is the process that accelerates tumor cell growth by creating a growth advantage for initiated cells leading to selective expansion of initiated cells. Promotion without initiation does not have a tumorigenic effect. Promotion requires a longer period. Mutations and

genotoxic events are not essential for promotion. Promotion is characterized by enzyme induction and growth. Tumor promotion is a reversible process at least in early stages. Thus the effect of promotion can vanish when the treatment with promoters is terminated.

Progression: Initiation and promotion only cause benign tumors. The malignant transformation of these cells is called progression. It is an accumulation of new mutations because more than one mutation is necessary for malignancy. The malignant cells show increased autonomy and the potential for metastasis. Progression is characterized by genome instability in which the mutated tumor suppressor gene p53 plays a prominent role (Schulte-Hermann 1999).

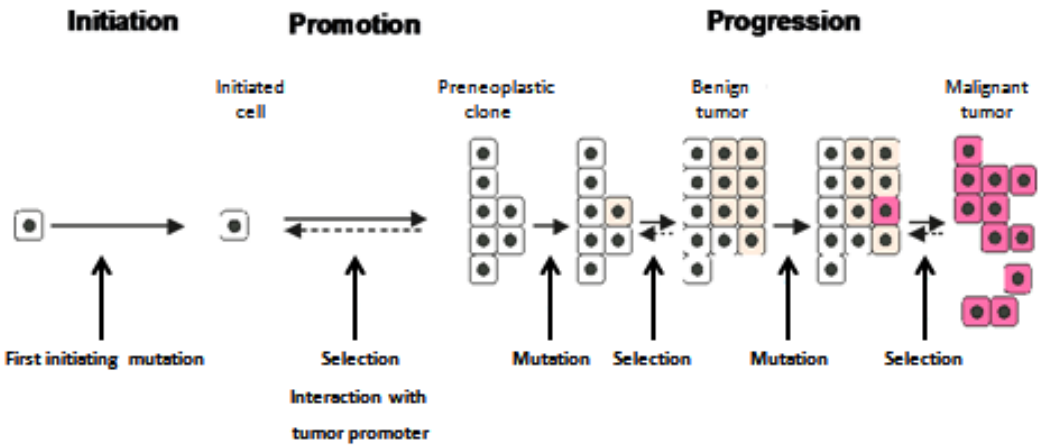


Figure 2.3.2.1 Initiation-promotion-progression model
(Modified Schulte-Hermann R. 2004. In Hiddemann W, Huber H, et al. (eds.): Die Onkologie. Berlin – Heidelberg, Germany: Springer Verlag)

2.3.3 The causes of cancer

The transformation into malignant tumor cells is characterized by mutations. These changes are caused by interaction between the genome of the single person and 3 different categories of external agents:

- Physical carcinogens: e.g. ionizing radiation, UV-light
- Chemical carcinogens: e.g. tobacco smoke, alcohol, asbestos
- Biological carcinogens: e.g. certain viruses such as Hepatitis B and C and certain types of Human Papilloma Virus

Ageing also increases the probability of cancer if they are carcinomas.

Risk factors of cancer are tobacco use, alcohol consumption, unhealthy nutrition, overweight, physical inactivity, air pollution and infection with certain viruses e.g. through sexual transmission (WHO 2011).

Although a person avoids general cancer risks he or she can develop cancer because of genetic predisposition. This means that a person is already born with a mutation which can cause cancer. An example for that are the breast cancer genes BRCA1 and BRCA1. Women who carry one of these mutated genes develop breast cancer with higher likelihood. The genetic predisposition is also possible for colorectal cancer (Cancer Research UK). However, cancer development is a very complex process. A germline mutation which causes predisposition for cancer does not lead automatically to cancer. In fact cancer is unlike classic genetic diseases such as cystic fibrosis a strong interaction between heredity and environmental factors. The reason for that is that additional mutations are necessary and environmental factors affect mutation rates. This explains why diets of large amounts of red meat lead to higher rates of colorectal cancer (Kinzler and Vogelstein 1996).

2.4 Anatomy of colon

The large intestine (Intestinum crassum) is 1.5-1.8m long and is located after the small intestine (Intestinum tenue). One segment of the large intestine is the colon which is located between the caecum and the rectum. The colon is divided into Colon ascendends, Colon transversum, Colon descendens and Colon sigmoideum. The colon is characterized by Taeniae coli which are 1cm broad thickenings of the outer longitudinal muscle layer and contraction rugae (Plicae semilunares coli). Characteristic protrusions (Haustra coli) emerge between 2 Plicae semilunares coli (Taschenatlas 2003).

The function of the Intestinum crassum is the resorption of water.

The intestine is generally composed of several layers of tissue: At the luminal side the Tunica mucosa resides. It consists of the Lamina epithelialis mucosae (epithelial layer), the Lamina propria mucusae (connective tissue including blood and lymphatic vessels, smooth muscle cells) and the lamina muscularis mucosae (smooth muscle cells). Next to the Tunica mucosa the Tela submucosa is located (blood and lymphatic vessels and Plexus submucosus as a part of the enteric nervous system). After the Tela submucosa the Tunica musularis is situated which consists of an inner circular layer of smooth muscle cells and an outer layer of longitudinal layer of smooth muscle cells. The Tunica musularis is important for peristalsis of the intestine and includes the Plexus myentericus as second part of the enteric nervous system. The last part of the intestine is the Tunica serosa which has a smooth surface in order to support movement of the intestine (see figure 2.4.1)

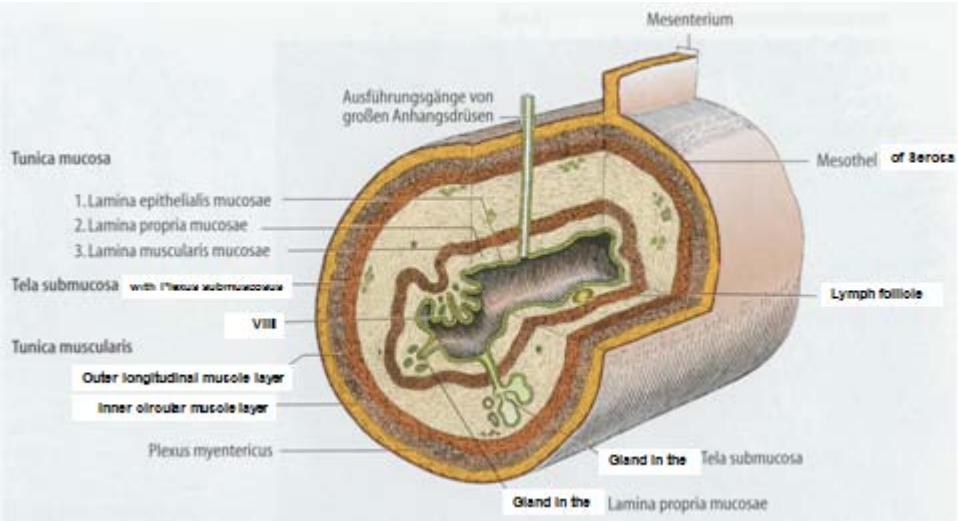


Figure 2.4.1 The layers of the intestine
(Junqueira 2002)

The Tunica mucosa of the small intestine is characterized by Villi intestinalis which are evaginations of the Lamina epithelialis and Lamina propria. Between the villi are crypts of Lieberkühn (glands) which sink into the Lamina propria (Junqueira 2002).

In contrast to the small intestine the mucosa of the colon does not have villi but a flat surface (Reya and Clevers 2005). The colon also has crypts which can be 0.5mm deep (Junqueira 2002).

2.5 Tumor stem cells, Wnt pathway and colorectal cancer development

The epithelium of the small intestine consists of differentiated cells and stem/progenitor cells. Spatially, the differentiated cells like enterocytes, enteroendocrine cells and goblet cells are located at the villi and the Paneth cells (produce antimicrobial agents) and stem/progenitor cells reside in the crypts.

In the colon stem/precursor cells are at the bottom 2/3 of the crypts and the differentiated cells cover the top third of the crypts and the surface of the lumen.

The life expectancy of an intestinal epithelial cell is less than a week. Thus an enormous amount of cells is generated per day at the bottom of a crypt and lost on the top. Only stem cells and Paneth cells can escape this continuous flow of cells.

Colon stem cells are located at the crypt bottom and they produce transit amplifying cells which rapidly proliferate and are able to differentiate in all concerning cell types populating the crypt. The tasks of the colon stem cells are self-renewal and differentiation but also regeneration of the epithelium after injury. Eventually, the committed progenitors enter cell cycle arrest and express differentiation markers when they arrive the top third of the crypts (see figure 2.5.1).

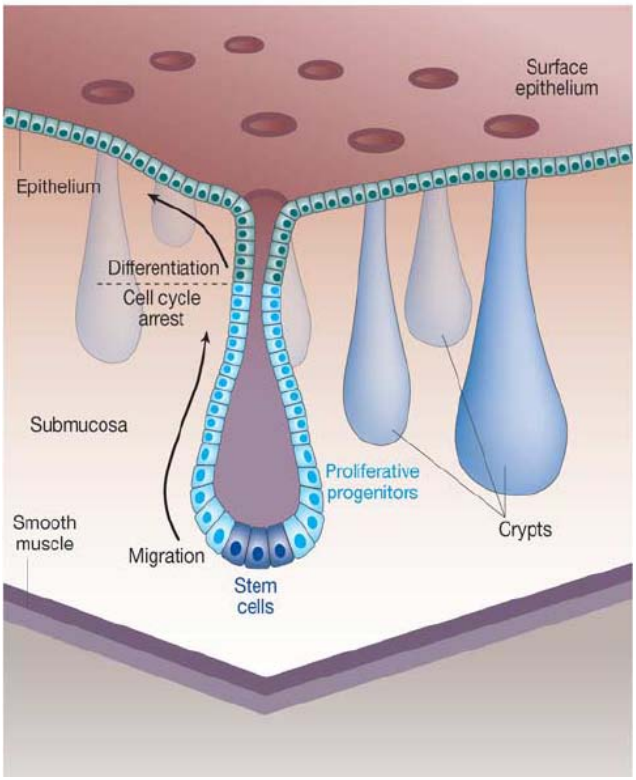


Figure 2.5.1 Colon stem cells
(Reya and Clevers 2005)

The canonical Wnt pathway is the dominant force in cell fate and drives the genetic programme in stem/progenitor cells of the crypts.

The central player of the Wnt pathway is β-catenin whose stability is regulated by a destruction complex. β-catenin also resides in cadherin cell-cell adhesions. Signalling is initiated when a Wnt ligand binds to a receptor complex which consists of Frizzled (serpentine receptor) and Lrp5/6 (LDL receptor family). In the absence of ligand the two scaffolding proteins complex APC (adenomatous polyposis coli) and axin bind to β-catenin. In this case GSK3 kinase phosphorylates the amino terminus of β-catenin. This phosphorylation leads to ubiquitination and subsequent proteasomal degradation. If a Wnt ligand binds the receptors GSK3 is inhibited and thus β-catenin accumulates and enters the nucleus where it binds the N-terminus of Tcf/Lef (DNA-binding protein). If β-catenin is absent Tcf/Lef is associated with co-repressors like Groucho

and this leads to repression of target genes. Tcf/Lef is converted into a transcriptional activator by binding to β-catenin (see figure 2.5.2).

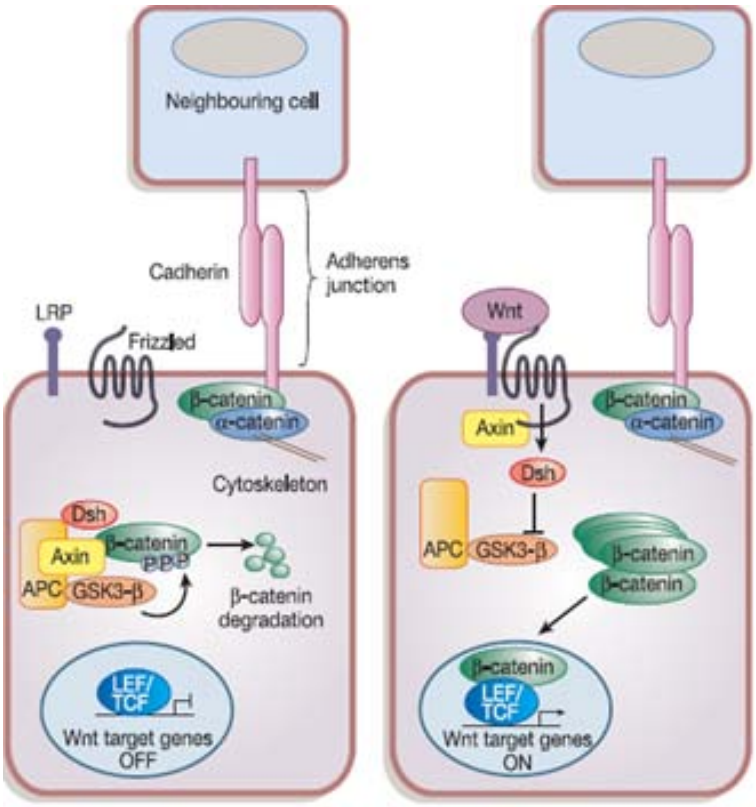


Figure 2.5.2 Wnt pathway
(Reya and Clevers 2005)

This is the important function of the Wnt pathway in colorectal stem/progenitor cells. Interestingly, the activation of the Wnt pathway also plays a crucial role in colorectal cancer.

APC was discovered as the underlying germ line mutation in familial adenomatous polyposis (FAP) which is an autosomal, dominantly inherited cancer syndrome affecting 1 in 7000 individuals. Those FAP patients who have one defective APC allele (chromosome 5q21 locus) frequently also lose the other allele by mutation or deletion and then develop a lot of adenomas (polyps) in their early life. If you examine a single adenoma they are clonal outgrowths of epithelial cells whose second APC allele is mutated or deleted (Kinzler and Vogelstein 1996; Reya and Clevers 2005).

The germline mutation of APC does not automatically lead to colorectal cancer but FAP patients have a higher risk to develop colorectal cancer. The rate-limiting step seems to be the somatic mutation of the wildtype APC allele (Kinzler and Vogelstein 1996).

Over time additional mutations of oncogenes or tumor suppressor genes accumulate in FAP patients and only those lead to colorectal carcinoma development.

APC is also lost in most sporadic colorectal cancers and consequently, tumor development is initiated with a mutational activation of the Wnt pathway. The inactivation of APC causes the aberrant stabilization of β-catenin and subsequently, the inappropriate activation of the Wnt pathway transforms the epithelial cells. DNA microarray analysis has shown that the same genetic programmes are activated through the Wnt pathway activation in colorectal cancer that are active in physiological stem/progenitor cells. When adenoma cells activate the Wnt pathway they maintain their progenitor status for an unlimited time. This feature allows them to exist for many years and enables the cells to acquire further mutations (Reya and Clevers 2005).

Thus, in most cases the initiating event of the intestinal neoplastic process is an APC mutation with the effects mentioned above. Subsequently, patients develop dysplastic aberrant crypt foci (ACF) and early adenoma. Other mutations occur during progression. 50% of colorectal carcinoma and an equal number of adenomas greater than 1 cm have K-RAS mutation but this mutation alone cannot lead to initiation of tumor development. Just one genetic event is necessary for activation of K-RAS. It occurs in a small adenoma and leads to a larger and stronger dysplastic tumor (intermediate adenoma) through clonal expansion. 70% of carcinomas lose several different tumor suppressors on

chromosome18q21 (e.g.DCC). Finally, a loss of a large portion of chromosome 17p is detected in more than 75% of colorectal carcinomas but this can be detected infrequently in adenomas. This region includes p53 which is an important tumor suppressor gene. Furthermore p53 mutations occur in the remaining allels of several colorectal cancers. Again the p53 mutation alone cannot initiate the neoplastic process. Interestingly, p53 mutation has a dominant negative effect and it leads to a selective growth advantage even if a wild-type p53 allele is still working. Loss of the wild-type p53 allele leads to the transition of adenomas to carcinomas. While the sequence of accumulation of mutations is not obligatory, they still occur in a preferred order that is characteristic for colorectal cancer. Thus APC seems the gatekeeper in colorectal cancer development (Fearon and Vogelstein 1990; Kinzler and Vogelstein 1996). See the sequential events of mutations in figure 2.5.3 and the different colorectal cancer stages in figures 2.5.4-2.5.5.

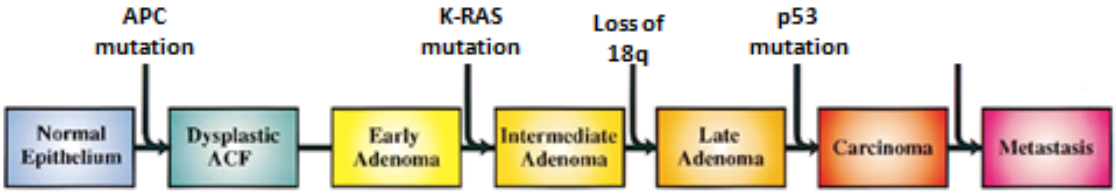


Figure 2.5.3 Sequential mutation events in colorectal cancer development
(Kinzler and Vogelstein 1996)

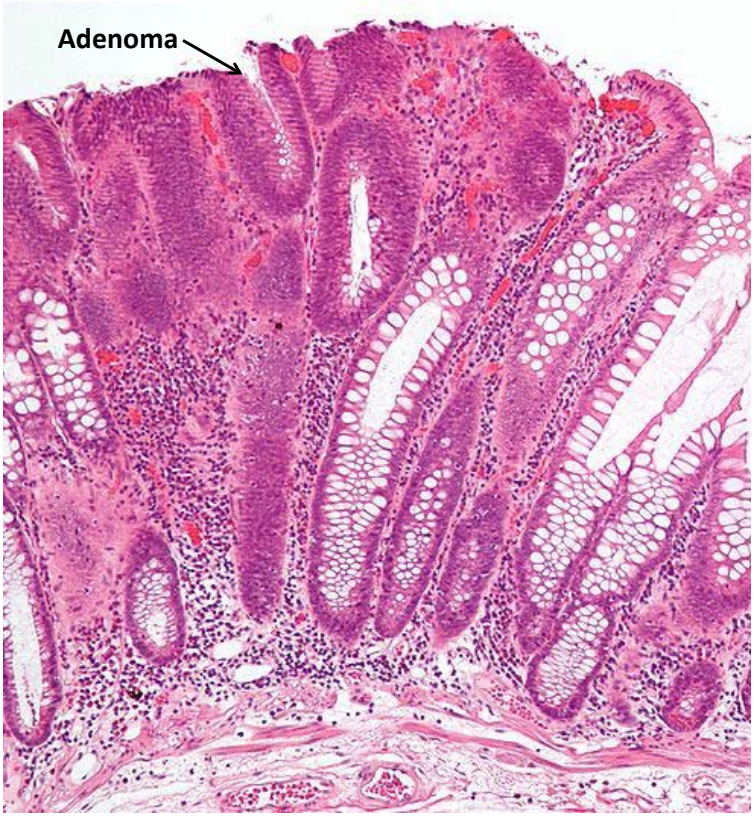


Figure 2.5.4 Adenomas surrounded by normal tissue
(adapted Wikipedia 2009)

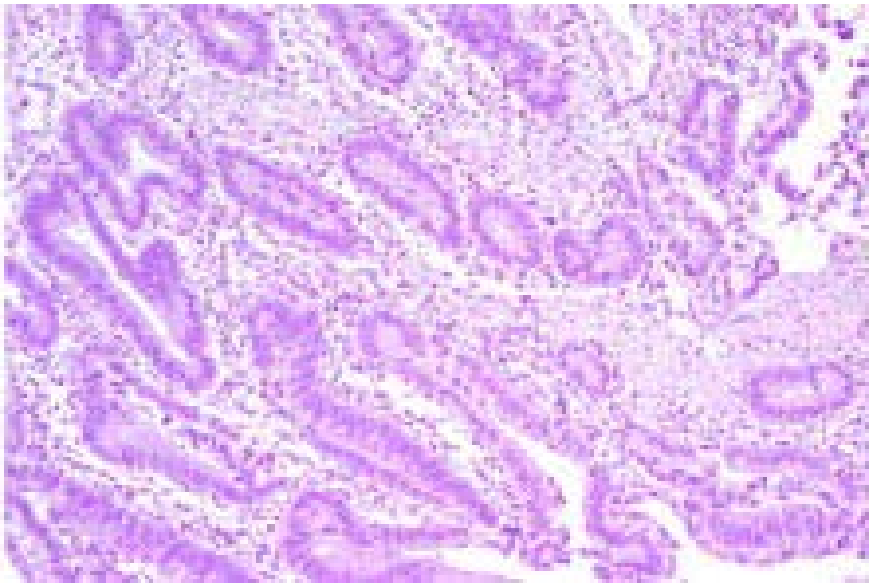


Figure 2.5.5 Colorectal carcinoma tissue
(Camarero et al. 2006)

2.6 Introduction of the diploma thesis topic

As mentioned above colon tumorigenesis is initiated by a mutation of the APC tumour suppressor gene which leads to prolonged and stronger activation of the WNT-pathway (Fearon and Vogelstein 1990). This causes a subsequent accumulation of CD44+ cells with higher survival potential in premalignant colorectal adenomas (Schulenburg et al. 2007). As mentioned above adenomas are benign neoplasms of epithelial tissue which arise from colonic crypts with mutated APC (Fearon and Vogelstein 1990). Thus the CD44+ subpopulation of the colorectal adenomas can progress to malignant colon carcinoma cells through subsequent mutations. CD44 is a WNT target gene and therefore it indicates an active WNT pathway (Schulenburg et al. 2007).

An activated WNT pathway was the main characteristic of a CD44+ subpopulation of the LT97 colon adenoma cells that displayed an extended survival potential (Schulenburg et al. 2007). In this context several survival-related genes have been found differentially regulated in CD44+ LT97 cells previous experiments. Among them were survivin, FGF18 und IGFBP3 (Schulenburg et al. 2011 manuscript).

Survivin (BIRC5) is an inhibitor of apoptosis that inactivates caspase activity and is a direct WNT target gene (Altieri 2008). FGF18 is also a known WNT target gene (Sonvilla et al. 2008) and a ligand of FGFR3-IIIc and FGFR4 (Zhang et al. 2006). It is important for cell proliferation, survival and migration of colonic tumor cells (Sonvilla et al. 2008). IGF binding protein 3 (IGFBP3) is a negative regulator of IGF1 signalling which stimulates growth and enhanced cell survival in HT29 and LT97 (Pollak 2008). Mediated via Akt (PKB) which phosphorylates and inactivates GSK3beta IGF-signaling can modulate wnt-pathway activity (Guessous et al. 2008).

It was observed that IGFBP3 was strongly down regulated in the CD44+ subpopulation of LT97 cells so that it is almost exclusively expressed in the CD44- subpopulation (Schulenburg et al. 2011 manuscript). It is assumed that the active WNT pathway negatively regulates IGFBP3 expression. In order to underpin this hypothesis sulindac is applied at HT29 and LT97 cells in this diploma thesis. Sulindac is a non-steriodal anti-inflammatory drug of the arylalkanoic acid class which has many effects including the inhibition of the WNT pathway and is used for chemoprevention of hereditary colon cancer (Koornstra et al. 2005). Although sulindac can down regulate the WNT pathway it is not specific enough to prove the hypothesis because it can have side effects which cause IGFBP3 up-regulation. Thus a more selective tool to shut down the WNT pathway was needed.

2.7 Aims of the diploma thesis

The hypothesis of the diploma thesis is that the active WNT pathway impacts negatively on the expression of IGFBP3. Thus the inactive WNT pathway shall increase IGFBP3 RNA and protein levels.

The aims of the diploma thesis are:

1. Sulindac treatment to inhibit wnt target genses such as surviving and FGF18 and to prove that this has also impacts on IGFBP3 RNA and protein levels in HT29 and LT97 cells.
2. Production of an adenovirus expressing a truncated ΔN-TCF4 gene to interrupt the β-catenin TCF/LEF interaction.
3. Specific inactivation of the wnt signalling using the pAd/CMV/V5-DEST-ΔN-TCF4 virus and investigation of the effects as above.

3. Results

3.1 Sulindac experiments

3.1.1 Results on the RNA level

HT29 and LT97 cells were cultivated and treated with sulindac according to **5.1** and **5.2** of the **Materials and methods** section. The RNA was extracted and analyzed according to **5.3 - 5.6** of the **Material and methods** section. Sybr® green I Real Time PCR was used to quantify survivin and IGFBP3 in the HT29 and LT97 samples. Taqman® probe kits were used to detect FGF18 and IGFBP3 in both cell lines.

The results are shown in figures 3.1.1.1 and 3.1.1.2 as percent values relative to the GAPDH house keeping gene and the untreated control group. Survivin was down-regulated to 28 ± 18.62% ($p = 0.0286$) in sulindac treated HT29 cells. In LT97 cells the impact was less pronounced but survivin was also clearly down-regulated to 61 ±3.55% ($p = 0.0286$) (see figure 3.1.1.1).

IGFBP3 shows a different picture. On the one hand IGFBP3 might be up-regulated in HT29 cells (134 % of the control level) but the standard deviation was too high providing a significant result. On the other hand it was almost unaffected in LT97.

Sulindac treatment had no effect on FGF18 in HT29 (107% of the control level). LT97 cells showed a down-regulation of FGF18 (65% of the control level) but the standard deviation was too high for a statistically significant result.

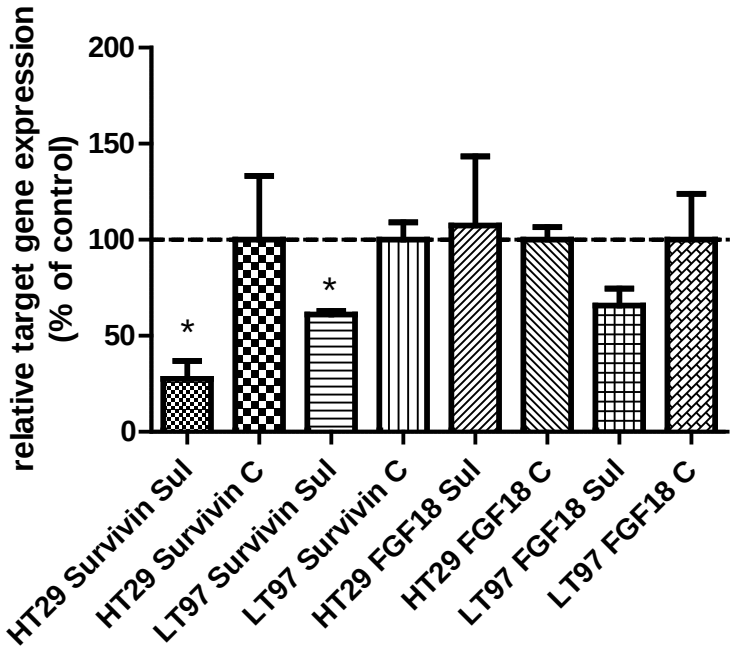


Figure 3.1.1.1: % in comparison with the control in sulindac treated HT29 and LT97 samples (Survivin, FGF18)
The value of 100 indicates the same value as the control.
* indicates statistically significant data

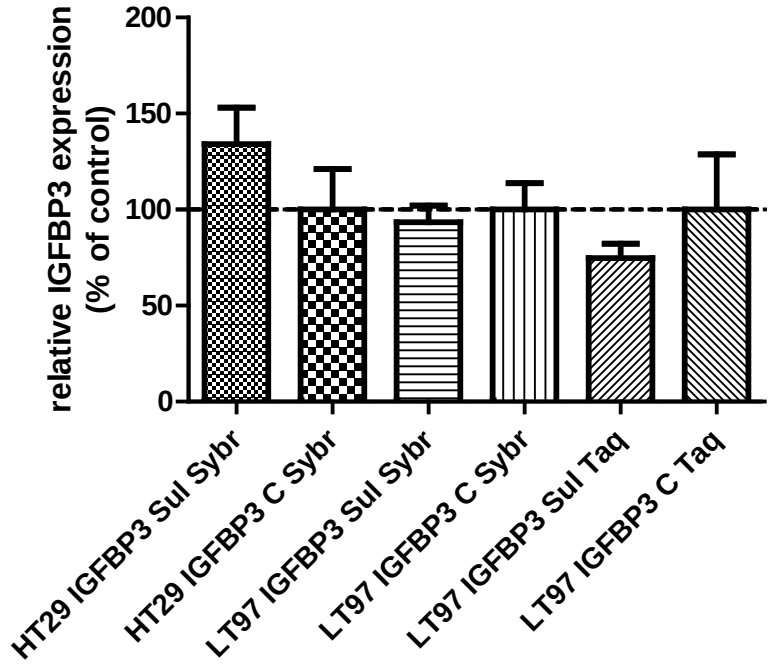


Figure 3.1.1.2: % in comparison with the control in sulindac treated HT29 and LT97 samples (IGFBP3)
The value of 100 indicates the same value as the control.

3.1.2 Results on the protein level

HT29 cells and LT97 were cultivated and treated with 10µM sulindac according to **5.1** and **5.2** of the **Materials and methods** section. The protein was extracted and analyzed by SDS PAGE and Western Blot according to **5.7 - 5.10** of the **Material and methods** section. Survivin was detected in both cell lines under sulindac and control conditions. β-Actin was used for standardization. The bands were analyzed with ImageQuant5.0. The results are in figure 3.1.2.1 and in figure3.1.2.2.

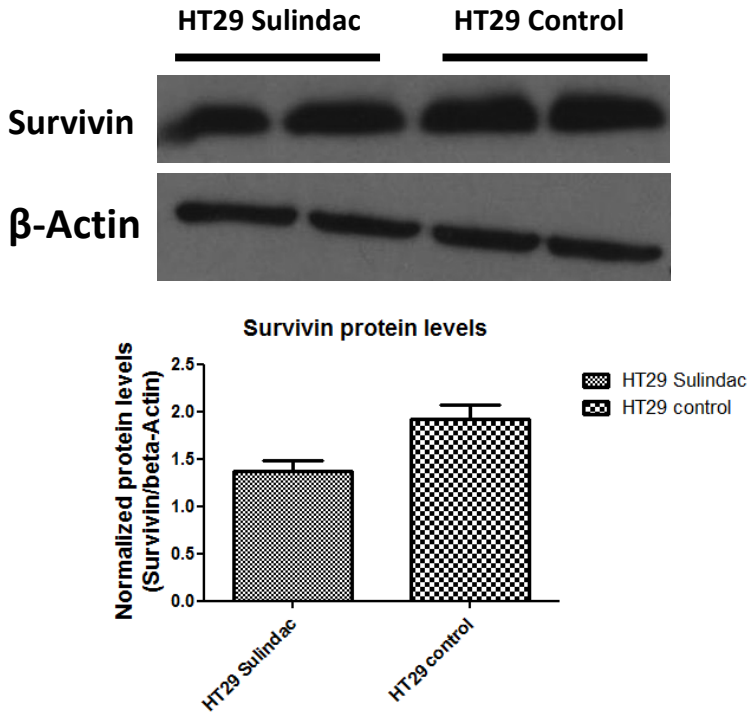


Figure 3.1.2.1 HT29 Survivin protein levels

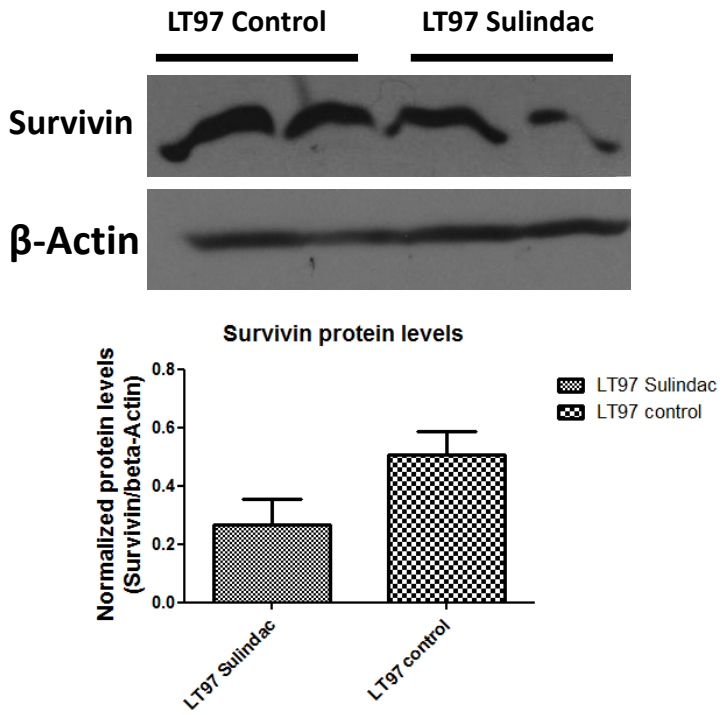


Figure 3.1.2.2 LT97 Survivin protein levels

According to figure 3.1.2.1 and figure 3.1.2.2 sulindac treatment decreased Survivin protein levels in HT29 and LT97 cells. These results were as expected because Survivin is a WNT target gene and Sulindac inhibits this signal pathway.

3.2 Transfection of HT29 cells with pbabe-puro-GFP

It was planned to transfect the pAAV-IRES-hrGFP-ΔN-Tcf4 plasmid (Holnthoner et al. 2002) in HT29 cells by lipofection in order to avoid cloning into an adenoviral vector and subsequent transduction. This possibility was tested with a pbabe-puro-GFP construct. For this purpose HT29 cells were transfected with 2µg and 4µg pbabe-puro-GFP plasmid in 6-well plates according to 5.13 in the materials and methods section. Control cells were not transfected. 24 hours after transfection the cells were prepared according to 5.14 in the materials and methods section and the GFP expression were evaluated by FACS analysis. The results are depicted in figure 3.3.1 and in table 3.3.1. Only a small fraction of cells could be transfected but at least 50% of cells shall express the pAAV-IRES-hrGFP-ΔN-Tcf4 in order to do experiments.

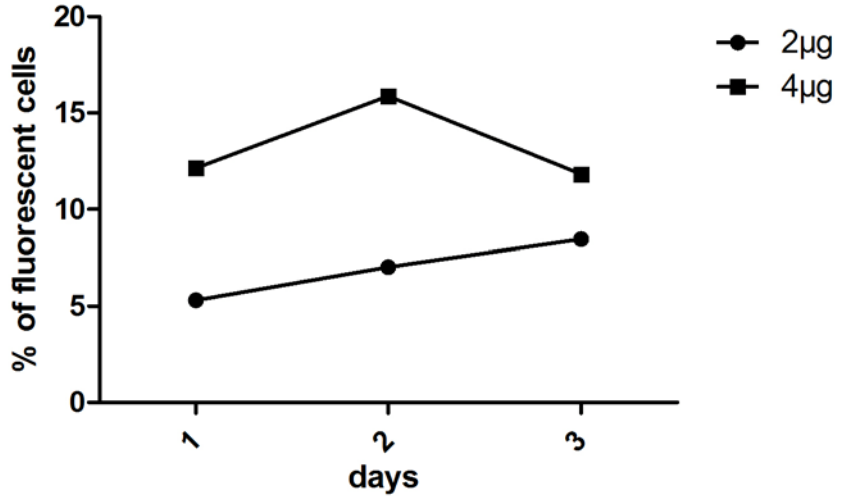


Figure 3.2.1: percentage of GFP expressing HT29 cells

Table 3.2.1: percentage of GFP expressing HT29 cells		
days	2µg plasmid	4µg plasmid
1	5.31	12.14
2	7.03	15.87
3	8.46	11.82

3.3 Production of adenoviral Tcf4-vector

The transfection of the pAAV-IRES-hrGFP-ΔN-Tcf4 or -VP16- ΔN-Tcf4 or -Tcf4wt plasmids (see figure 3.3.1) (Holnthoner et al. 2002) were not possible according to the results in 3.2 and hence the ΔN-Tcf4 or -VP16-ΔN-Tcf4 or -Tcf4wildtype fragments were cloned into an adenoviral vector. For this purpose the fragments were cut out of the pAAV-IRES-hrGFP backbone and cloned into a pENTR vector which delivers the insert into the final adenoviral expression vector by gateway cloning.

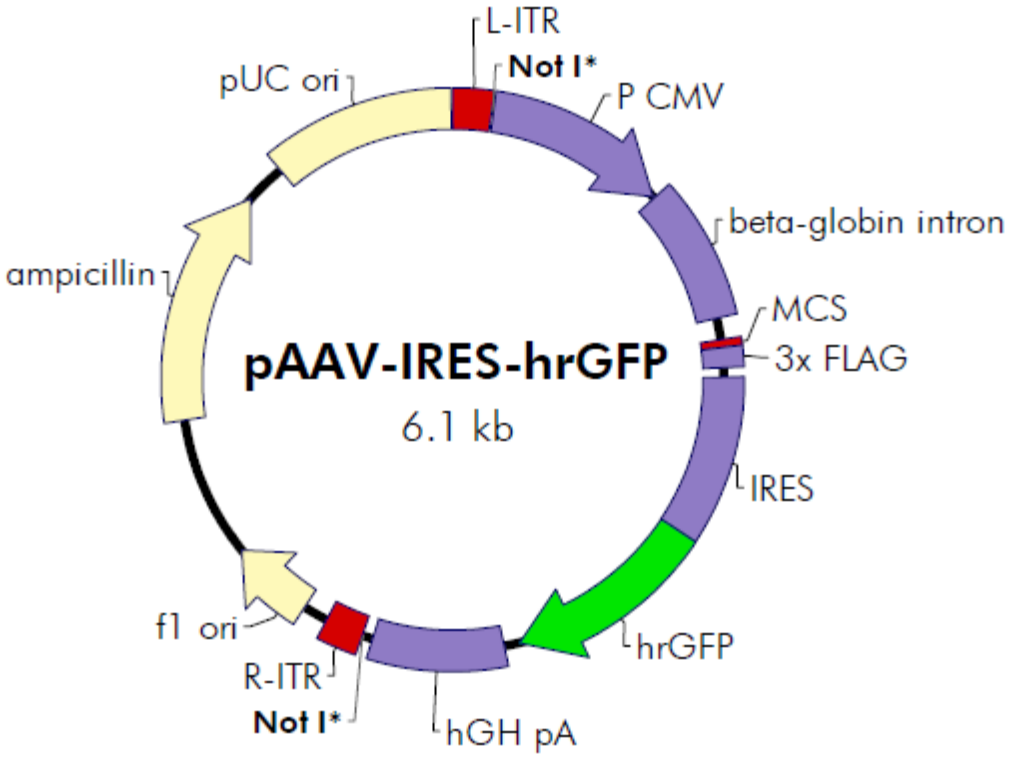


Figure 3.3.1 pAAV-IRES-hrGFP: ΔN-Tcf4, VP16- ΔN-Tcf4 and Tcf4wildtype are inserted into the MCS

3.3.1 Preparation for re-cloning

First of all the pAAV-IRES-hrGFP-ΔN-Tcf4 or -VP16- ΔN-Tcf4 or -Tcf4wildtype plasmids were transformed and amplified according to 5.12 in the material and methods section. The lengths of the inserts are in table 3.3.1.1.

Table 3.3.1.1 insert lengths	
Insert	app. length
ΔN-Tcf4	1700bp
VP16- ΔN-Tcf4	1950bp
Tcf4 wildtype	1800bp

See miniprep results for pAAV-IRES-hrGFP-ΔN-Tcf4 in table 3.3.1.2.

Table 3.3.1.2 pAAV-IRES-hrGFP-ΔN-Tcf4 samples 1 and 2	
Sample	Concentration (ng/µl)
Sample 1	251.14
Sample 2	170.27

The pAAV-IRES-hrGFP-ΔN-Tcf4 plasmid was analyzed with the restriction enzymes EcoRI and XhoI (Fermentas) and 10x buffer H (Roche).

See the results for the digest of pAAV-IRES-hrGFP-ΔN-Tcf4 sample 1 and 2 at figure 3.3.1.1. The digest of both samples had bands at1700bp and therefore they included ΔN-Tcf4.

Figure 3.3.1.1 pAAV-IRES-hrGFP- ΔN-Tcf4 sample 1 and 2 digest with EcoRI and XhoI

Sample 1 were amplified and isolated with Midiprep (see results in table 3.3.1.3).

Table 3.3.1.3 pAAV-IRES-hrGFP-ΔN-Tcf4 samples 1 and 2

Sample	Concentration (ng/µl)
Aliquot 1	200
Aliquot 2	74

The pAAV-IRES-hrGFP-VP16-ΔN-Tcf4 and -Tcf4wt plasmids were isolated by miniprep (see results in table 3.3.1.4) and analyzed with the restriction enzymes EcoRI and XhoI (Fermentas). See the resulting products in figure 3.3.1.2 and table 3.3.1.5. Midiprep aliquot 1 of pAAV-IRES-hrGFP-ΔN-Tcf4 were also tested and verified.

The pAAV-IRES-hrGFP-VP16-ΔN-Tcf4 samples showed bands at 250bp if they were digested with EcoRI because VP16 is flanked by these restriction sites. Thus the double digest of these samples also had bands at 1700bp because of the remaining ΔN-Tcf4. The double digest of pAAV-IRES-hrGFP-Tcf4wt samples had bands at 1800bp.

The pAAV-IRES-hrGFP-VP16-ΔN-Tcf4 sample 2 and pAAV-IRES-hrGFP-Tcf4wt sample 2 showed that clearly and midiprep was done with them (see midiprep results in table 3.3.1.6)

Table 3.3.1.4 pAAV-IRES-hrGFP-VP16-ΔN-Tcf4 and -Tcf4wt

Sample	Concentration (ng/µl)
VP16 S1	275.58
VP16 S2	248.33
Tcf4 wt S1	251.33
Tcf4 wt S2	82.25

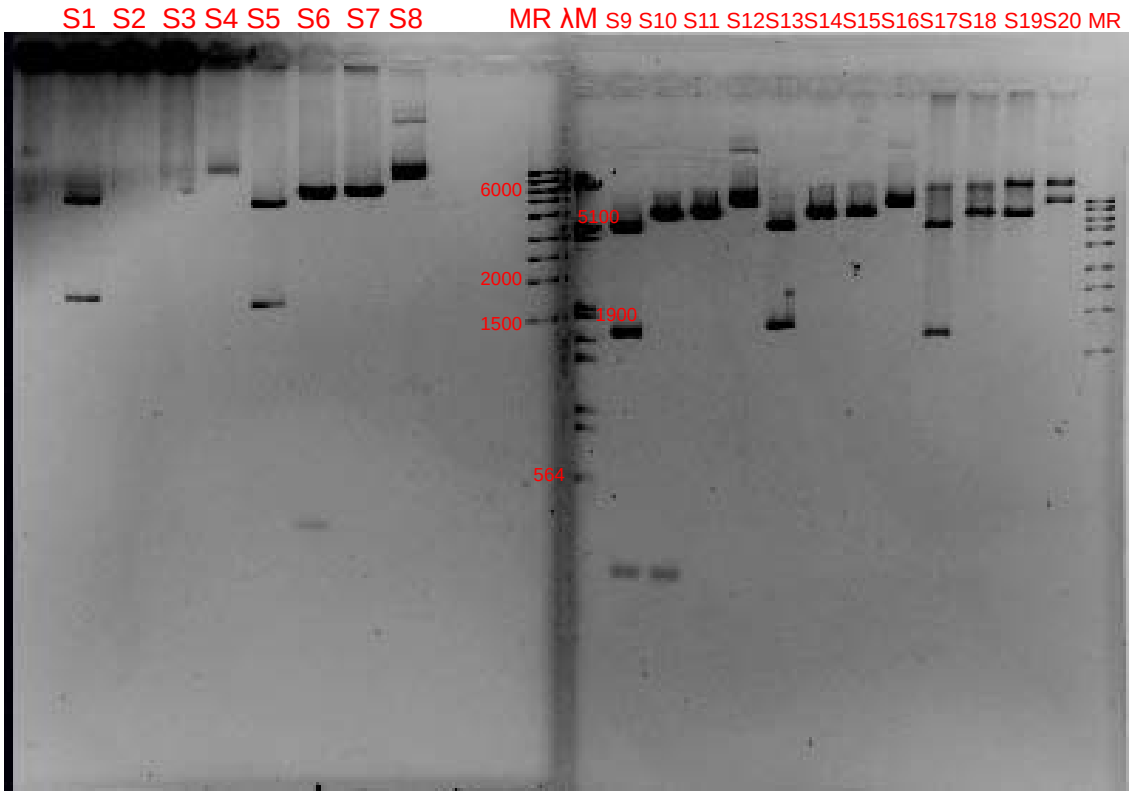


Figure 3.3.1.2 EcoRI and XhoI restriction digest

Table 3.3.1.5 sample description of figure 3.3.1.2

Sample number	Sample details
S1	Tcf4wt sample1 EcoRI and XhoI
S2	Tcf4wt sample1 EcoRI
S3	Tcf4wt sample1 XhoI
S4	Tcf4wt sample1 no restriction enzymes
S5	VP16-Δ-Tcf4 sample1 EcoRI and XhoI
S6	VP16-Δ-Tcf4 sample1 EcoRI
S7	VP16-Δ-Tcf4 sample1 XhoI
S8	VP16-Δ-Tcf4 sample1 no restriction enzymes
S9	VP16-Δ-Tcf4 sample2 Eco RI and XhoI
S10	VP16-Δ-Tcf4 sample2 EcoRI
S11	VP16-Δ-Tcf4 sample2 XhoI
S12	VP16-Δ-Tcf4 sample2 no restriction enzymes
S13	Tcf4wt sample2 EcoRI and XhoI
S14	Tcf4wt sample2 EcoRI
S15	Tcf4wt sample2 XhoI
S16	Tcf4wt sample2 no restriction enzymes
S17	Δ-Tcf4 aliquot1 EcoRI and XhoI
S18	Δ-Tcf4 aliquot1 EcoRI
S19	Δ-Tcf4 aliquot1 XhoI
S20	Δ-Tcf4 aliquot1 no restriction enzymes
λM	Figure 5.12.4.1 Lambda 3 marker
MR	Figure 5.12.4.2 Mass Ruler™ DNA Ladder High Range

Table 3.3.1.6 midiprep of pAAV-IRES-hrGFP-VP16-ΔN-Tcf4 S2 and-Tcf4wt S2

Sample	Concentration (ng/µl)
pAAV-IRES-hrGFP-VP16-ΔN-Tcf4 S2 aliquot 1	136
pAAV-IRES-hrGFP-Tcf4wt S2 aliquot 1	211

Midiprep of pAAV-IRES-hrGFP-VP16-ΔN-Tcf4 sample 2 and –Tcf4wt sample 2 were tested with restriction digests of EcoRI and XhoI (Fermentas). Furthermore midiprep samples of pAAV-IRES-hrGFP-ΔN-Tcf4,-VP16-ΔN-Tcf4 and -Tcf4wt were analyzed with single restriction enzyme digests of NotI, BglII (10xFast Digest® Buffer, Fermentas) and BamHI (10x buffer B). See the resulting gel photos in figure 3.3.1.3, figure 3.3.1.4 and table 3.3.1.7. The expected fragment lengths are in table 3.3.1.8.

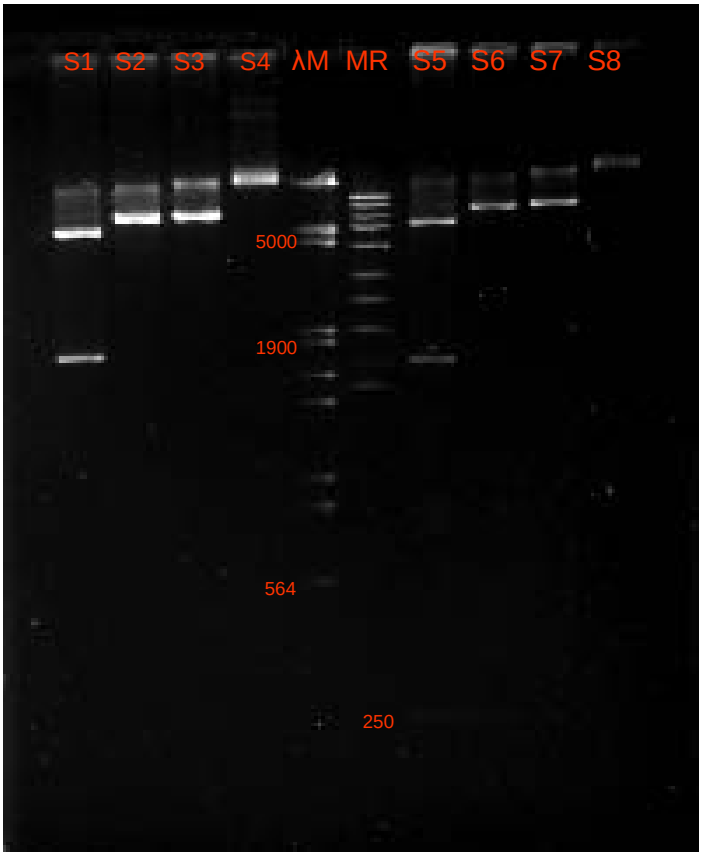


Figure 3.3.1.3 EcoRI, XhoI and NotI restriction digests

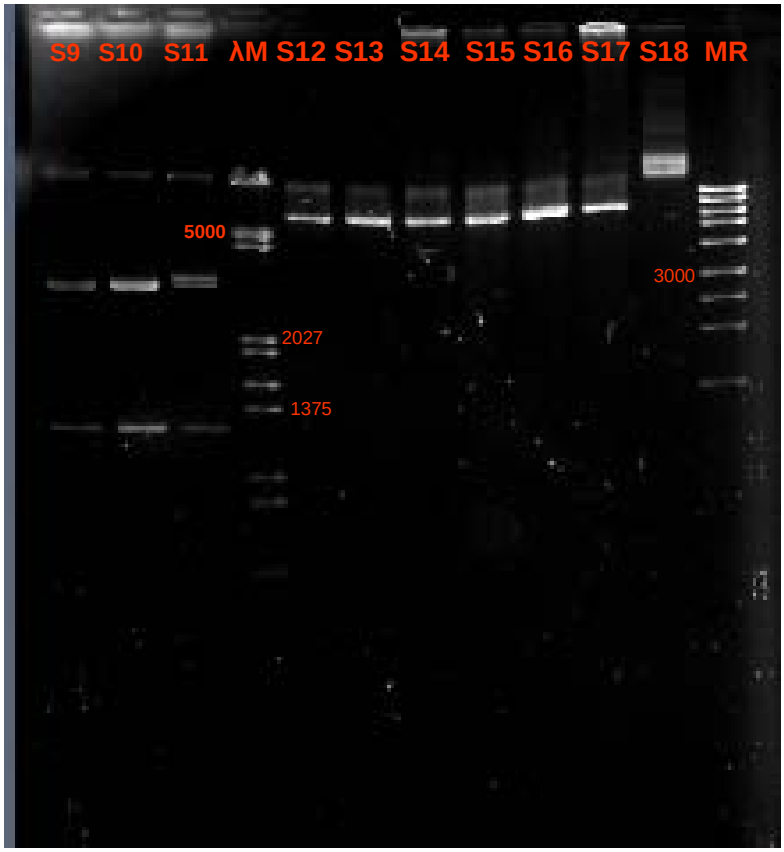


Figure 3.3.1.4 NotI, BamHI and BglII restriction digest

Table 3.3.1.7 Sample description of figure 3.3.1.3 and 3.3.1.4	
Sample number	Sample details
S1	Midiprep sample Tcf4wt EcoRI and XhoI
S2	Midiprep sample Tcf4wt EcoRI
S3	Midiprep sample Tcf4wt XhoI
S4	Midiprep sample Tcf4wt no restriction enzymes
S5	Midiprep sample VP16-ΔN-Tcf4 EcoRI and XhoI
S6	Midiprep sample VP16-ΔN-Tcf4 EcoRI
S7	Midiprep sample VP16-ΔN-Tcf4 XhoI
S8	Midiprep sample VP16-ΔN-Tcf4 no restriction enzymes
S9	Midiprep sample ΔN-Tcf4 NotI
S10	Midiprep sample Tcf4wt NotI
S11	Midiprep sample VP16-ΔN-Tcf4 NotI
S12	Midiprep sample ΔN-Tcf4 BamHI
S13	Midiprep sample Tcf4wt BamHI
S14	Midiprep sample VP16-ΔN-Tcf4 BamHI
S15	Midiprep sample ΔN-Tcf4 BglII
S16	Midiprep sample Tcf4wt BglII
S17	Midiprep sample VP16-ΔN-Tcf4 BglII
S18	Midiprep sample ΔN-Tcf4 no restriction enzymes
λM	Figure 5.12.4.1 Lambda 3 marker
MR	Figure 5.12.4.2 Mass Ruler™ DNA Ladder High Range

Table 3.3.1.8 Expected cutting lengths	
Restriction enzymes	Fragments (bp)
NotI (3 fragments)	1230; 3538 Tcf4wt: 3824 ΔN-Tcf4: 3724 VP16-ΔN-Tcf4: 3974
BamHI (1 fragment) BglII (1 fragment)	Tcf4wt: 7900 ΔN-Tcf4: 7800 VP16-ΔN-Tcf4: 8050

The aim was to excise the Tcf4wt, ΔN-Tcf4 and VP16-ΔN-Tcf4 inserts with BamHI and XhoI from the of pAAV-IRES-hrGFP vector. Furthermore another attempt was to cut the Tcf4wt, ΔN-Tcf4 and VP16-ΔN-Tcf4 inserts together with 3x Flag + hrGFP with BamHI and BglII out of the rest vector.

Subsequently, 5µg midiprep samples of pAAV-IRES-hrGFP-Tcf4wt, -ΔN-Tcf4 and -VP16-ΔN-Tcf4 were digested with BamHI and XhoI or BamHI and BglII (see 5.15.1). Aliquots of these large digests were tested.

Additionally, single cuts and no cuts were analyzed. See the resulting gel photos in figure 3.3.1.5, figure 3.3.1.6 and table 3.3.1.9. The expected fragment lengths for the double digest are in table 3.3.1.10. The insert Tcf4t, ΔN-Tcf4 and VP16-ΔN-Tcf4 was cut out successfully in samples 1-3.

The attempt to cut out Tcf4wt, ΔN-Tcf4 and VP16-ΔN-Tcf4 together with 3x Flag + hrGFP with BamHI and BglII failed because the products were 1000bp shorter than predicted from the sequence (see table 3.3.1.10).

It was likely that at least parts of hrGFP were absent in the pAAV plasmids because it was expected that BamHI and BglII excised approximately 1000bp bigger fragments.

Thus the samples 1-3 were used for the further procedures.

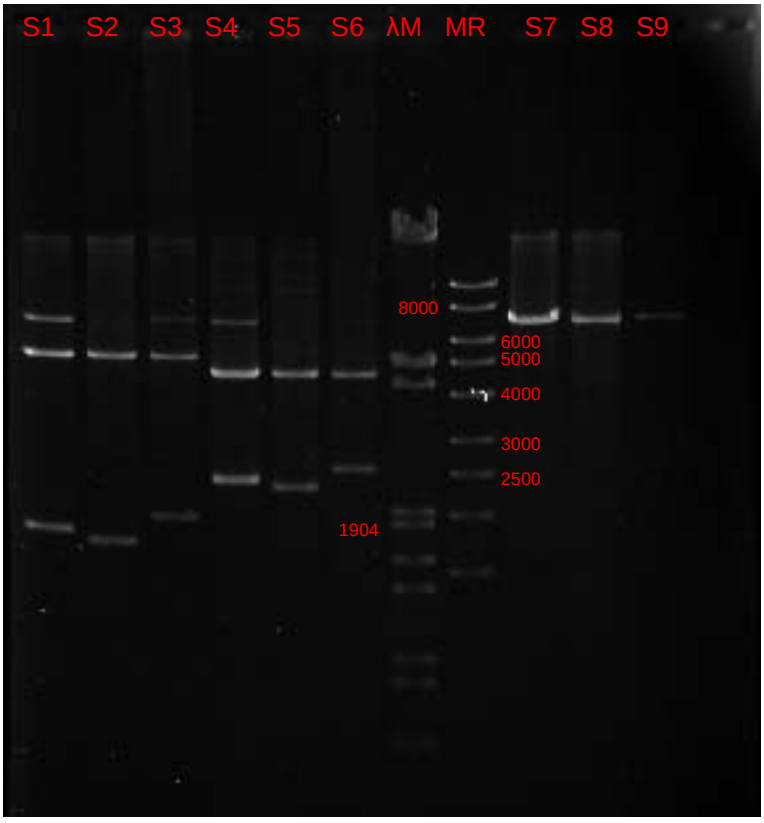


Figure 3.3.1.5 restriction digests of pAAV-IRES-hrGFP-Tcf4wt, -ΔN-Tcf4 and -VP16-ΔN-Tcf4

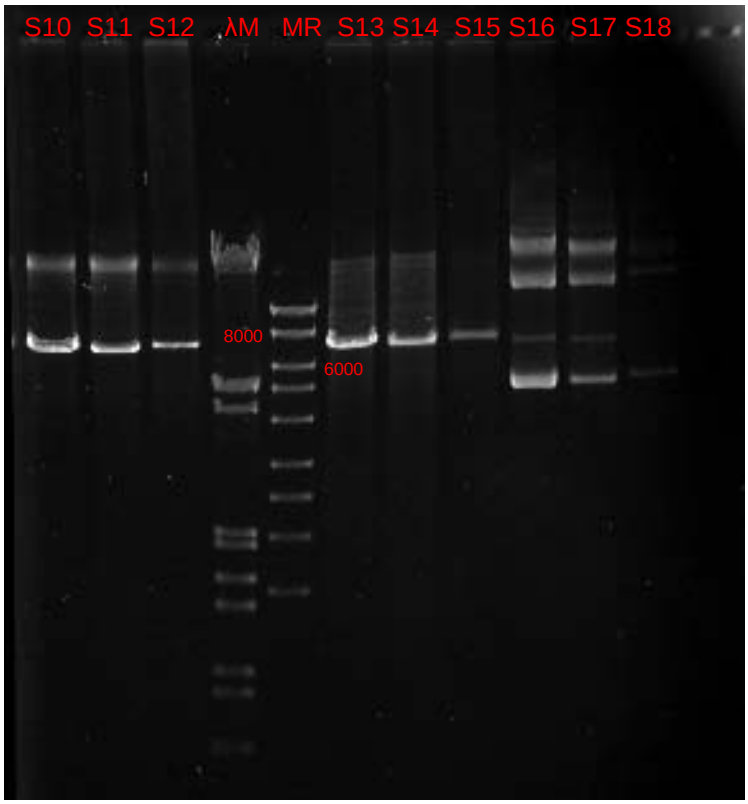


Figure 3.3.1.6 restriction digests of pAAV-IRES-hrGFP-Tcf4wt, -ΔN-Tcf4 and -VP16-ΔN-Tcf4

Table 3.3.1.9 Sample description of figure 3.3.1.5 and 3.3.1.6	
Sample number	Sample details
S1	Midiprep sample Tcf4wt BamHI and XhoI, 10x buffer B
S2	Midiprep sample ΔN-Tcf4 BamHI and XhoI, 10x buffer B
S3	Midiprep sample VP16-ΔN-Tcf4 BamHI and XhoI, 10x buffer B
S4	Midiprep sample Tcf4wt BamHI and BglII, fast digest
S5	Midiprep sample ΔN-Tcf4 BamHI and BglII, fast digest
S6	Midiprep sample VP16-ΔN-Tcf4 BamHI and BglII, fast digest
S7	Midiprep sample Tcf4wt BamHI, fast digest
S8	Midiprep sample ΔN-Tcf4 BamHI, fast digest
S9	Midiprep sample VP16-ΔN-Tcf4 BamHI, fast digest
S10	Midiprep sample Tcf4wt XhoI, 10x buffer B
S11	Midiprep sample ΔN-Tcf4 XhoI, 10x buffer B
S12	Midiprep sample VP16-ΔN-Tcf4 XhoI, 10x buffer B
S13	Midiprep sample Tcf4wt BglII, fast digest
S14	Midiprep sample ΔN-Tcf4 BglII, fast digest
S15	Midiprep sample VP16-ΔN-Tcf4 BglII, fast digest
S16	Midiprep sample Tcf4wt no restriction enzymes
S17	Midiprep sample ΔN-Tcf4 no restriction enzymes
S18	Midiprep sample VP16-ΔN-Tcf4 no restriction enzymes
λM	Figure 5.12.4.1 Lambda 3 marker
MR	Figure 5.12.4.2 Mass Ruler™ DNA Ladder High Range

Table 3.3.1.10 expected cutting lengths for double digest	
Restriction enzymes	Predicted fragments (bp)
BamHI + XhoI (2 fragements in each sample)	6100; Tcf4wt: 1800 ΔN-Tcf4: 1700 VP16-ΔN-Tcf4: 1950
BamHI + BglII (2 fragments in each sample)	4612; Tcf4wt: 3288 ΔN-Tcf4: 3188 VP16-ΔN-Tcf4: 3438

3.3.2 pENTR vector cloning

3.3.2.1 pENTR/D-TOPO® vector cloning

Subsequently, it was tried to clone Tcf4wildtype, ΔN-Tcf4 and VP16-ΔN-Tcf4 into the pENTR/D-TOPO® because this pENTR clone procedure was common in the Holzmann lab. The pENTR/D-TOPO® vector is depicted in figure 3.3.2.1.1.

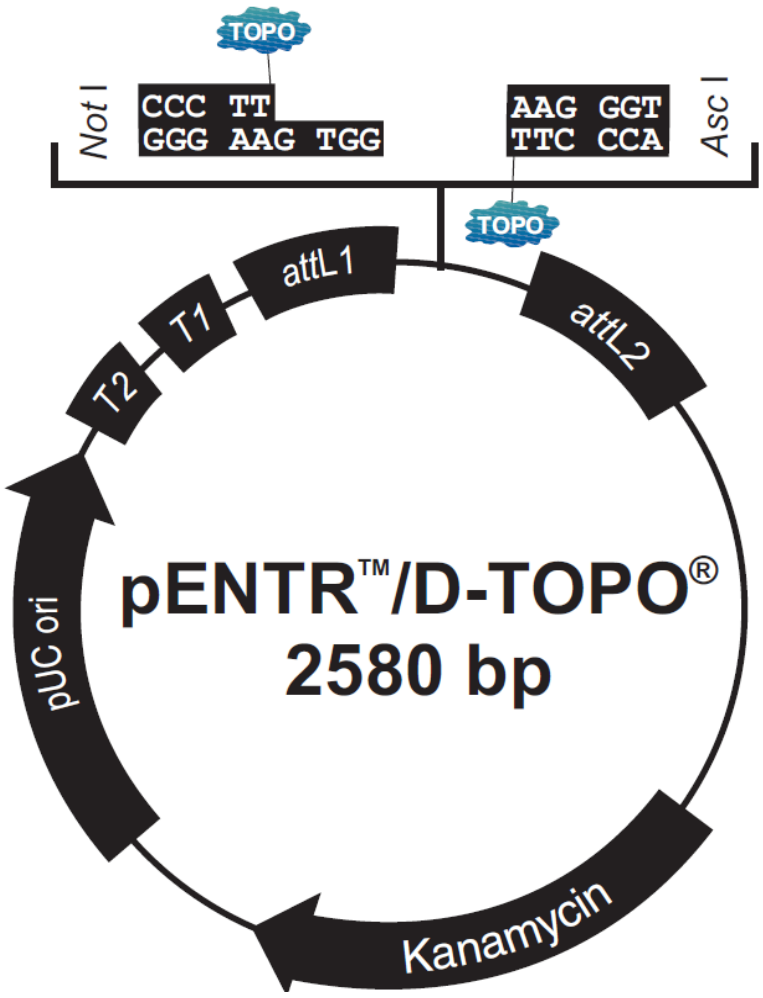


Figure 3.3.2.1.1 pENTR/D-TOPO® vector

For this purpose samples 1-3 of the digest in table 3.3.1.9 were treated with phosphatase according to 5.15.2. The samples contained 4µg DNA which was approximately 3 picomoles DNA ends. Thus 0.03u CIAP was necessary and 5µl of the phosphatase treatment master mix was taken. After that the success of the phosphatase treatment was tested with a re-ligation check according to 5.15.3. 5µl of the phosphatase treated samples were transformed into 160µl competent TOP10 cells together with a pUC19 control. The results of the re-ligation check are in table 3.3.2.1.1. Only the phosphatase treatment of sample 1 (pAAV-IRES-hrGFP-Tcf4wt) was successful because the colony numbers of the control were 10 times higher than the phosphatase treated sample. Sample 2 and 3 had approximately equal colony numbers. The used DNA amounts are listed in table 3.3.2.1.2. The ratio of the DNA amount of the controls and the phosphatase treated samples was approximately 100:80 in the re-ligation check. Thus this could not interfere significantly with the comparison of the colony number of controls and phosphatase treated samples. The phosphatase seems to work inefficiently. pAAV-IRES-hrGFP-Tcf4wt restriction digest (figure 3.3.1.5) was incomplete and there was a big band at approximate 7000bp which showed that only 1 restriction enzyme cut there (see figure 3.3.1.5). Subsequently, pAAV-IRES-hrGFP-Tcf4wt restriction digest (figure 3.3.1.5) had fewer ends than the other samples and thus phosphatase could work better there. On the other hand figure 3.3.1.5 shows that more DNA was digested of pAAV-IRES-hrGFP-Tcf4wt.

Table 3.3.2.1.1 Results of the re-ligation check

Sample	Colonies 1:50	Colonies of undiluted rest
Phosphatase treatment of sample 1	5	200
Untreated sample 1 control	57	1000
Phosphatase treatment of sample 2	7	200-300
Untreated sample 2 control	9	approx. 50
Phosphatase treatment of sample 3	15	approx. 100
Untreated sample 3 control	14	approx. 100

Table 3.3.2.1.2 DNA amounts		
Procedures	Control	Treated samples
Restriction digest	5µg	5µg
Phosphatase treatment		4µg (80ng/µl)
Ligation	0.1µg (10ng/µl)	80ng (8ng/µl)
Transformation	50ng	40ng

The phosphatase treated constructs were converted into blunt ends according to 5.15.4.

Sample 1 (Tcf4wt) which was treated with phosphatase and converted into blunt ends was cloned into the pENTR/D-TOPO® (kanamycin resistance) according to 5.15.5. Subsequently, the resulting vector was transformed into 200µl competent TOP10 bacteria together with a pUC19 control plasmid (ampicillin resistance) and a pENTR/D-TOPO®-IIIC control plasmid. The resulting colony numbers are listed in table 3.3.2.1.3. The transformation rates are in table 3.3.2.1.4. pUC19 control gave much more colonies than pENTR/D-TOPO®-IIIC control. The transformation rate of pENTR/D-TOPO®-IIIC control was lower. pENTR/D-TOPO® constructs had a decreased transformation efficiency, especially if transformation was used for selection as in the case of the produced pENTR/D-TOPO®-Tcf4wt plasmid. The 2 colonies of pENTR/D-TOPO®-Tcf4wt undiluted plate were picked and transferred in 14ml tubes which are filled with 7ml liquid LB and kanamycin and then they were shaken at 200 rpm at 37°C overnight. DNA was isolated by miniprep (see results in table 3.3.2.1.5), digested with EcoRI and EcoRV (2µl miniprep samples) and analyzed by 1% agarose gel electrophoresis (see figure 3.3.2.1.2 and table 3.3.2.1.6).

Table 3.3.2.1.3 Colony numbers		
Sample	1:50 dilution	Rest
pUC19 control	65	Lawn
pENTR-IIIC control	2	28
pENTR-Tcf4wt	0	2

Table 3.3.2.1.4 Transformation rates	
Plate	Colony numbers at 1µg
pUC19 1:50 dilution (20pg)	3250000
pENTR-IIIC 1:50 dilution (20pg)	100000
pENTR-IIIC rest (1ng)	28000

Table 3.3.2.1.5 Miniprep results of possible pENTR-Tcf4wt	
Sample	Concentration (ng/µl)
pENTR-Tcf4wt Colony1	92.84
pENTR-Tcf4wt Colony2	88.34

The pENTR/D-TOPO®-IIIC control transformation results (see table 3.3.2.1.4) indicated a decreased transformation efficiency in addition to the lower chance that correct pENTR/D-TOPO®-Tcf4wt plamids could be ligated.

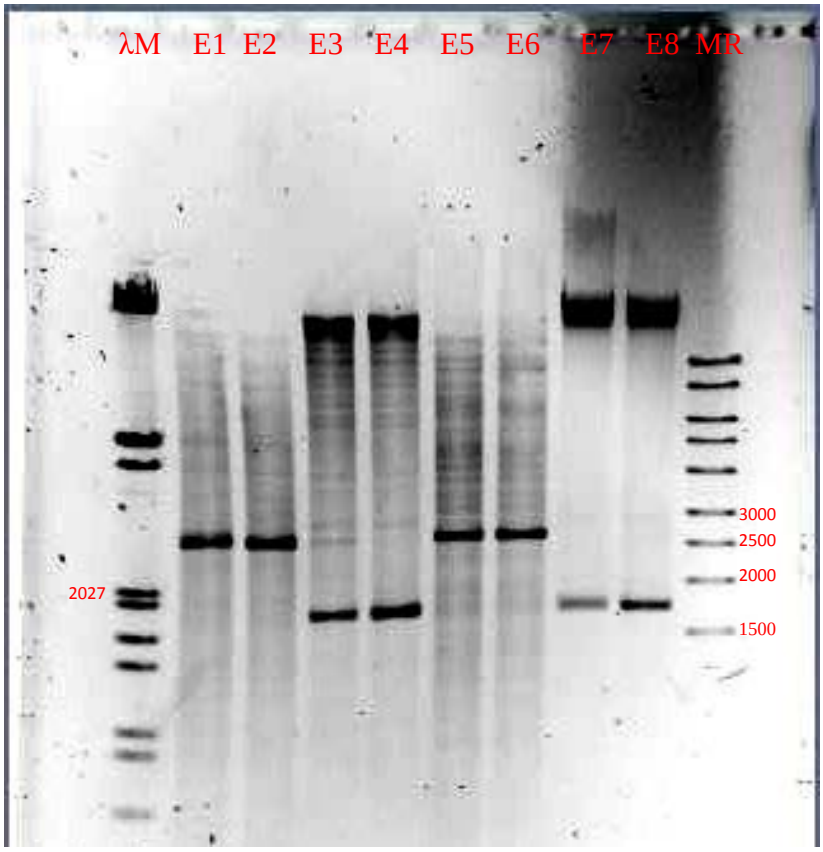


Figure 3.3.2.1.2 Digest of first production attempt of pENTR/D-TOPO-TCF4wt

Table 3.3.2.1.6 Sample description of figure 3.3.2.1.2

Sample number	Sample details
E1	pENTR/D-TOPO®-Tcf4wt colony1 EcoRI, EcoRV, buffer B
E2	pENTR/D-TOPO®-Tcf4wt colony2 EcoRI, EcoRV, buffer B
E3	pENTR/D-TOPO®-Tcf4wt colony1 EcoRI, buffer B
E4	pENTR/D-TOPO®-Tcf4wt colony2 EcoRI, buffer B
E5	pENTR/D-TOPO®-Tcf4wt colony1 EcoRV, buffer B
E6	pENTR/D-TOPO®-Tcf4wt colony2 EcoRV, buffer B
E7	pENTR/D-TOPO®-Tcf4wt colony1 no restriction enzymes
E8	pENTR/D-TOPO®-Tcf4wt colony2 no restriction enzymes
λM	Figure 5.12.4.1 Lambda 3 marker
MR	Figure 5.12.4.2 Mass Ruler™ DNA Ladder High Range

EcoRI only cut once in the insert and EcoRV cut once in the pENTR/D-TOPO® vector and in the correct pENTR/D-TOPO®-Tcf4wt sample. The expected fragments for the empty pENTR/D-TOPO® vector and pENTR/D-TOPO® +insert are listed in table 3.3.2.1.7.

Table 3.3.2.1.7 Expected fragments for EcoRI and EcoRV digests	
Restriction enzymes	Fragments (bp)
EcoRV (cut once in vector) or EcoRI	Empty vector: 2576 (EcoRI does not cut) Vector + Tcf4wt: 4387 Vector + ΔN-Tcf4: 4297 Vector + VP16-ΔN-Tcf4: 4546
EcoRV and EcoRI (cuts in insert)	Empty vector: 2576 Vector + Tcf4wt sense: 1939; 2448 Vector+ Tcf4wt antisense: 133; 4254 Vector + ΔN-Tcf4 sense: 1849; 2448 Vector + ΔN-Tcf4 antisense: 133; 4164 Vector + VP16-ΔN-Tcf4 sense: 249; 1849; 2448 Vector + VP16-ΔN-Tcf4 antisense: 249; 133; 4164

Figure 3.3.2.1.2 showed that pENTR/D-TOPO® vector did not ligate with Tcf4wt. Thus the procedure had to be changed in order to gain pENTR/D-TOPO®-Tcf4wt, -ΔN-Tcf4 and -VP16-ΔN-Tcf4. It was decided to elute the digested fragments from the gel in order to gain purified products which have higher phosphatase treatment efficiency.

The large digest was repeated: 5µg midiprep samples of pAAV-IRES-hrGFP-Tcf4wt, -ΔN-Tcf4 and -VP16-ΔN-Tcf4 were digested with BamHI and XhoI (see 5.15.1). Aliquots of these large digests were tested. Additionally, single cuts and no cuts were analyzed. See the resulting gel photos in figure 3.3.2.1.3 and table 3.3.2.1.8. The expected fragment lengths for the double digest are in table 3.3.1.10.

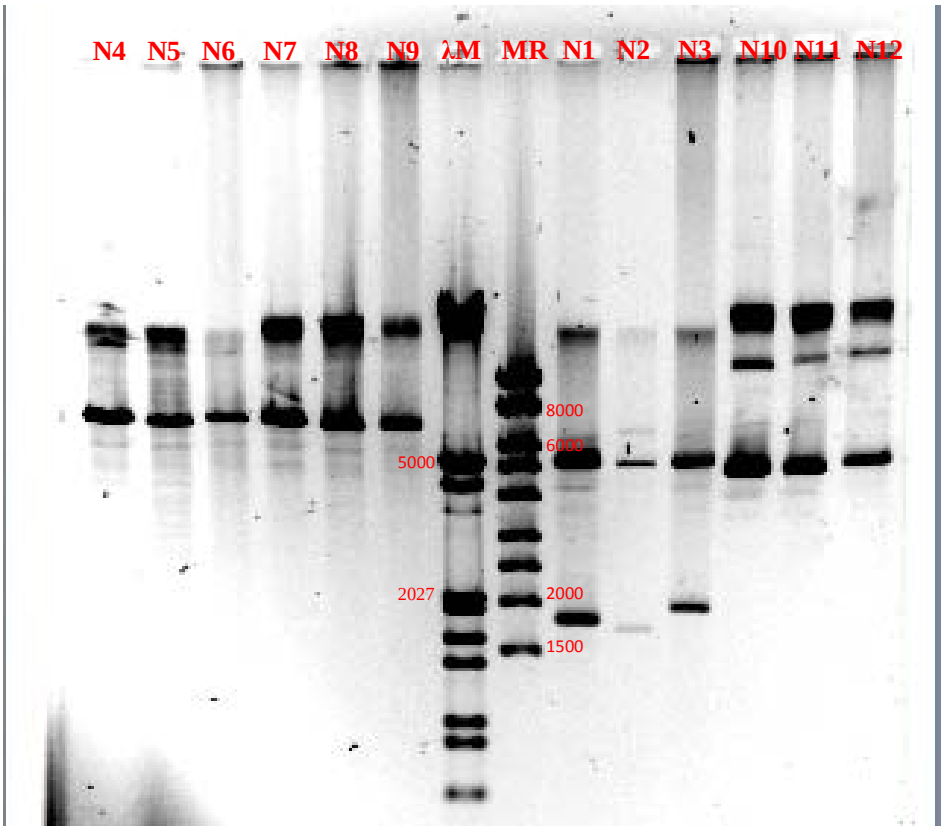


Figure 3.3.2.1.3 restriction digests of pAAV-IRES-hrGFP-Tcf4wt, -ΔN-Tcf4 and -VP16-ΔN-Tcf4

Table 3.3.2.1.8 Sample description of figure 3.3.2.1.3

Sample number	Sample details
N1	Midiprep sample Tcf4wt BamHI and XhoI, buffer B
N2	Midiprep sample ΔN-Tcf4 BamHI and XhoI, buffer B
N3	Midiprep sample VP16-ΔN-Tcf4 BamHI and XhoI, buffer B
N4	Midiprep sample Tcf4wt BamHI, buffer B
N5	Midiprep sample ΔN-Tcf4 BamHI, buffer B
N6	Midiprep sample VP16-ΔN-Tcf4 BamHI, buffer B
N7	Midiprep sample Tcf4wt XhoI, buffer B
N8	Midiprep sample ΔN-Tcf4 XhoI, buffer B
N9	Midiprep sample VP16-ΔN-Tcf4 XhoI, buffer B
N10	Midiprep sample Tcf4wt no restriction enzymes
N11	Midiprep sample ΔN-Tcf4 no restriction enzymes
N12	Midiprep sample VP16-ΔN-Tcf4 no restriction enzymes
λM	Figure 5.12.4.1 Lambda 3 marker
MR	Figure 5.12.4.2 Mass Ruler™ DNA Ladder High Range

The repetition of the large digest of pAAV-IRES-hrGFP-ΔN-Tcf4 failed and was repeated successfully again (see figure 3.3.2.1.4 and table 3.3.2.1.9). The volume of this large digest was smaller than the other digests.

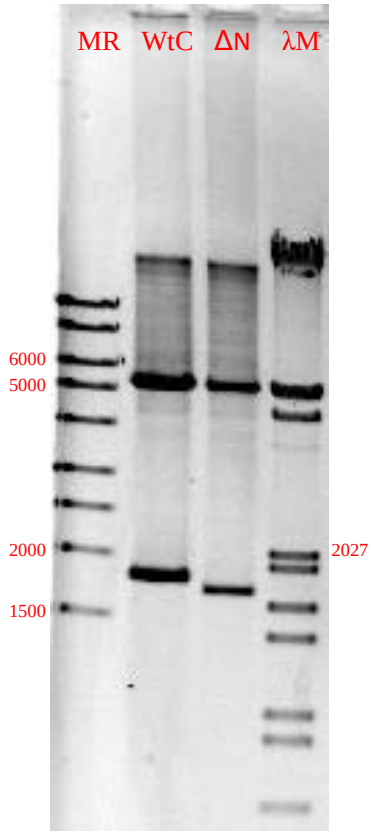


Figure 3.3.2.1.4 restriction digest of pAAV-IRES-hrGFP-ΔN-Tcf4

Table 3.3.2.1.9 Sample description of figure 3.3.2.1.4

Sample number	Sample details
WtC	Midiprep sample Tcf4wt Control. BamHI and XhoI, buffer B
ΔN	Midiprep sample ΔN-Tcf4 BamHI and XhoI, buffer B
λM	Figure 5.12.4.1 Lambda 3 marker
MR	Figure 5.12.4.2 Mass Ruler™ DNA Ladder High Range

Subsequently, the restriction digests N1, N3 (see figure 3.3.2.1.3) and ΔN (N1: 30µl; N3: 40µl; ΔN: 40µl) were loaded on an agarose gel and the bands of Tcf4wt, ΔN-Tcf4 and VP16-ΔN-Tcf4 are cut successfully out of the gel according to 5.15.5 (see figure 3.3.2.1.5 and table 3.3.2.1.10). Subsequently, the DNA was extracted according to 5.15.5 and 2µl samples are checked on a 1% agarose gel (see figure 3.3.2.1.6 and table 3.3.2.1.11). The DNA amount of each sample was estimated by comparing the band intensity with those of the used markers and measured with Nanodrop (see figure 5.12.4.1 Lambda 3 marker, 48502bp

and figure 5.12.4.2 Mass Ruler™ DNA Ladder High Range). The estimated DNA amounts are depicted in table 3.3.2.1.12. After that a phosphatase treatment was done with the gel extracted samples according to 5.15.2. 2µl 0.001u/µl CIAP dilution (1:1000) was used for the phosphatase reaction mix at the Tcf4wt gel extracted sample. 1µl 0.001u/µl CIAP dilution was used for phosphatase reaction mix at the ΔN-Tcf4 gel extracted sample and the VP16-ΔN-Tcf4 gel extracted sample.

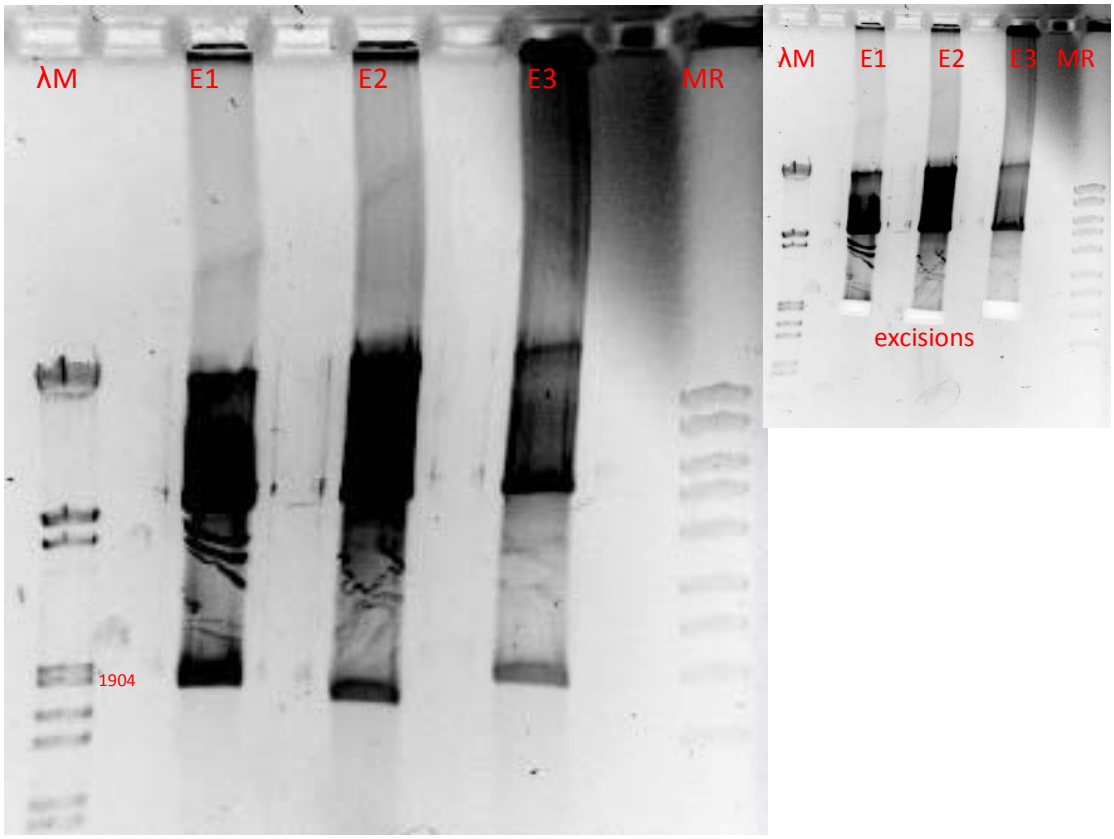


Figure 3.3.2.1.5 Gel excision of Tcf4wt, ΔN-Tcf4 and VP16-ΔN-Tcf4: left photo: before gel excision; right photo: after gel excision

Table 3.3.2.1.10 Sample description of figure 3.3.2.1.5

Sample number	Sample details
E1	30µl N1
E2	40µl ΔN
E3	40µl N3
λM	Figure 5.12.4.1 Lambda 3 marker
MR	Figure 5.12.4.2 Mass Ruler™ DNA Ladder High Range

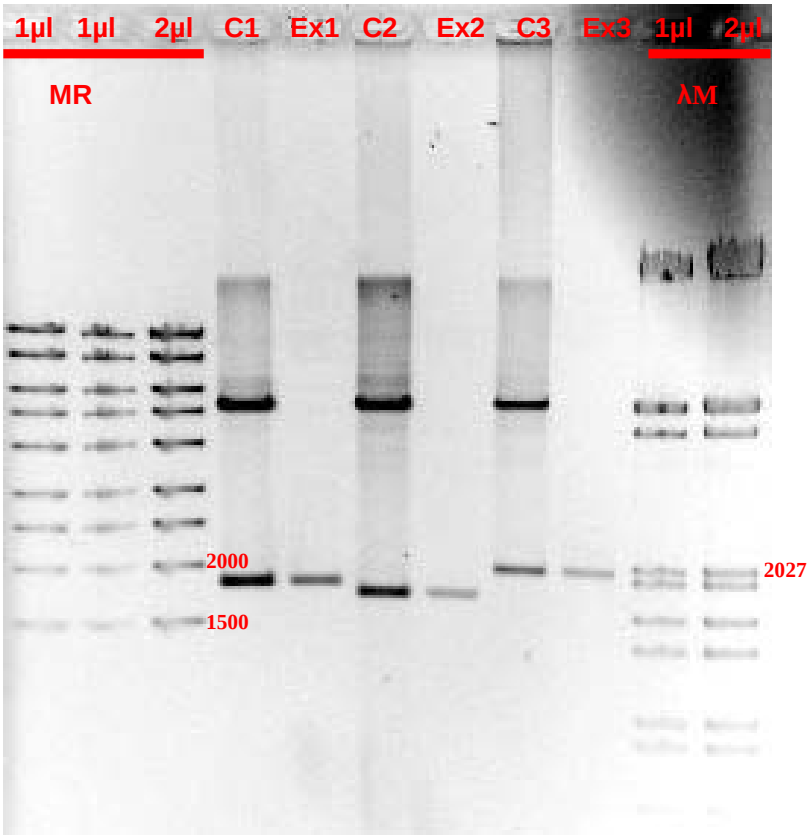


Figure 3.3.2.1.6 Gel extraction check

Table 3.3.2.1.11 Sample description of figure 3.3.2.1.6	
Sample number	Sample details
C1	2µl N1
Ex1	2µl E1
C2	2µl ΔN
Ex2	2µl E2
C3	2µl N3
Ex3	2µl E3
λM	Figure 5.12.4.1 Lambda 3 marker
MR	Figure 5.12.4.2 Mass Ruler™ DNA Ladder High Range

Table 3.3.2.1.12 Estimated DNA amounts of gel extracted samples	
ng/µl	Sample details
7	Tcf4wt gel extracted
4	ΔN-Tcf4 gel extracted
4	VP16-ΔN-Tcf4 gel extracted

Subsequently, 5.8ng phosphatase treated ΔN-Tcf4 sample was cloned into the pENTR/D-TOPO® according to 5.15.5. The resulting vector was transformed into 20µl commercial competent TOP10 E.coli (invitrogen) together with the pUC19 control (ampicillin resistance) and pENTR/D-TOPO®-IIIC control (see 5.12.2.2). The transformation rate of these cells is 100 times higher (10^8 colonies/µg DNA) than self-made competent TOP10 E.coli. The resulting colony numbers are listed in table 3.3.2.1.13. The transformation rates are in table 3.3.2.1.14. Much more colonies could be gained at this transformation of a TOPO® cloning reaction if you compare the results of table 3.3.2.1.13 with those of table 3.3.2.1.3. The pENTR-IIIC control colony numbers of table 3.3.2.1.13 dropped significantly in comparison to table 3.3.2.1.3.

Table 3.3.2.1.13 Colony numbers

Sample	1:50 dilution	Rest
pUC19 control	137	Lawn
pENTR-IIIC control	0	14
pENTR-ΔN-Tcf4	0	42

Table 3.3.2.1.14 Transformation rates

Plate	Colony numbers at 1µg
pUC19 1:50 dilution (20pg)	6850000
pENTR-IIIC rest (1ng)	14000
pENTR- ΔN-Tcf4 rest (5.8ng)	7224

4 colonies of the pENTR/D-TOPO®-ΔN-Tcf4 rest plate were picked and shaken at 200 rpm at 37°C overnight. Next day the DNA were isolated by miniprep and analyzed by electrophoresis. The expected fragments of the EcoRI and EcoRV restriction digest check are in table 3.3.2.1.7. The results are in figure 3.3.2.1.7 and table 3.3.2.1.15.

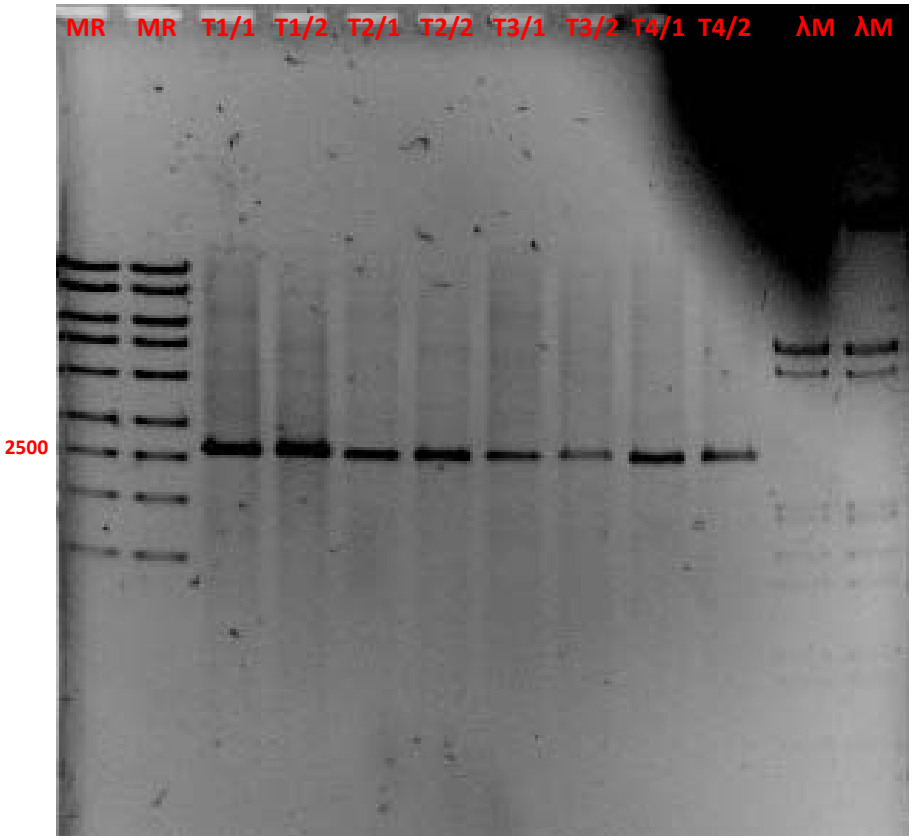


Figure 3.3.2.1.7 Digest of first production attempt of pENTR/D-TOPO-ΔN-Tcf4

Table 3.3.2.1.15 Sample description of figure 3.3.2.1.7	
Sample number	Sample details
T1/1	pENTR/D-TOPO®-ΔN-Tcf4 colony1 EcoRV, buffer B
T1/2	pENTR/D-TOPO®-ΔN-Tcf4 colony1 EcoRI, EcoRV, buffer B
T2/1	pENTR/D-TOPO®-ΔN-Tcf4 colony2 EcoRV, buffer B
T2/2	pENTR/D-TOPO®-ΔN-Tcf4 colony2 EcoRI, EcoRV buffer B
T3/1	pENTR/D-TOPO®-ΔN-Tcf4 colony3 EcoRV, buffer B
T3/2	pENTR/D-TOPO®-ΔN-Tcf4 colony3 EcoRI, EcoRV, buffer B
T4/1	pENTR/D-TOPO®-ΔN-Tcf4 colony4 EcoRV, buffer B
T4/2	pENTR/D-TOPO®-ΔN-Tcf4 colony4 EcoRI, EcoRV, buffer B
λM	Figure 5.12.4.1 Lambda 3 marker
MR	Figure 5.12.4.2 Mass Ruler™ DNA Ladder High Range

There were only bands at 2500bp in figure 3.3.2.1.7. Thus the samples only included empty pENTR/D-TOPO® vectors. 18 colonies of the pENTR/D-TOPO®-ΔN-Tcf4 rest plate were picked again in order to find vectors with insert. Next day samples T5-T13 were pooled in one miniprep and samples T14-T22 in another one. 400µl of each overnight culture were taken in order to gain 2 pooled miniprep samples. The STET

boiling plasmid miniprep was used for this step (see 5.12.3). After that the 2 miniprep samples were analyzed by EcoRI and EcoRV restriction digests and subsequent electrophoresis. These digests gave very strange results. Subsequently, DNA was isolated from the T5-T22 samples by STET minipreps, digested with EcoRI (10xbuffer H, Roche) and analyzed by electrophoresis.

Additionally, a streak of pENTR-1A vector stock was done and 2 colonies were picked and cultivated in 6ml LB + kanamcyin overnight culture. Again DNA was isolated by STET minipreps from these 2 pENTR-1A samples, digested with BamHI (10x buffer B, Roche) and analyzed by electrophoresis.

The subsequent gel photos of the digests are in figure 3.3.2.1.8, figure 3.3.2.1.9, figure 3.3.2.1.10 and table 3.3.2.1.16.

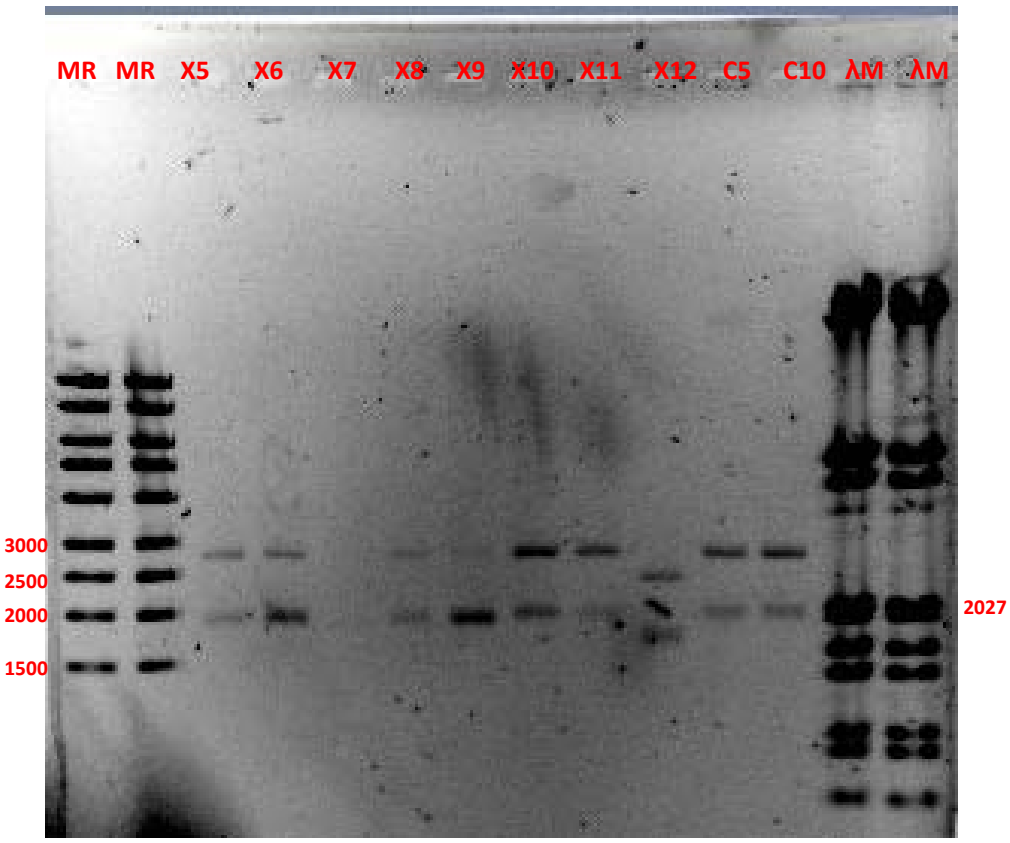


Figure 3.3.2.1.8 Restriction digests of T5-T12

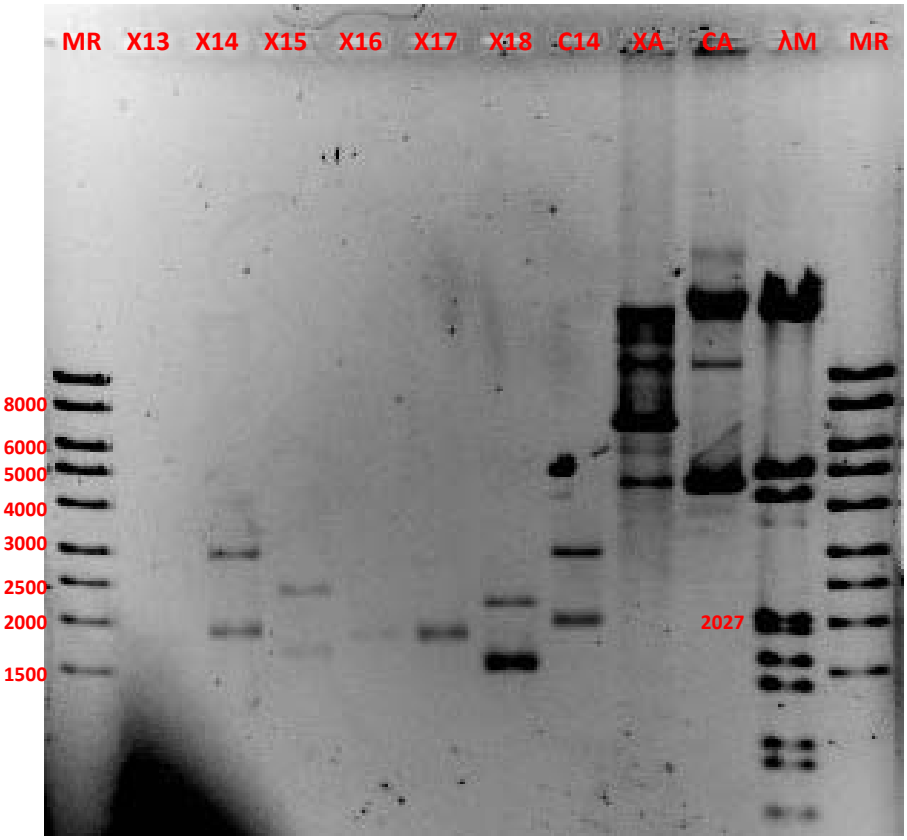


Figure 3.3.2.1.9 Restriction digests of T13-T18

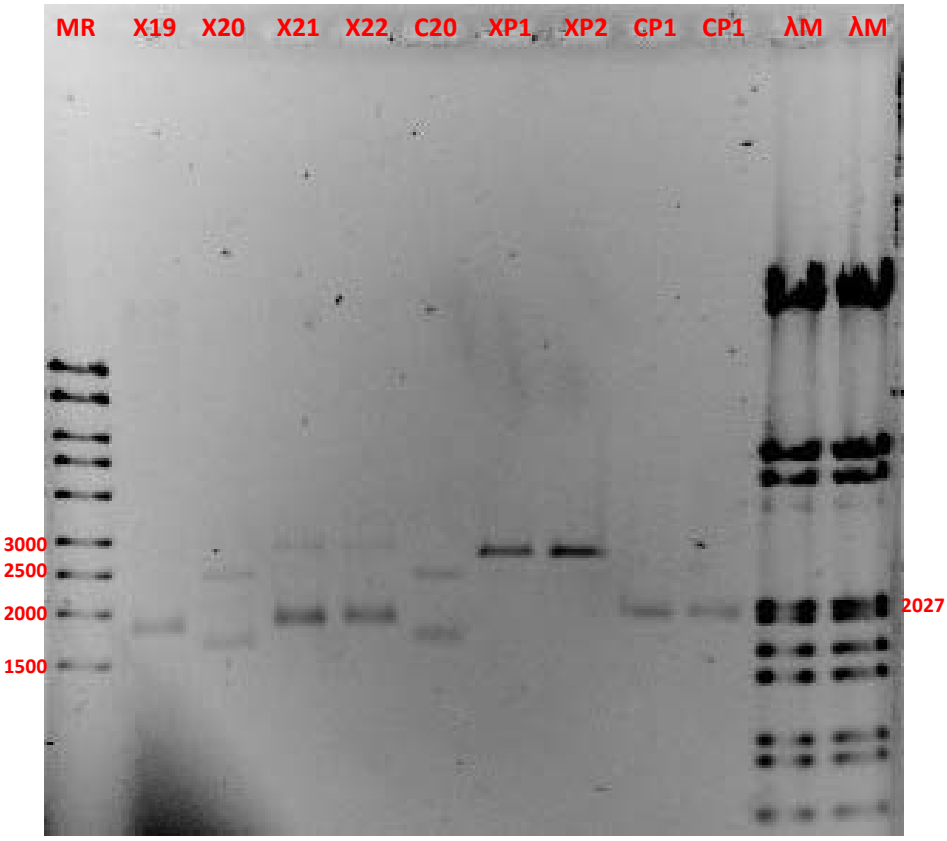


Figure 3.3.2.1.10 Restriction digests of T19-T2 and pENTR-1A

Table 3.3.2.1.16 sample description of figure 3.3.2.1.8-10	
Sample number	Sample details
X5-X22	Sample T5-T22, EcoRI, buffer H
C5	Sample T5, no restriction enzymes
C10	Sample T10, no restriction enzymes
C14	Sample T14, no restriction enzymes
C20	Sample T20, no restriction enzymes
XA	pAAV-IRES-hrGFP-deltaN-Tcf4, EcoRI, buffer H
CA	pAAV-IRES-hrGFP-deltaN-Tcf4, no restriction enzymes
XP1	pENTR-1A colony 1, BamHI, buffer B
XP2	pENTR-1A colony 2, BamHI, buffer B
CP1	pENTR-1A colony 1, no restriction enzymes
CP2	pENTR-1A colony 2, no restriction enzymes
λM	Figure 5.12.4.1 Lambda 3 marker
MR	Figure 5.12.4.2 Mass Ruler™ DNA Ladder High Range

X5-X22 did not give positive results which would be at 4297bp (see table 3.4.1.16 **)** . Positive samples could not be detected. Subsequently, the samples were classified in 3 groups. Group 1 had bands at 2500bp and 1900bp (see X20, X12, X15 and X18). Group 2 had bands at 3000bp and 2000bp (see X5-X11, X14, X21 and X22). Group 3 had bands at 2000 (see X16, X17 and X19). 1 sample was chosen from each of the 3 groups: T17, T15 and T11. 2µl of these samples were digested with NruI (fast digest buffer) and analysed with electrophoresis (see figure 3.3.2.1.11 and table 3.3.2.1.17). No positive constructs could be detected. The bands were at 2500bp and below that. Thus they were empty pENTR/D-TOPO® vectors and fragments of those. Therefore the TOPO® cloning failed and it was decided to use a different pENTR vector.

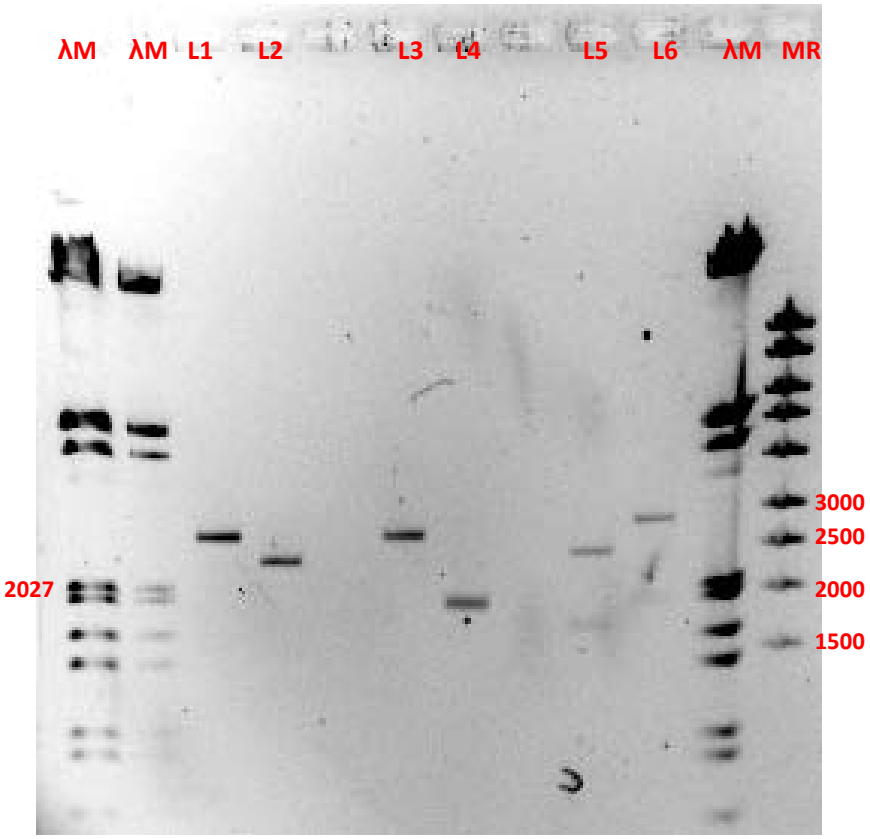


Figure 3.3.2.1.11 NruI digest of T17, T15 and T11

Table 3.3.2.1.17 Sample description of figure 3.3.2.1.11

Sample number	Sample details
L1	Sample T17, NruI, fast digest buffer
L2	Sample T15, NruI, fast digest buffer
L3	Sample T11, NruI, fast digest buffer
L4	Sample T17, no restriction enzymes
L5	Sample T15, no restriction enzymes
L6	Sample T11, no restriction enzymes
λM	Figure 5.12.4.1 Lambda 3 marker
MR	Figure 5.12.4.2 Mass Ruler™ DNA Ladder High Range

3.3.2.2 pENTR-1A vector cloning

It was tried to clone the Tcf4wildtype, ΔN-Tcf4 and VP16-ΔN-Tcf4 fragments into the pENTR-1A vector (see figure 3.3.2.2.1). Both pENTR-1A samples of figure 3.3.2.1.10 had bands at 2700bp (see XP1 and XP2) which were expected. It was estimated that pENTR-1A colony 2 sample had 20ng/µl according to the gel photo.

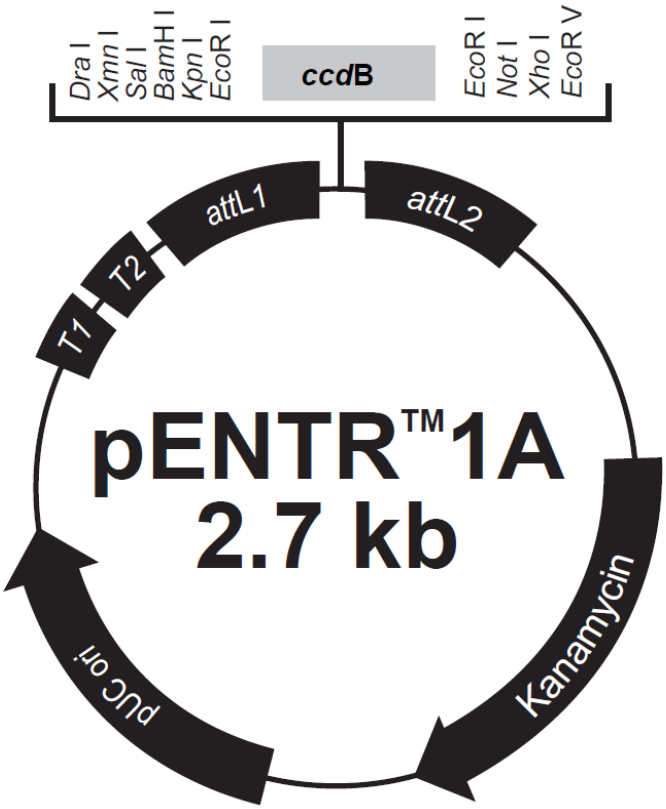


Figure 3.3.2.2.1 pENTR-1A vector

Subsequently, 2 different methods are tried to clone ΔN-Tcf4 into the pENTR-1A vector.

The first method(“quick method”):

1µl pENTR-1A (20ng) and 1µl pAAV-IRES-hrGFP- ΔN-Tcf4 sample (200ng) were digested together with BamHI and XhoI in one 1.5ml Eppendorf tube and analysed by electrophoresis (see figure 3.3.2.2.2 and table 3.3.2.2.1). Subsequently, 5µl of the combined digest was ligated according to 5.16.2 and bacteria transformed according to 5.16.3 with pUC19 control (ampicillin resistance) and pENTR/D-TOPO®-IIIC control. This method failed for the ΔN-Tcf4 construct, while the pUC19 control had a transformation efficiency of 1530000 colonies/µg. The restriction digest of figure 3.3.2.2.2 showed that the restriction enzymes do not work properly and that the DNA amount was too low.

The second method (“sophisticated method”):

pAAV-IRES-hrGFP- ΔN-Tcf4 sample (200ng/µl) was diluted 1:10 in order to have equal concentrations with pENTR-1A colony 2 sample. Both constructs were digested with 1µl BamHI and 1µl XhoI. The large double digests included 200ng DNA of each construct in 50µl reaction mix. These digests were analysed with electrophoresis (see figure 3.3.2.2.2 and table 3.3.2.2.1).

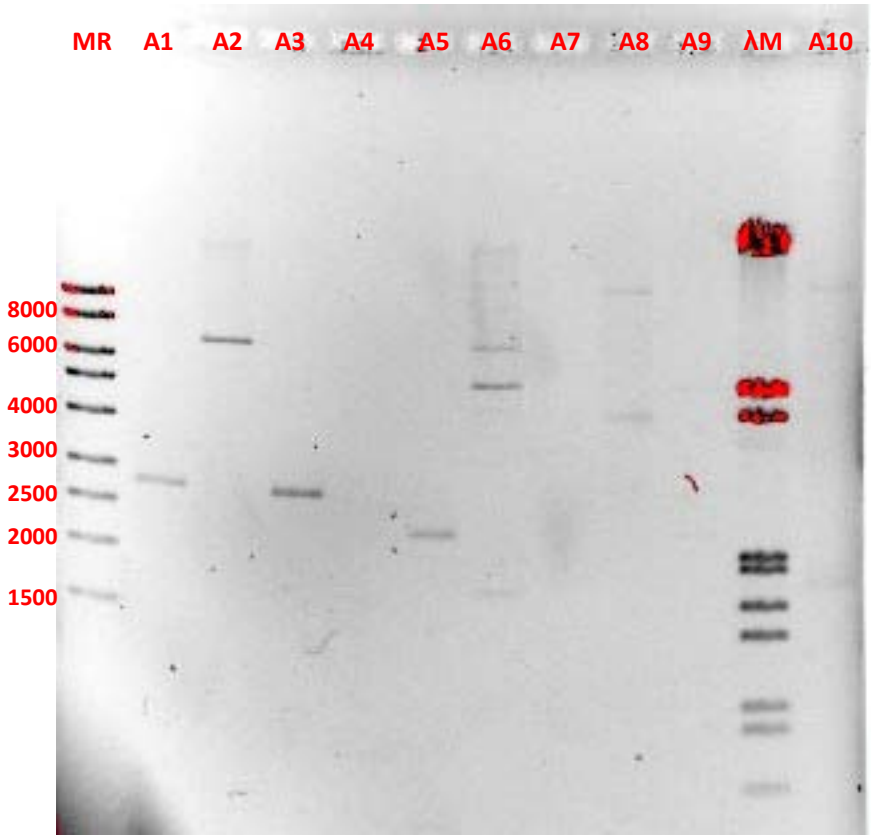


Figure 3.3.2.2.2 pENTR-1A cloning digests

Table 3.3.2.2.1 Sample description of figure 3.3.2.2.2

Sample number	Sample details
A1	pENTR-1A colony2, BamHI, buffer B
A2	pAAV-IRES-hrGFP- ΔN-Tcf4, BamHI, buffer B
A3	pENTR-1A colony2 sample, XhoI, buffer B
A4	pAAV-IRES-hrGFP- ΔN-Tcf4, XhoI, buffer B
A5	pENTR-1A colony2 sample, BamHI, XhoI, buffer B
A6	pAAV-IRES-hrGFP- ΔN-Tcf4, BamHI, XhoI, buffer B
A7	pENTR-1A colony2, no restriction enzymes
A8	pAAV-IRES-hrGFP- ΔN-Tcf4, no restriction enzymes
A9	pENTR-1A colony2, pAAV-IRES-hrGFP- ΔN-Tcf4, BamHI, XhoI, buffer B
A10	pENTR-1A colony2, pAAV-IRES-hrGFP- ΔN-Tcf4, no restriction enzymes
λM	Figure 5.12.4.1 Lambda 3 marker
MR	Figure 5.12.4.2 Mass Ruler™ DNA Ladder High Range

The digests of pENTR-1A cloning did not work properly. Digestion products in lanes A1-A3 displayed the bands that had been expected. Thus the restriction enzyme XhoI worked clearly in A3 but it did not in A4. The double digest with BamHI and XhoI shown in A5 produced a lower molecular weight band as expected. 450bp were removed from the 2700bp pENTR-1A vector. The double digest of the construct A6 resulted in a band at 5000bp that represents the pAAV-IRES-hrGFP without the ΔN-Tcf4 insert. There was a weak band at 7000bp which indicated that either the BamHI or XhoI cut was incomplete. On the gel a weak band could be detected at 1700bp representing the ΔN-Tcf4. In lane A8 the uncut pAAV-IRES-hrGFP- ΔN-Tcf4 could be seen. In lane A9 very weak bands were observed which showed pAAV-IRES-hrGFP without the ΔN-Tcf4 insert and the smaller pENTR-1A but ΔN-Tcf4 alone could not be detected. The reason for that could be that only 1µl of the quick method digest was loaded on the gel.

The digests of the sophisticated method of pENTR-1A was adapted:

pENTR-1A colony glycerol stock was picked and the DNA was extracted by midiprep (result for aliquot 1: 220ng/µl). 5µg of both constructs were double digested with 3µl BamHI (Fermentas, #ER0051, 10u/µl) and 3µl XhoI (Fermentas, #ER0691, 10u/µl) in separate 50µl reaction mix. Additionally, 200ng of pENTR-1A were double digested in a 10µl reaction mix. 600ng of pAAV-IRES-hrGFP- ΔN-Tcf4 was digested with the same 10µl double digest. 200ng and 600ng were digested with 5u of each restriction enzyme. Analysis depicted in figure 3.3.2.2.3 and table 3.3.2.2.2. M1 and M2 seemed to be completely digested, while M5 and M6 were not totally digested. Both 5µg DNA digests were only partially cut. This can be explained by the used units of the restriction enzymes. The enzyme activity of BamHI and XhoI are defined: 1u cuts 1µg λ DNA at 37°C for 1hour. Thereby, BamHI has 5 restriction sites and XhoI 1

restriction site in the λ phage genome. Thus BamHI has a 5fold restriction enzyme activity in comparison to XhoI. If this unit definitions are related to the pAAV-IRES-hrGFP-ΔN-Tcf4 construct (approx.7000bp) and the pENTR-1A vector (2700bp) XhoI needs more units in order to cut completely than it is used in the restriction digest of figure 3.3.2.2.3 but BamHI is used in excess. 35u of XhoI has to be used at the pAAV-IRES-hrGFP-ΔN-Tcf4 construct and 90u for pENTR-1A rather than 30u. M9 and M10 were totally digested and were used for the subsequent ligation reaction.

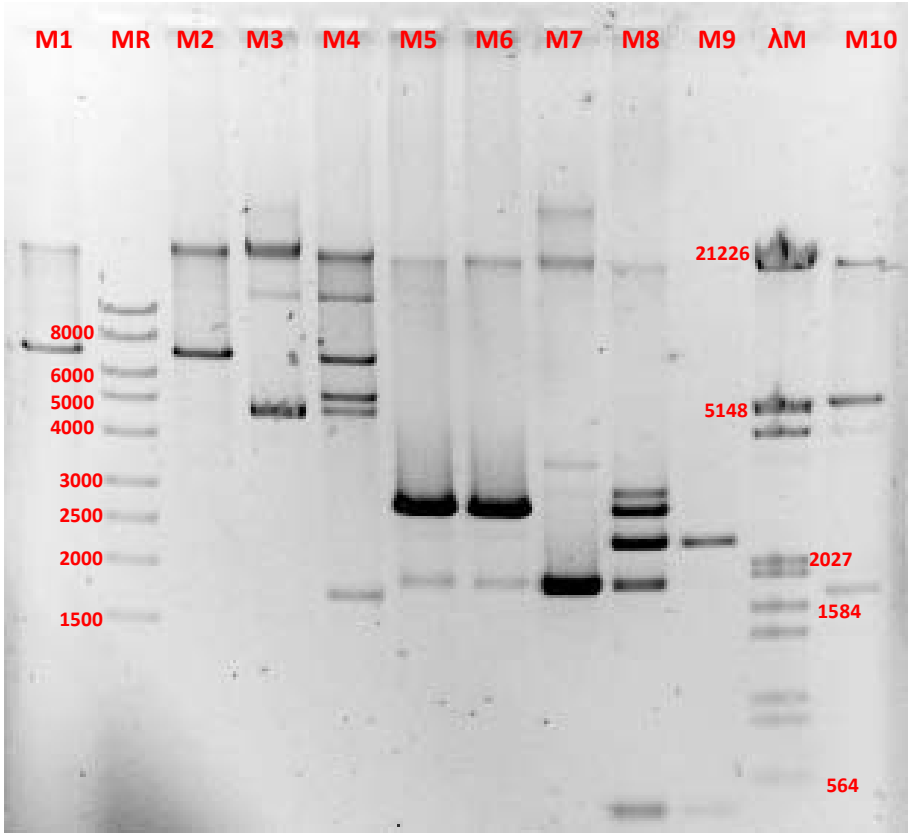


Figure 3.3.2.2.3 BamHI and XhoI digests of 5µg pENTR-1A and pAAV-IRES-hrGFP-ΔN-Tcf4

Table 3.3.2.2.2 Sample description of figure 3.3.2.2.3	
Sample number	Sample details
M1	pAAV-IRES-hrGFP- ΔN-Tcf4, BamHI, buffer B
M2	pAAV-IRES-hrGFP- ΔN-Tcf4, XhoI, buffer B
M3	pAAV-IRES-hrGFP- ΔN-Tcf4, no restriction enzymes
M4	pAAV-IRES-hrGFP- ΔN-Tcf4,BamHI, XhoI, buffer B, 5µg DNA
M5	pENTR-1A colony2 aliquot1, BamHI, buffer B
M6	pENTR-1A colony2 aliquot1, XhoI, buffer B
M7	pENTR-1A colony2 aliquot1, no restriction enzymes
M8	pENTR-1A colony2 aliquot1, BamHI, XhoI, buffer B, 5µg DNA
M9	pENTR-1A colony2 aliquot1, BamHI, XhoI, buffer B, 220ng DNA
M10	pAAV-IRES-hrGFP- ΔN-Tcf4,BamHI, XhoI, buffer B, 600ng DNA
λM	Figure 5.12.4.1 Lambda 3 marker
MR	Figure 5.12.4.2 Mass Ruler™ DNA Ladder High Range

1µl M9 (22ng) and 4µl M10 (240ng) were ligated according to 5.16.2 and bacteria transformed according to 5.12. Negative ligation controls of M9 and M10 alone as well as 1ng pUC19 and 1ng pEGFP-C1 (kanamycin resistance, Clontech) as positive control were also used for transformation. The results are in tables 3.3.2.2.3 and 3.3.2.2.4. The ligation was inefficient. The pEGFP-1C positive control showed expected transformation rates and this fact indicated that kanamycin plates were not the problem of the low transformation rates. The 1 clone of the pENTR-1A- ΔN-Tcf4 was picked and cultivated overnight. A streak of the picked clone was done. A clone was picked from the streak and cultivated overnight. The DNA of the streak clone sample was isolated by miniprep with column according to 5.12.3. The DNA concentration was very low at 25ng/µl. 4µl of the sample was digested by BamHI and XhoI and analysed by electrophoresis. Bands could not be detected on the gel.

Table 3.3.2.2.3 Colony numbers		
Sample	1:50 dilution	Rest
pUC19 control	6	Lawn
pEGFP-C1	30	Approx.1000
Vector negative control		0
Insert negative control		0
pENTR-1A-ΔN-Tcf4	0	1

Table 3.3.2.2.4 Transformation rates	
Plate	Colony numbers at 1µg
pUC19 1:50 dilution (20pg)	300000
GFP-C1 1:50 (20pg)	1500000
GFP-C1 Rest (1ng)	1000000

In order to identify the supposed problem with the ligase, 0.5µl T4 DNA ligase (Fermentas, 30u/µl) and the 10x T4 DNA Ligase Buffer of another lab (test 1) was used to ligate 10ng pAAV-IRES-hrGFP- ΔN-Tcf4 which was digested with XhoI. Additionally, the same restriction digest sample was ligated with T4 DNA ligase (Fermentas, 5u/µl) of the Prof.Holzmann lab in combination with 10x T4 DNA Ligase Buffer of the other lab (test 2). As a control the sample mix without ligase was used (ligation negative control). See the results in table 3.3.2.2.5 and table 3.3.2.2.6.

Table 3.3.2.2.5 Colony numbers		
Sample	1:50 dilution	Rest
pUC19 control	31	
Test1	2	71
Test2	55	Approx.1000
Ligation negative control		5

Table 3.3.2.2.6 Transformation rates	
Plate	Colony numbers at 1µg
pUC19 1:50 dilution (20pg)	1550000
Test1 1:50 (200pg)	10000
Test1 Rest (10ng)	7100
Test2 1:50 (200pg)	275000
Test2 Rest (10ng)	100000
Ligation negative control (10ng)	500

The ligation control experiment showed that the ligase buffer of the Prof.Holzmann caused problems. A possible explanation was that the ATP was decayed.

According to the results of the ligation control experiment 2µl pENTR-1A vector (220ng/µl) was double digested with 0.5µl BamHI and 0.5µl XhoI in a 10µl digest. Subsequently, 3µl pAAV-IRES-hrGFP- ΔN-Tcf4 (200ng/µl) was digested in the same manner. 5µl of the vector digest was dephosphorylated with1µl CIAP dilution (0.01u/µl) according to 5.16.2. After that 3 different ligations were done according to 5.16.2 successful ligation mix. α ligation included 1µl desphosphorylated pENTR-1A digest (20ng) and 4µl pAAV-IRES-hrGFP- ΔN-Tcf4 digest (240ng). β ligation had the same composition like α ligation excepting 1µl phosphorylated pENTR-1A digest (44ng) instead of the dephosphorylated vector.

γ ligation was an adaption of α ligation: 2µl of the vector and 6µl of the insert were included. The ligation samples were transformed in competent E.coli cells and the results are in table 3.3.2.2.7 and 3.3.2.2.8. 1ng pUC19 was heat shocked for 35 and 90 seconds as controls in addition to the 1ng pEGFP-C1 control (kanamycin resistance, Clontech).

Only the β ligation was successful and produced colonies. Interestingly, the dephosphorylated samples failed. It could be that the dephosphorylation procedure destroyed the sticky ends resulting in an inhibition of ligation. 4 colonies of the β ligation were picked from the rest plate and cultivated overnight. Next day STET miniprep was done to isolate DNA. The yield was between 70 and 250ng/µl. Subsequently, 2µl miniprep samples were digested with 0.5µl BamHI alone and the same amount of enzyme restriction enzyme was also used in combination with 0.5µl XhoI (buffer B). The results are summarized in figure 3.3.2.2.4 and table 3.3.2.2.9. The expected fragments are in table 3.3.2.2.10.

Table 3.3.2.2.7 Colony numbers		
Sample	1:50 dilution	Rest
pUC19 control 35s	Uncountable	
pUC19 control 90s	Uncountable	
GFP-C1 control	24	
α ligation	0	0
β ligation	6	116
γ ligation	0	0

Table 3.3.2.2.8 Transformation rates	
Plate	Colony numbers at 1µg
GFP-C1 control (1ng)	1200000
β ligation (10ng) 1:50 aliquot	30000
β ligation (10ng)	11600

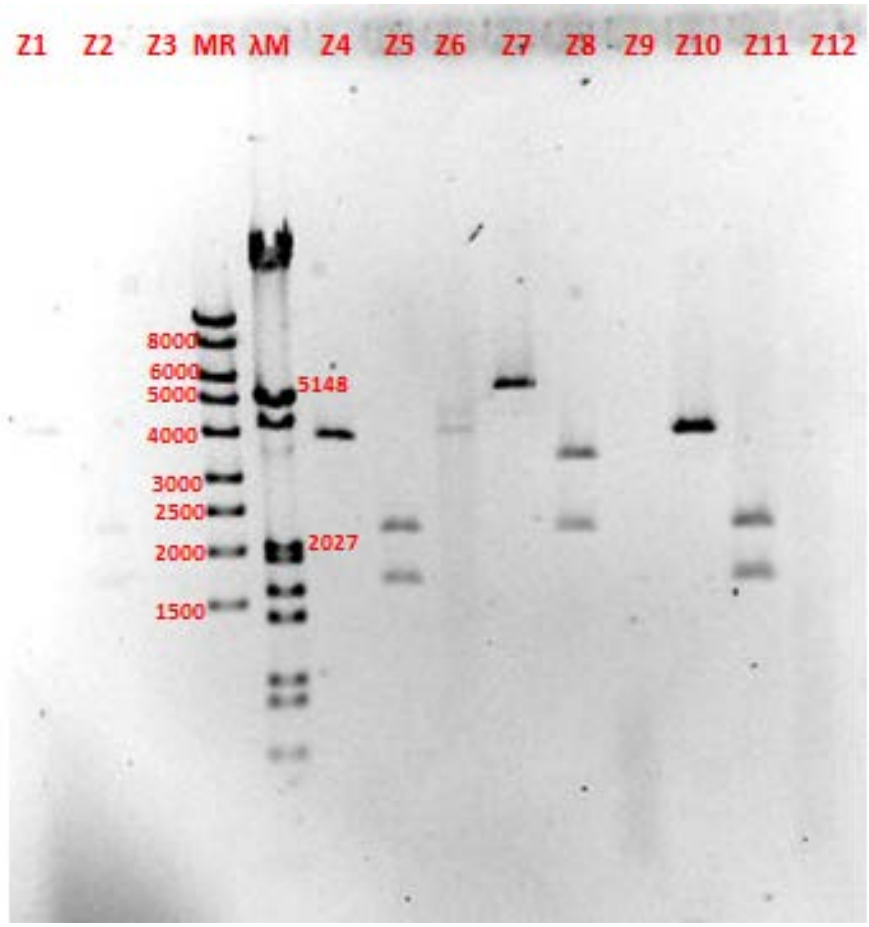


Figure 3.3.2.2.4 restriction digest for identification of pAAV-IRES-hrGFP- ΔN-Tcf4 clones

Table 3.3.2.2.9 Sample description of figure 3.3.2.2.4	
Sample number	Sample details
Z1	pENTR-1A- ΔN-Tcf4 colony 1, BamHI
Z2	pENTR-1A - ΔN-Tcf4 colony1, BamHI, XhoI
Z3	pENTR-1A - ΔN-Tcf4 colony 1, no restriction enzymes
Z4	pENTR-1A- ΔN-Tcf4 colony 2, BamHI
Z5	pENTR-1A - ΔN-Tcf4 colony 2, BamHI, XhoI
Z6	pENTR-1A - ΔN-Tcf4 colony 2, no restriction enzymes
Z7	pENTR-1A- ΔN-Tcf4 colony 3, BamHI
Z8	pENTR-1A - ΔN-Tcf4 colony 3, BamHI, XhoI
Z9	pENTR-1A - ΔN-Tcf4 colony 3, no restriction enzymes
Z10	pENTR-1A- ΔN-Tcf4 colony 4, BamHI
Z11	pENTR-1A - ΔN-Tcf4 colony 4, BamHI, XhoI
Z12	pENTR-1A - ΔN-Tcf4 colony 4, no restriction enzymes
λM	Figure 5.12.4.1 Lambda 3 marker
MR	Figure 5.12.4.2 Mass Ruler™ DNA Ladder High Range

Table 3.3.2.2.10 Expected fragments for BamHI and XhoI digests	
Restriction enzymes	Constructs and fragments (bp)
BamHI	Vector + ΔN-Tcf4: 3995
BamHI and XhoI	Vector + ΔN-Tcf4: 1728; 2267

pENTR-1A- ΔN-Tcf4 colony 2 and colony 4 samples showed the correct bands as expected. Colony 1 showed only weak bands which were also correct. Colony 3 had bands which were shifted too higher molecular weight and thus they were incorrect.

Subsequently, BamHI and XhoI double digests of 600ng pAAV-IRES-hrGFP-VP16-ΔN-Tcf4 and pAAV-IRES-hrGFP-Tcf4wt and 440ng pENTR-1A were obtained similar to the previous procedure with pAAV-IRES-hrGFP- ΔN-Tcf4. After that the samples were ligated and transformed according to the same procedure above. The results are listed in table 3.3.2.2.11 and table 3.3.2.2.12.

Table 3.3.2.2.11 Colony numbers		
Sample	1:50 dilution	Rest
pUC19 control	Uncountable	Uncountable
VP16 ligation	28	Approx.1000
Wt ligation	86	Approx. >1000

Table 3.3.2.2.12 Transformation rates	
Plate	Colony numbers at 1µg
VP16 ligation (22ng) 1:50 aliquot	63636
Wt ligation (22ng) 1:50 aliquot	195454

The yield of colonies was much higher than in the previous β ligation. 2 colonies were picked from each of the 1:50 aliquot plates of VP16 ligation and Wt ligation and cultivated overnight. Subsequently, minipreps with column were done with the samples according to 5.12.3. Additionally, an overnight culture was cultivated from the β ligation glycerine stock of pENTR-1A- ΔN-Tcf4 colony 4 and a subsequent miniprep with column was done. The yields of the miniprep samples were between 60 and 100ng/µl. After that 2µl of each miniprep sample was tested with 3 different restriction digests as above. The results are shown in figure 3.3.2.2.5, figure 3.3.2.2.6 and table 3.3.2.2.13. The expected fragments of the digests are listed in table 3.3.2.2.14.

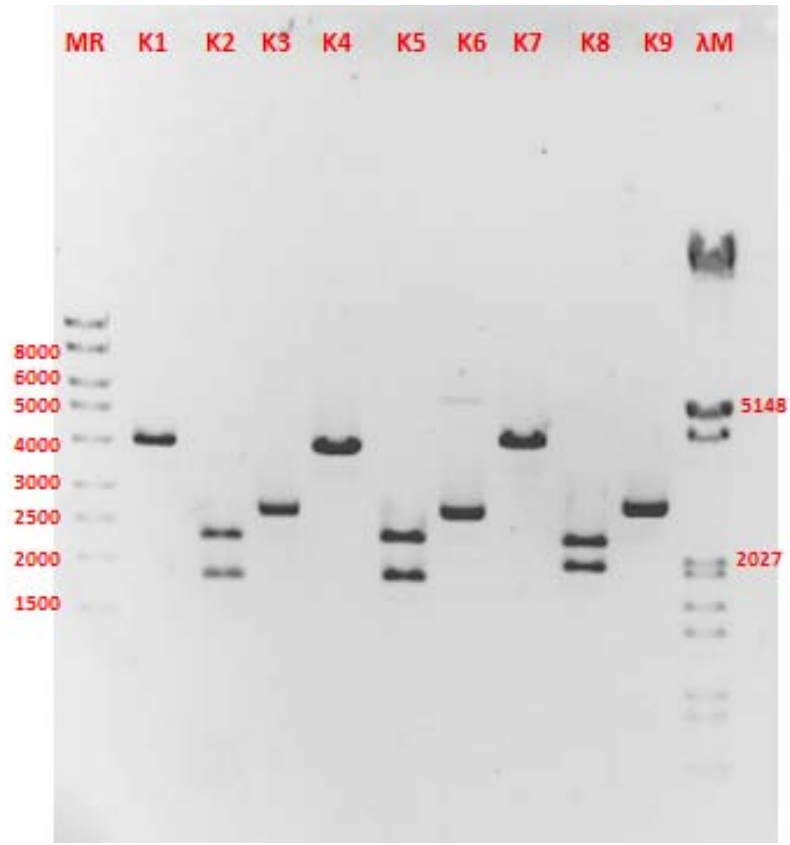


Figure 3.3.2.2.5 restriction digests of VP16 ligation and wt ligation

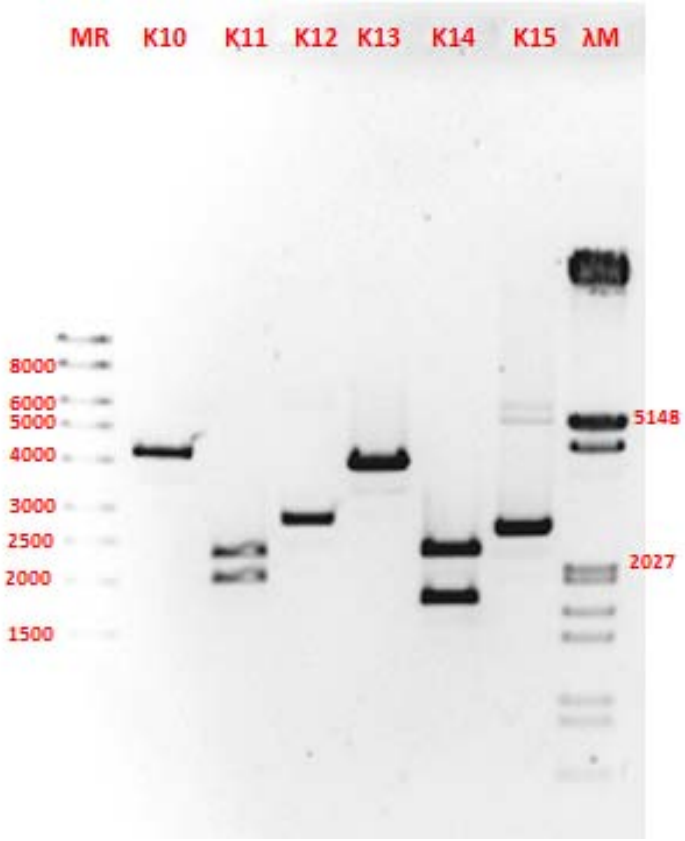


Figure 3.3.2.2.6 restriction digests of VP16 ligation and wt ligation

Table 3.3.2.2.13 Sample description of figure 3.3.2.2.5-6	
Sample number	Sample details
K1	pENTR-1A-Tcf4wt colony 1, XhoI
K2	pENTR-1A -Tcf4wt colony1, BamHI, XhoI
K3	pENTR-1A -Tcf4wt colony 1, no restriction enzymes
K4	pENTR-1A-Tcf4wt colony 2, XhoI
K5	pENTR-1A -Tcf4wt colony 2, BamHI, XhoI
K6	pENTR-1A -Tcf4wt colony 2, no restriction enzymes
K7	pENTR-1A-VP16-ΔN-Tcf4 colony 1, XhoI
K8	pENTR-1A-VP16-ΔN-Tcf4 colony 1, BamHI, XhoI
K9	pENTR-1A –VP16-ΔN-Tcf4 colony 1, no restriction enzymes
K10	pENTR-1A-VP16-ΔN-Tcf4 colony 2, XhoI
K11	pENTR-1A-VP16-ΔN-Tcf4 colony 2, BamHI, XhoI
K12	pENTR-1A-VP16-ΔN-Tcf4 colony 2, no restriction enzymes
K13	pENTR-1A-ΔN-Tcf4 colony 4, XhoI
K14	pENTR-1A-ΔN-Tcf4 colony 4, BamHI, XhoI
K15	pENTR-1A-ΔN-Tcf4 colony 4, no restriction enzymes
λM	Figure 5.12.4.1 Lambda 3 marker
MR	Figure 5.12.4.2 Mass Ruler™ DNA Ladder High Range

Table 3.3.2.2.14 Expected fragments for BamHI and XhoI digests	
Restriction enzymes	Constructs and fragments (bp)
XhoI	Vector + Tcf4wt: 4085 Vector + VP16- ΔN-Tcf4: 4244
BamHI and XhoI	Vector + Tcf4wt: 1818; 2267 Vector + VP16-ΔN-Tcf4: 1977; 2267

Both colonies of pENTR-1A-Tcf4wt and pENTR-1A-VP16-ΔN-Tcf4 yielded the expected fragments in the digest. The miniprep of pENTR-1A-ΔN-Tcf4 colony 4 also produced weak bands that were not expected indicating minimal traces of another product. In order to gain pure pENTR-1A-ΔN-Tcf4 bacteria from the glycerol stock were streaked onto a plate to produce single colonies that were picked and DNA obtained by a miniprep with column according to 5.12.3 with the concerning overnight cultures. The 2 miniprep samples were digested with BamHI and XhoI as above. Additionally, double digests of pENTR-1A-Tcf4wt colony 1 and pENTR-1A-VP16-ΔN-Tcf4 colony 1 were produced. The samples were analyzed by gel electrophoresis. The results are depicted in figure 3.3.2.2.7 and table 3.3.2.2.15. The expected fragments are listed in table 3.3.2.2.10 and 3.3.2.2.14.

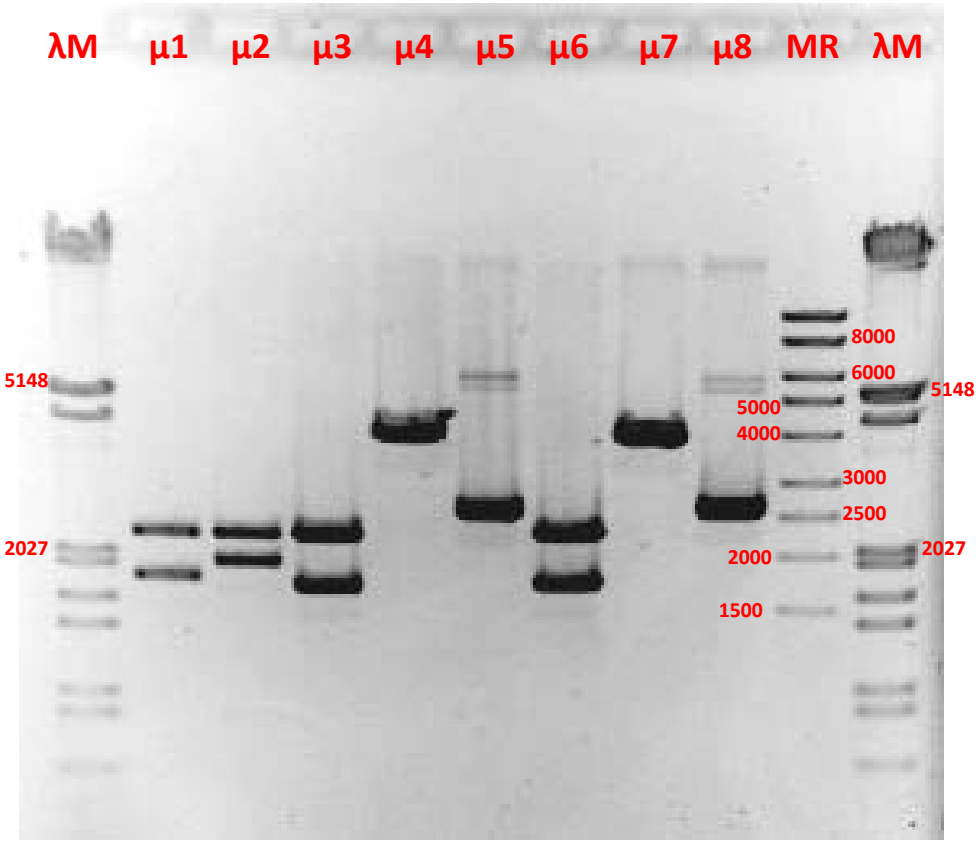


Figure 3.3.2.2.7 restriction digests of the streak samples

Table 3.3.2.2.15 Sample description of figure 3.3.2.2.7	
Sample number	Sample details
µ1	pENTR-1A-Tcf4wt colony 1, BamHI, XhoI
µ2	pENTR-1A-VP16-ΔN-Tcf4 colony 1, BamHI, XhoI
µ3	pENTR-1A-ΔN-Tcf4 colony 4 streak colony1 BamHI, XhoI
µ4	pENTR-1A-ΔN-Tcf4 colony 4 streak colony1, XhoI
µ5	pENTR-1A-ΔN-Tcf4 colony 4 streak colony1, no restriction enzymes
µ6	pENTR-1A-ΔN-Tcf4 colony 4 streak colony2 BamHI, XhoI
µ7	pENTR-1A-ΔN-Tcf4 colony 4 streak colony2, XhoI
µ8	pENTR-1A-ΔN-Tcf4 colony 4 streak colony2, no restriction enzymes
λM	Figure 5.12.4.1 Lambda 3 marker
MR	Figure 5.12.4.2 Mass Ruler™ DNA Ladder High Range

The predicted bands were obtained in all samples demonstrating that the constructs had the correct sequence and were suitable for construction of the adenoviral constructs.

See the resulting pENTR1A-insert clones in figures 3.3.2.2.8-10.

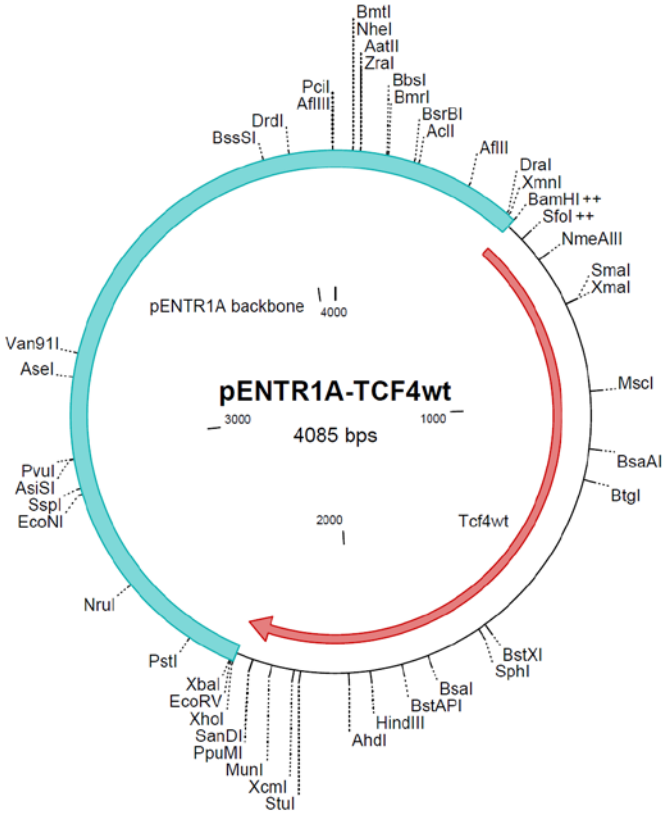


Figure 3.3.2.2.8 pENTR1A-TCF4wt

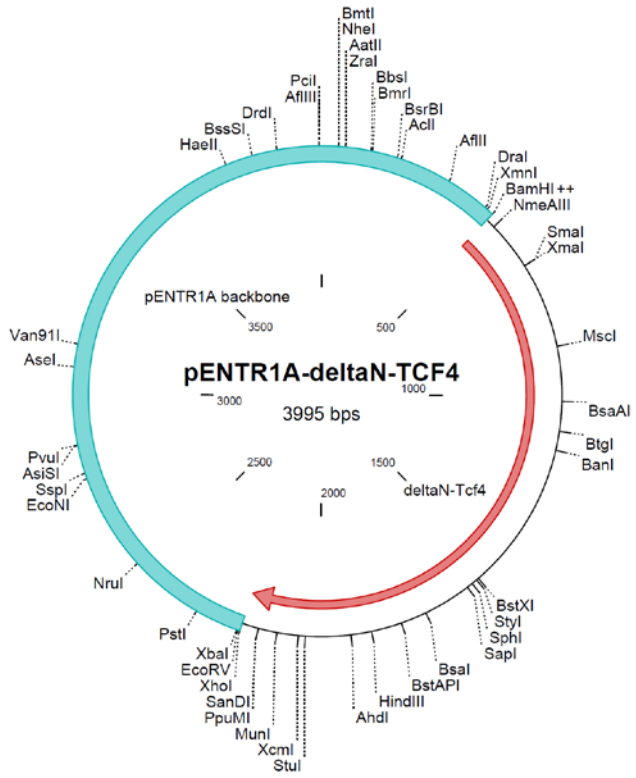


Figure 3.3.2.2.9 pENTR1A-deltaN-TCF4

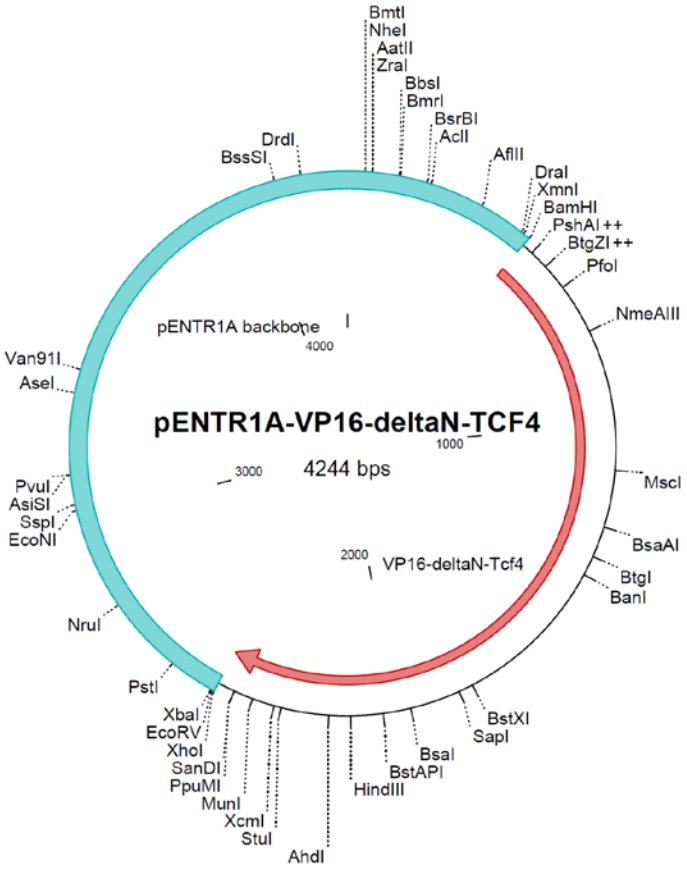


Figure 3.3.2.2.10 pENTR1A-VP16-deltaN-TCF4

3.3.3 pAd/CMV/V5-DEST vector cloning:

After pENTR cloning the inserts had to be recombined into the adenoviral expression vector pAd/CMV/V5-DEST™ vector (see figure 3.3.3.1) by the LR reaction.

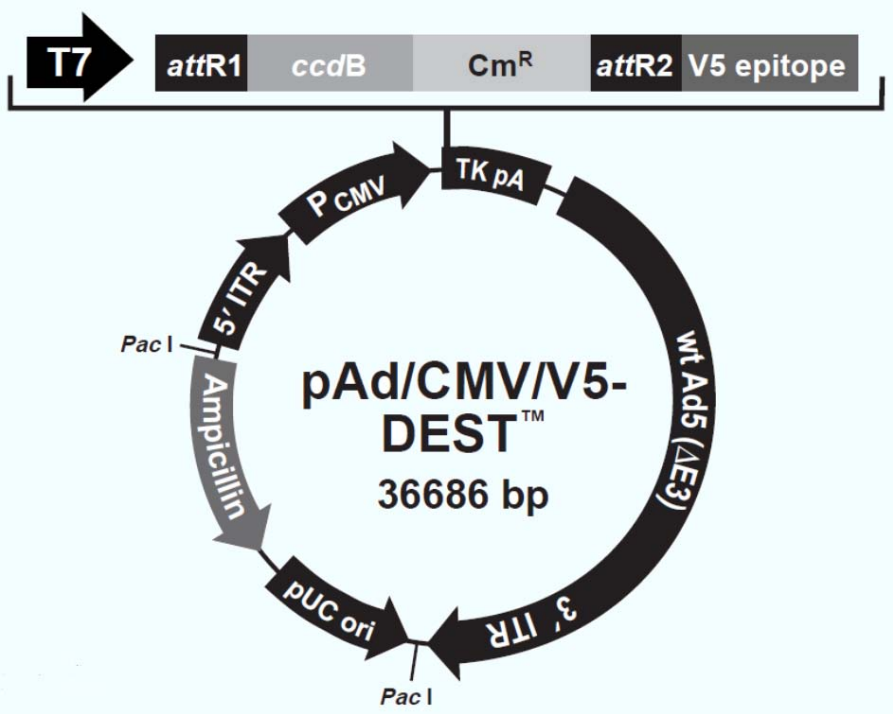


Figure 3.3.3.1 pAd/CMV/V5-DEST™ vector

The Tcf4wt, ΔN-Tcf4, VP16-ΔN-Tcf4 inserts were flanked by attL1 and attL2 recombination sites of the pENTR1A vector and those sites were recombined into the pAd/CMV/V5-DEST™ vector (invitrogen, see figure 3.3.3.1) by Gateway® LR Clonase™ II reaction (invitrogen) according to 5.17. The LR reaction is depicted in figure 3.3.3.2.

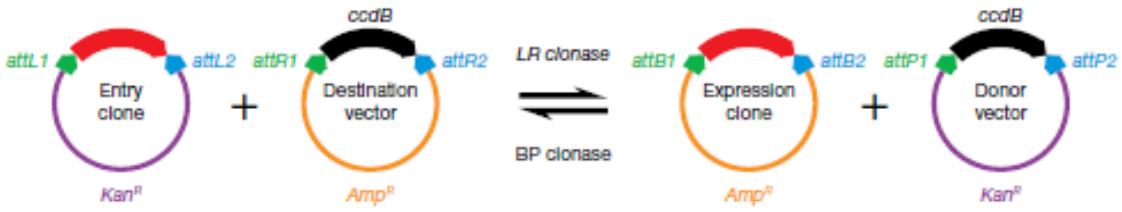


Figure 3.3.3.2 overview of LR reaction

3µl pENTR-1A-Tcf4wt colony1 and 3µl pENTR-1A –VP16-ΔN-Tcf4 colony1 miniprep samples (about 150ng) were recombined with 1µl pAd/CMV/V5-DEST™ vector in a half mix according to 5.17. 2µl pENTR-1A-ΔN-Tcf4 colony 4 streak colony2 was also combined with the destination vector in a full mix according to 5.17. The resulting half gateway mixes samples were transformed in commercial TOP10 E.coli according to 5.12.2. Additionally, half of the mix of pAd/CMV/V5-DEST- ΔN-Tcf4 was transformed in Chinese hyper-competent cells according to 5.12.2. pUC19 controls were used for both competent cell types. The results are in table 3.3.3.1 and table 3.3.3.2.
2 colonies per plate were picked and cultivated in 7ml Chinese SOC/AMP overnight. Subsequently, DNA was obtained by miniprep with column.

Table 3.3.3.1 Colony numbers

Sample	1:50 dilution	1:100 dilution	Rest
pUC19 Chinese		50	
PUC19 commercial	Several 100		Uncountable
Tcf4wt			7
VP16			16
ΔTcf4 Chinese			6
ΔTcf4 commercial			22

Table 3.3.3.2 Transformation rates

Plate	Colony numbers at 1µg
pUC19 Chinese (10pg) 1:100	5000000
pUC19 bought (20pg) 1:50	More than 10000000
Tcf4wt (150ng)	46
VP16 (150ng)	105
ΔTcf4 Chinese	39
ΔTcf4 bought	145

4µl of the miniprep samples and 2µl of the empty pAd/CMV/V5-DEST™ vector were digested with 0.5µl XhoI and buffer H (Roche) and then analyzed by electrophoresis. The results are shown in figure 3.3.3.3, figure 3.3.3.4 and table 3.3.3.3. The expected fragments are in table 3.3.3.4.

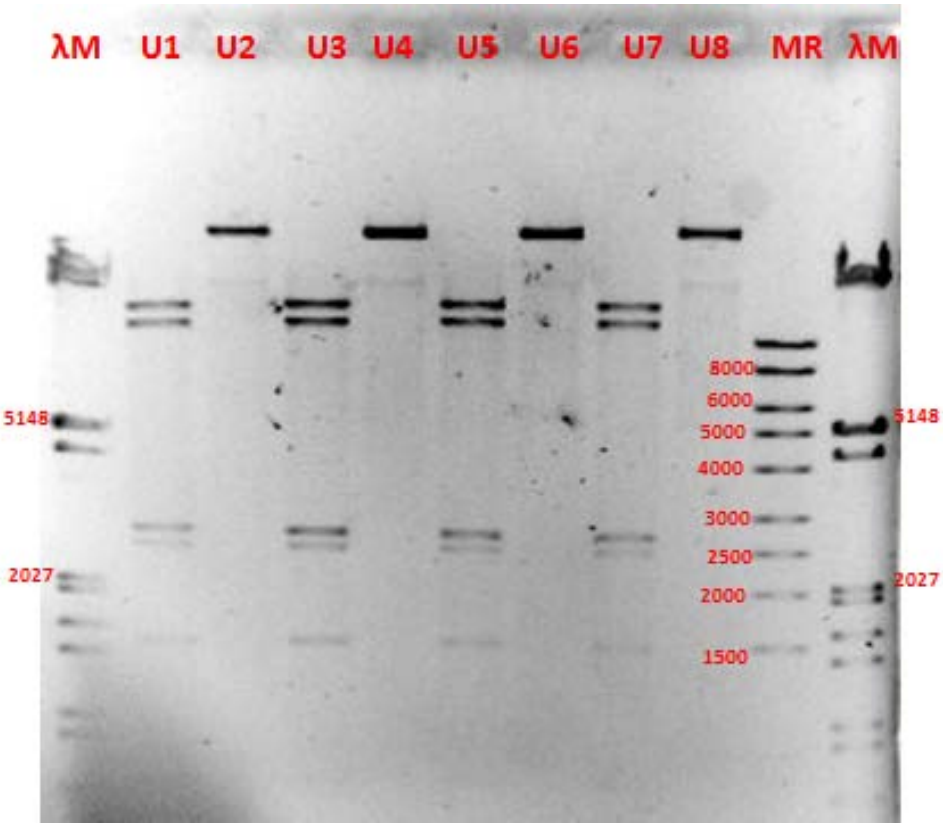


Figure 3.3.3.3 XhoI digest of LR-reaction samples

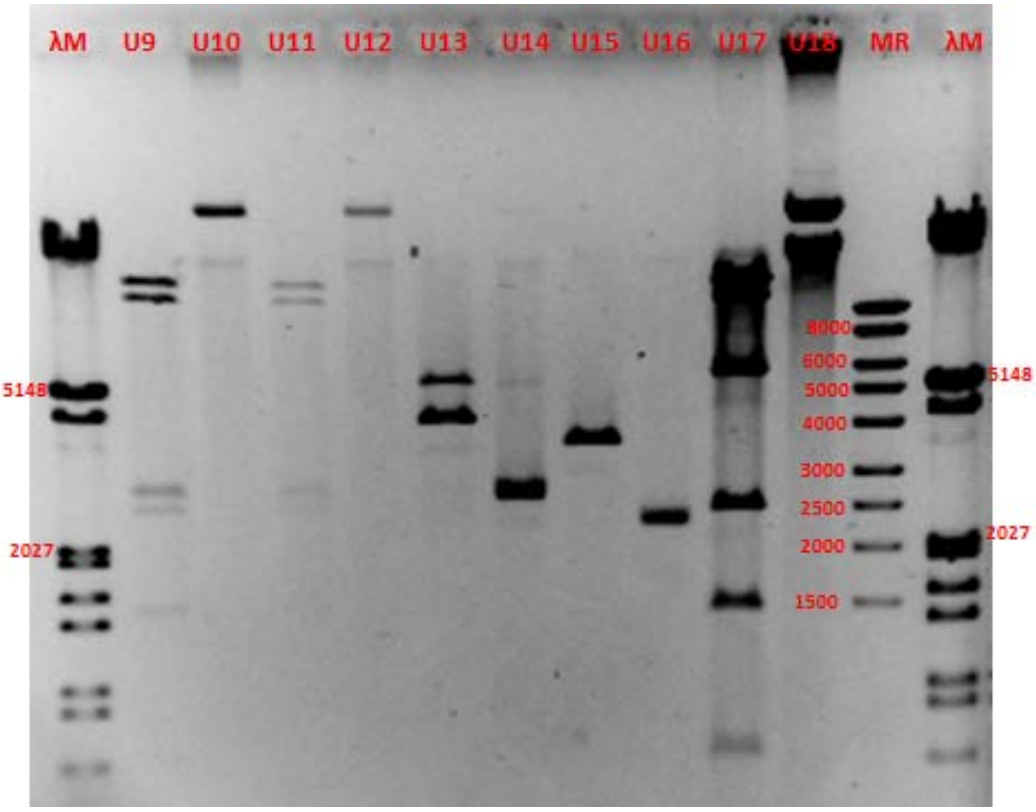


Figure 3.3.3.4 XhoI digest of LR-reaction samples

Table 3.3.3.3 Sample description of figure 3.3.3.3-4

Sample number	Sample details
U1	pAd/CMV/V5-DEST- ΔN-Tcf4 colony1 Chinese, XhoI
U2	pAd/CMV/V5-DEST- ΔN-Tcf4 colony1 Chinese, no restriction enzymes
U3	pAd/CMV/V5-DEST- ΔN-Tcf4 colony2 Chinese, XhoI
U4	pAd/CMV/V5-DEST- ΔN-Tcf4 colony2 Chinese, no restriction enzymes
U5	pAd/CMV/V5-DEST- ΔN-Tcf4 colony1 commercial cells, XhoI
U6	pAd/CMV/V5-DEST- ΔN-Tcf4 colony1 commercial cells, no restriction enzymes
U7	pAd/CMV/V5-DEST- ΔN-Tcf4 colony2 commercial cells, XhoI
U8	pAd/CMV/V5-DEST- ΔN-Tcf4 colony1 commercial cells, no restriction enzymes
U9	pAd/CMV/V5-DEST-Tcf4wt colony1, XhoI
U10	pAd/CMV/V5-DEST-Tcf4wt colony1, no restriction enzymes
U11	pAd/CMV/V5-DEST-Tcf4wt colony2, XhoI
U12	pAd/CMV/V5-DEST-Tcf4wt colony2, no restriction enzymes
U13	pAd/CMV/V5-DEST-VP16- ΔN-Tcf4 colony1, XhoI
U14	pAd/CMV/V5-DEST-VP16- ΔN-Tcf4 colony1, no restriction enzymes
U15	pAd/CMV/V5-DEST-VP16- ΔN-Tcf4 colony2, XhoI
U16	pAd/CMV/V5-DEST-VP16- ΔN-Tcf4 colony2, no restriction enzymes
U17	pAd/CMV/V5-DEST, XhoI
U18	pAd/CMV/V5-DEST, no restriction enzymes
λM	Figure 5.12.4.1 Lambda 3 marker
MR	Figure 5.12.4.2 Mass Ruler™ DNA Ladder High Range

Table 3.3.3.4 Expected fragments for XhoI digest

Construct	fragments (bp)
pAd/CMV/V5-DEST-ΔN-Tcf4	2699; 2698; 2466; 1445; 595; 14502; 11832
pAd/CMV/V5-DEST-Tcf4wt	2789; 2698; 2466; 1445; 595; 14502; 11832
pAd/CMV/V5-DEST-VP16- ΔN-Tcf4	2948; 2698; 2466; 1445; 595; 14502; 11832
pAd/CMV/V5-DEST	485; 5846; 2466; 1445; 595; 14502; 11347

The fragments of pAd/CMV/V5-DEST- ΔN-Tcf4 and pAd/CMV/V5-DEST-Tcf4wt samples and of the empty pAd/CMV/V5-DEST were as expected. pAd/CMV/V5-DEST-VP16- ΔN-Tcf4 produced different fragments as expected.

Subsequently, 7ml LB/AMP cultures were obtained from pAd/CMV/V5-DEST-ΔN-Tcf4 colony2 Chinese and colony 1commercial cells samples. The same was done with pAd/CMV/V5-DEST-Tcf4wt colony1. In order to identify bacteria containing the correct pAd/CMV/V5-DEST-VP16-ΔN-Tcf4 construct 8 colonies were picked. Next day STET boiling plasmid miniprep were performed and the samples were checked with XhoI as above. The results are summarized in figure 3.3.3.5, figure 3.3.3.6 and table 3.3.3.5.

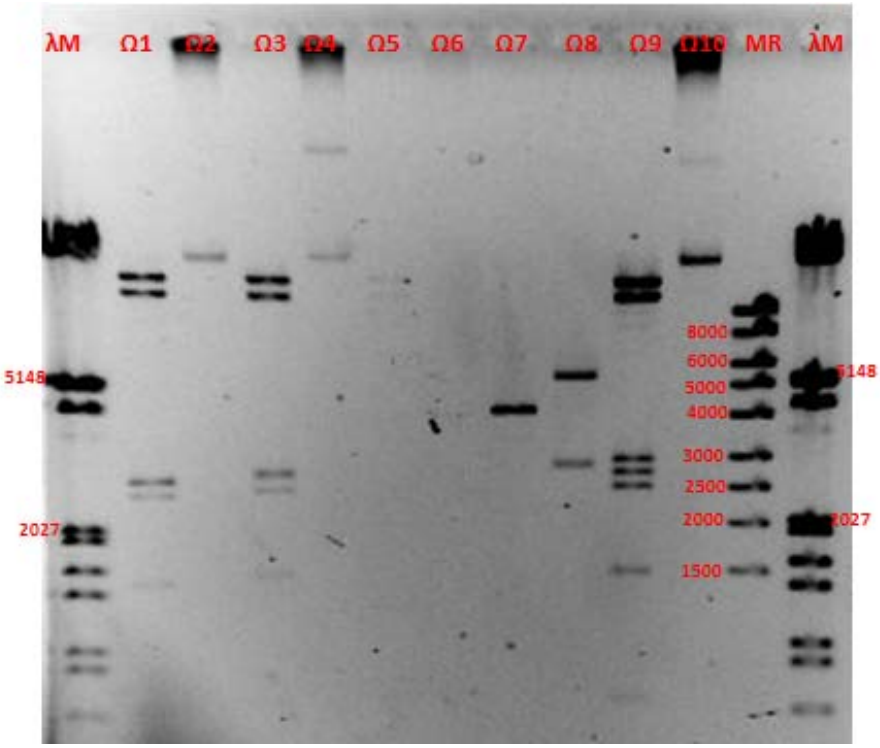


Figure 3.3.3.5 XhoI digest of different pAd/CMV/V5-DEST-VP16- ΔN-Tcf4 clones

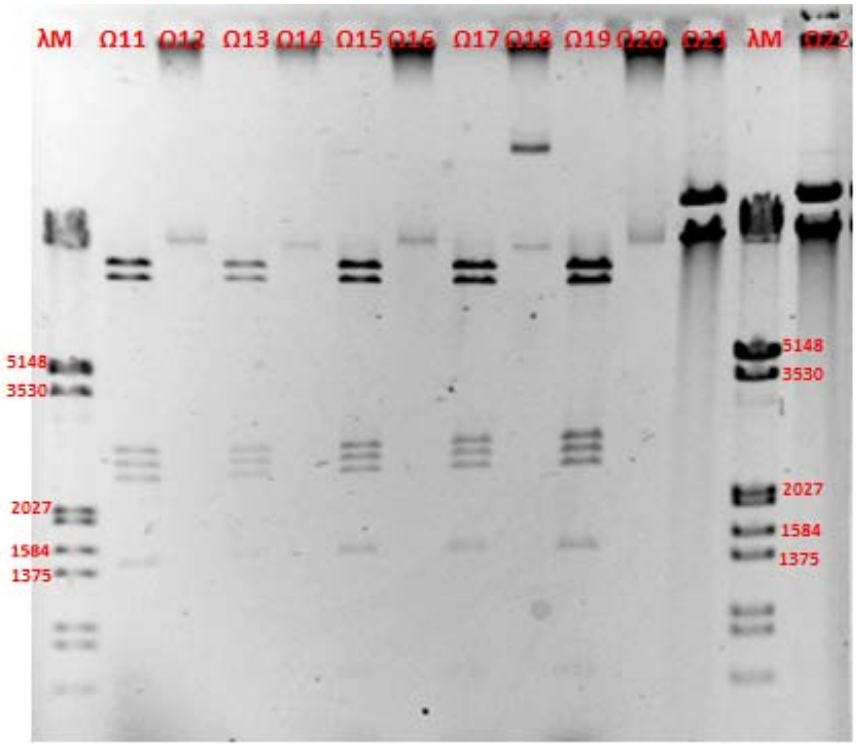


Figure 3.3.3.6 XhoI digest of different pAd/CMV/V5-DEST-VP16- ΔN-Tcf4 clones

Table 3.3.3.5 Sample description of figure 3.3.3.5-6	
Sample number	Sample details
Ω1	pAd/CMV/V5-DEST- ΔN-Tcf4 colony1 commercial cells, XhoI
Ω2	pAd/CMV/V5-DEST- ΔN-Tcf4 colony1 commercialt cells, no restriction enzymes
Ω3	pAd/CMV/V5-DEST-Tcf4wt colony1, XhoI
Ω4	pAd/CMV/V5-DEST-Tcf4wt colony1, no restriction enzymes
Ω5	pAd/CMV/V5-DEST-VP16- ΔN-Tcf4 colony3, XhoI
Ω6	pAd/CMV/V5-DEST-VP16- ΔN-Tcf4 colony3, no restriction enzymes
Ω7	pAd/CMV/V5-DEST-VP16- ΔN-Tcf4 colony4, XhoI
Ω8	pAd/CMV/V5-DEST-VP16- ΔN-Tcf4 colony4, no restriction enzymes
Ω9	pAd/CMV/V5-DEST-VP16- ΔN-Tcf4 colony5, XhoI
Ω10	pAd/CMV/V5-DEST-VP16- ΔN-Tcf4 colony5, no restriction enzymes
Ω11	pAd/CMV/V5-DEST-VP16- ΔN-Tcf4 colony6, XhoI
Ω12	pAd/CMV/V5-DEST-VP16- ΔN-Tcf4 colony6, no restriction enzymes
Ω13	pAd/CMV/V5-DEST-VP16- ΔN-Tcf4 colony7, XhoI
Ω14	pAd/CMV/V5-DEST-VP16- ΔN-Tcf4 colony7, no restriction enzymes
Ω15	pAd/CMV/V5-DEST-VP16- ΔN-Tcf4 colony8, XhoI
Ω16	pAd/CMV/V5-DEST-VP16- ΔN-Tcf4 colony8, no restriction enzymes
Ω17	pAd/CMV/V5-DEST-VP16- ΔN-Tcf4 colony9, XhoI
Ω18	pAd/CMV/V5-DEST-VP16- ΔN-Tcf4 colony9, no restriction enzymes
Ω19	pAd/CMV/V5-DEST-VP16- ΔN-Tcf4 colony10, XhoI
Ω20	pAd/CMV/V5-DEST-VP16- ΔN-Tcf4 colony10, no restriction enzymes
Ω21	pAd/CMV/V5-DEST, XhoI
Ω22	pAd/CMV/V5-DEST, no restriction enzymes
λM	Figure 5.12.4.1 Lambda 3 marker
MR	Figure 5.12.4.2 Mass Ruler™ DNA Ladder High Range

pAd/CMV/V5-DEST-VP16- ΔN-Tcf4 colony3 and 4 did not produce the predicted bands but colony5 -10 digests resulted in the expected bands. pAd/CMV/V5-DEST- ΔN-Tcf4 colony1commercial cells and pAd/CMV/V5-DEST-Tcf4wt colony1 had bands as predicted.

Figure 3.3.3.5 and figure 3.3.3.6 also proved that 3 different adenoviral constructs were produced.

Finally, glycerol stocks were made of bacteria habouring pAd/CMV/V5-DEST- ΔN-Tcf4 colony1 bought cells, pAd/CMV/V5-DEST-Tcf4wt colony1 and pAd/CMV/V5-DEST-VP16- ΔN-Tcf4 colony5 and DNA was isolated by midiprep.

The constructed pAd/CMV/V5-DEST-insert vectors are shown in figure 3.3.3.7-9.

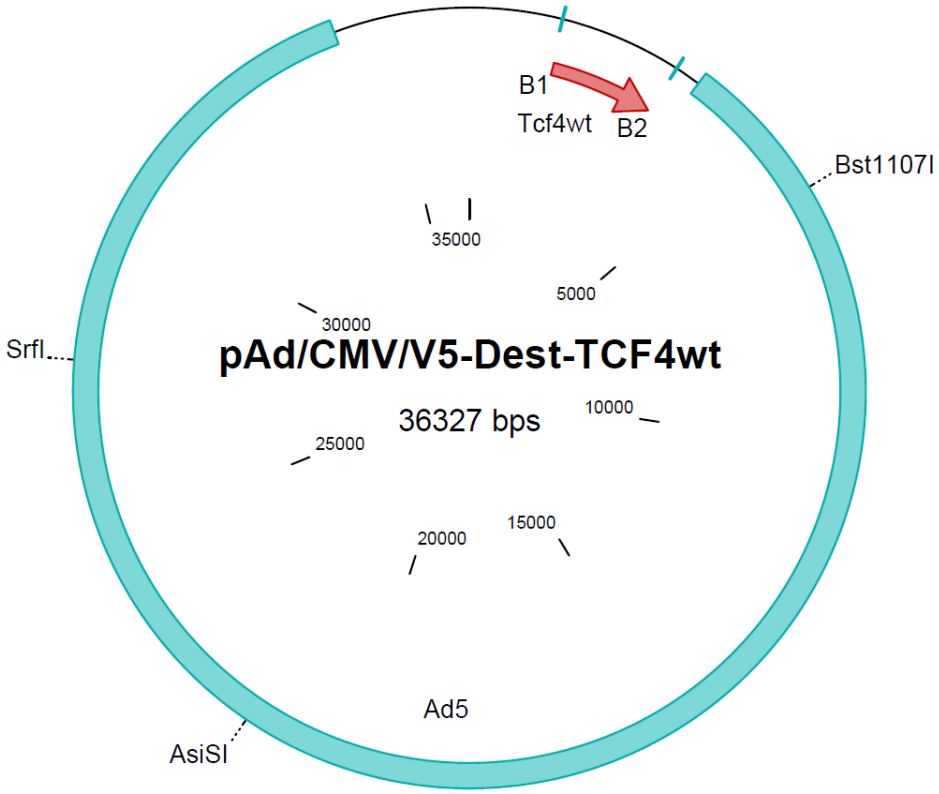


Figure 3.3.3.7 pAd/CMV/V5-Dest-TCF4wt

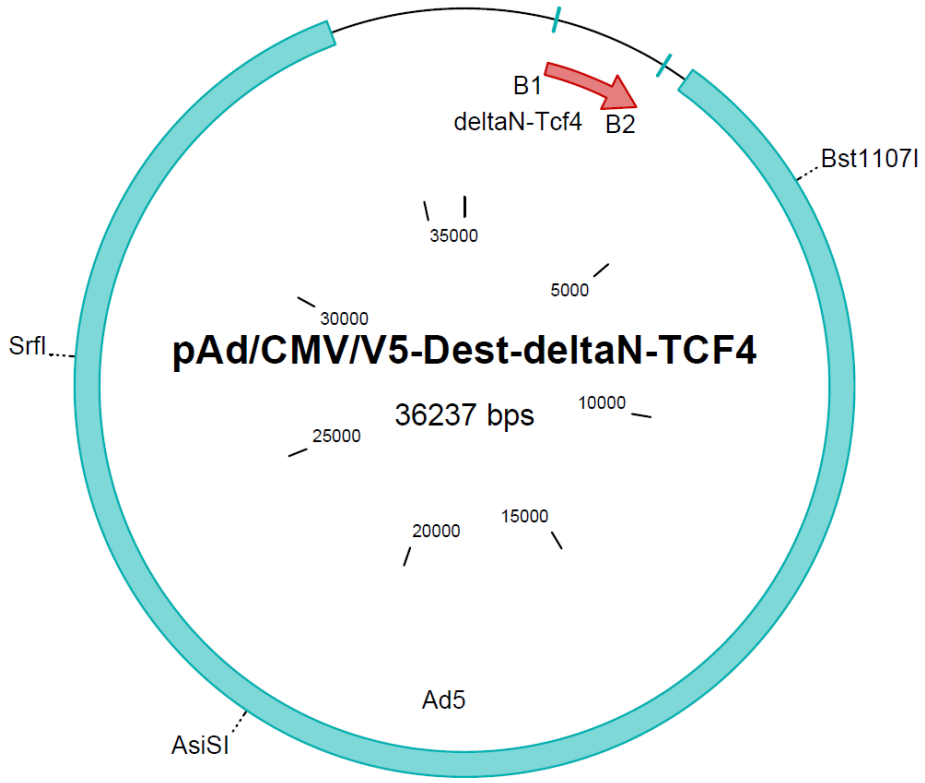


Figure 3.3.3.8 pAd/CMV/V5-Dest-deltaN-TCF4

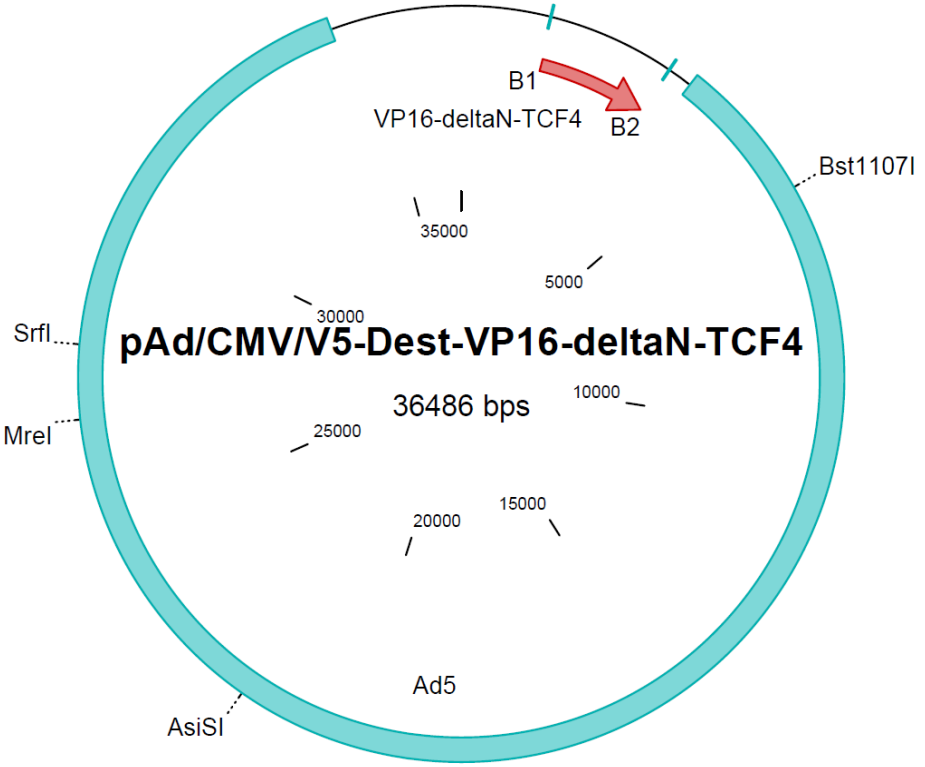


Figure 3.3.3.9 pAd/CMV/V5-Dest-VP16-deltaN-TCF4

3.3.4 Virus production in HEK293 cells

The 3 pAd/CMV/V5-DEST-insert midiprep samples were digested with PacI and the plasmids were checked and precipitated according to 5.18. The results are depicted in figure 3.3.4.1-2 and table 3.3.4.1-2. The expected fragments of the PacI digest were 2074bp and about 34000bp.

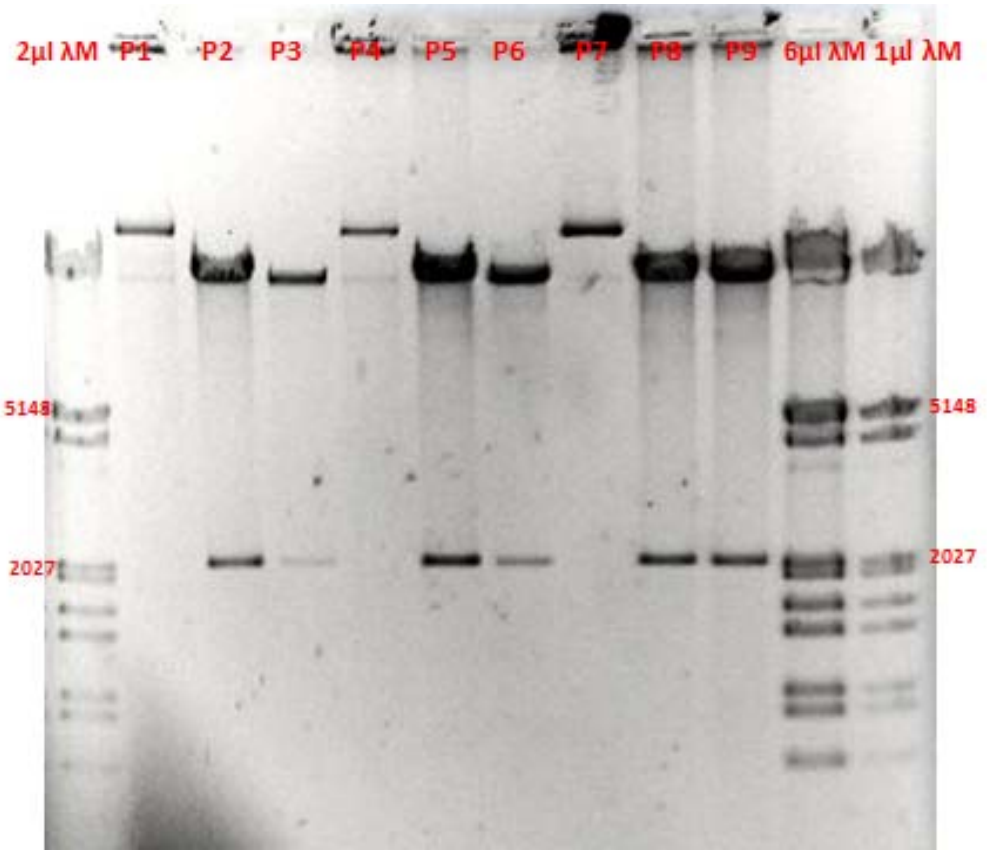


Figure 3.3.4.1 PacI digest

Table 3.3.4.1 Sample description of figure 3.3.4.1

Sample number	Sample details
P1	pAd/CMV/V5-DEST-Tcf4wt, no restriction enzymes
P2	pAd/CMV/V5-DEST-Tcf4wt, PacI, before precipitation
P3	pAd/CMV/V5-DEST-Tcf4wt, PacI, after precipitation
P4	pAd/CMV/V5-DEST- ΔN-Tcf4, no restriction enzymes
P5	pAd/CMV/V5-DEST- ΔN-Tcf4, PacI, before precipitation
P6	pAd/CMV/V5-DEST- ΔN-Tcf4, PacI, after precipitation
P7	pAd/CMV/V5-DEST-VP16- ΔN-Tcf4, no restriction enzymes
P8	pAd/CMV/V5-DEST- VP16-ΔN-Tcf4, PacI, before precipitation
P9	pAd/CMV/V5-DEST- VP16-ΔN-Tcf4, PacI, after precipitation
λM	Figure 5.12.4.1 Lambda 3 marker

All digests showed clear bands at 2000bp as expected. Nanodrop measurements showed that pAd/CMV/V5-DEST- VP16-ΔN-Tcf4 had 250ng/µl after precipitation and this was enough for transfection. pAd/CMV/V5-DEST-ΔN-Tcf4 and pAd/CMV/V5-DEST-Tcf4wt had 30-50ng/µl yield after precipitation and these constructs were digested and precipitated again with higher yield (about 150-200ng/µl). Subsequently, the linearized adenoviral constructs were transfected into HEK293 cells according to 5.18. After 3 days the cells were cracked according to 5.18. A cytopathic effect could not be detected.

It was decided to go on with the pAd/CMV/V5-DEST-ΔN-Tcf4. Subsequently, 100µl, 200µl and 400µl of the cracked sample were added to HEK293 cells in 6-well-plates and a cytopathic effect was observed at day 3 and at day4 the cells were cracked (passage 2). HEK293 cells were transfected with 500µl and 250µl lysate in 2*10cm Pds and a cytopathic effect was detected again at day 3. The plates were cracked separately (passage 3) and a standard PCR for pAd/CMV/V5-DEST-insert according to 5.18.6 was performed in order to prove that pAd/CMV/V5-DEST-ΔN-Tcf4 was in the lysates. The expected PCR product lengths are listed in table 3.3.4.2. The PCR products were tested by electrophoresis (see figure 3.3.4.2 and table 3.3.4.3).

Table 3.3.4.2 PCR product lengths

Constructs	Lenghts
pAd/CMV/V5-DEST-empty	2368bps
pAd/CMV/V5-DEST-VP16-ΔN-Tcf4	2168bps
pAd/CMV/V5-DEST- ΔN-Tcf4	1919bps
pAd/CMV/V5-DEST-Tcf4wt	2009bps

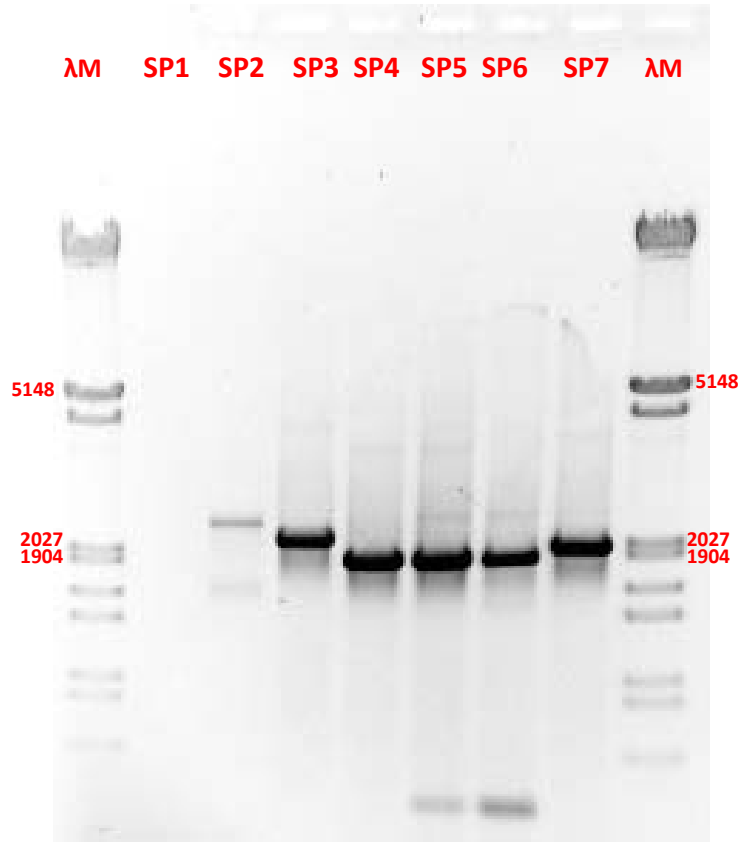
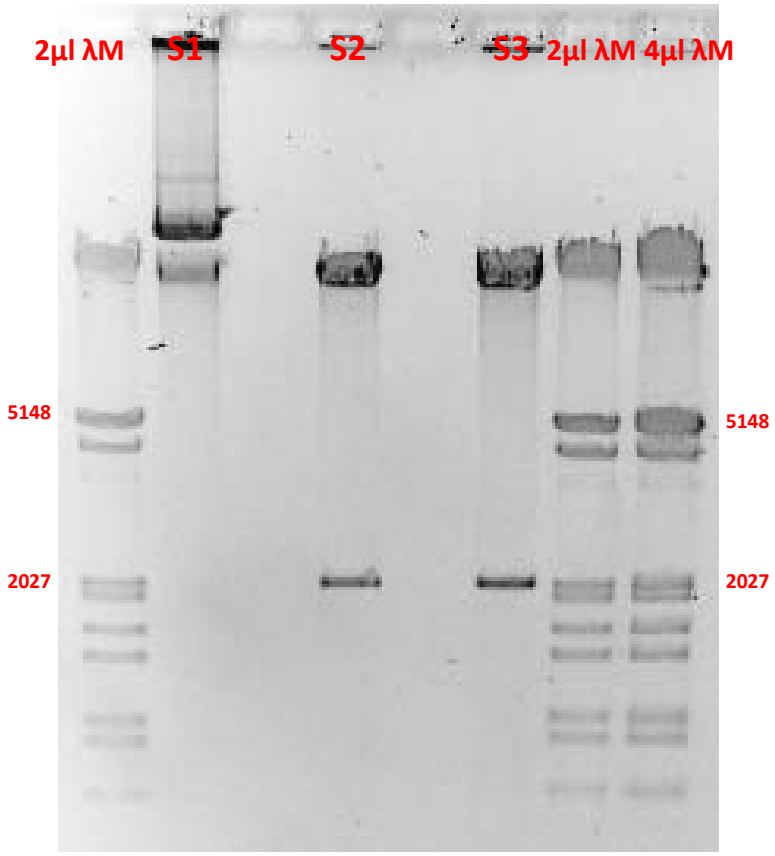


Figure 3.3.4.2 Control PCR of pAd/CMV/V5-DEST-ΔN-Tcf4 virus

Table 3.3.4.3 Sample description of figure 3.3.4.2

Sample number	Sample details
SP1	NTC
SP2	pAd/CMV/V5-DEST-empty control
SP3	pAd/CMV/V5-DEST- VP16-ΔN-Tcf4 plasmid
SP4	pAd/CMV/V5-DEST- ΔN-Tcf4 plasmid
SP5	pAd/CMV/V5-DEST- ΔN-Tcf4 virus sample 500µl plate
SP6	pAd/CMV/V5-DEST- ΔN-Tcf4 virus sample 250µl plate
SP7	pAd/CMV/V5-DEST-Tcf4wt plasmid
λM	Figure 5.12.4.1 Lambda 3 marker

Additionally, a new PacI digest of pAd/CMV/V5-DEST-ΔN-Tcf4 was performed and precipitated as above. The yield was about 230ng/µl after precipitation. The digest was also analyzed with gel electrophoresis. The results are depicted in figure 3.3.4.3 and table 3.3.4.4.



3.3.4.3 pAd/CMV/V5-DEST-ΔN-Tcf4 PacI digest

Table 3.3.4.4 Sample description of figure 3.3.4.3

Sample number	Sample details
S1	pAd/CMV/V5-DEST-ΔN-Tcf4, no restriction enzymes
S2	pAd/CMV/V5-DEST-ΔN-Tcf4, PacI, before precipitation
S3	pAd/CMV/V5-DEST-ΔN-Tcf4, PacI, after precipitation
λM	Figure 5.12.4.1 Lambda 3 marker

Subsequently, the transfection of the HEK293 cells were performed with 10µl and 5µl of the precipitated PacI digest in 2 wells of a 6-well-plate according to 5.18. After 5 days the cells were cracked according to 5.18 because a cytopathic effect might be observed. Subsequently, HEK293 cells were transfected with 500µl and 250µl lysate in 2*10cm Pds but a cytopathic effect could not be detected after 7 days and the attempt was discarded.

The bands of figure 3.3.4.2 showed clearly that the HEK293-virus lysate contained the pAd/CMV/V5-DEST- ΔN-Tcf4 construct. Subsequently, 3*10cm Pds with HEK293 cells were incubated with 100µl, 200µl and 300µl HEK293-virus lysate of the 500µl plate (passage 3). After 24 hours a strong cytopathic effect could be observed and they were cracked. The propagation of viral particles needs at least 48 hours and thus the passage had to be repeated with a smaller amount of lysate. The repetition of the reinfection with 30µl HEK293-virus lysate of the 500µl plate (passage 3) causes a cytopathic effect after 24 hours again and they are cracked on the second day. 250µl of these passage 4 lysate were incubated on each 2x15cm plates with $8x10^8$ HEK293 cells. After 2 days a strong cytopathic effect was observed and the cells were cracked. Subsequently, the passage 5 lysate was tested again with PCR according to 5.18.6. The expected PCR product lengths are listed in table 3.3.4.2. The PCR products were analysed by electrophoresis (see figure 3.3.4.4 and table 3.3.4.5) but no viral particles could be detected (see no band at SP5).

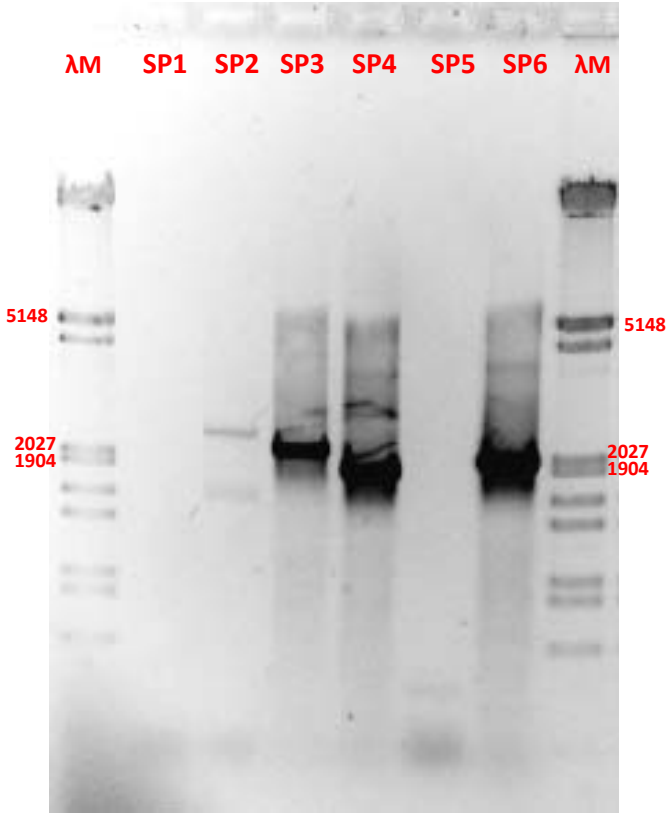


Figure 3.3.4.4 2nd control PCR of pAd/CMV/V5-DEST-ΔN-Tcf4

Table 3.3.4.5 Sample description of figure 3.3.4.4 and figure3.3.4.5	
Sample number	Sample details
SP1	NTC
SP2	pAd/CMV/V5-DEST-empty control
SP3	pAd/CMV/V5-DEST- VP16-ΔN-Tcf4 plasmid
SP4	pAd/CMV/V5-DEST- ΔN-Tcf4 plasmid
SP5	pAd/CMV/V5-DEST- ΔN-Tcf4 virus passage 5 in figure 3.4.1.34
	pAd/CMV/V5-DEST- ΔN-Tcf4 virus passage 4 in figure 3.4.1.35
SP6	pAd/CMV/V5-DEST-Tcf4wt plasmid
λM	Figure 5.12.4.1 Lambda 3 marker

The problem could be that the cytopathic effect was too strong and too early. This causes that most viral particles were delivered in the medium but they could not be harvested by the cracking procedure.

After that 2x15cm Pds with HEK293 cells were reinfected with 30µl HEK293-virus lysate of the 500µl plate (passage 3) and a strong cytopathic effect could be observed at day 2. This new passage 4 was tested again with PCR according to 5.18.6 and analysed by electrophoresis (see figure 3.3.4.5 and table 3.3.4.5). Again virus DNA could not be detected.

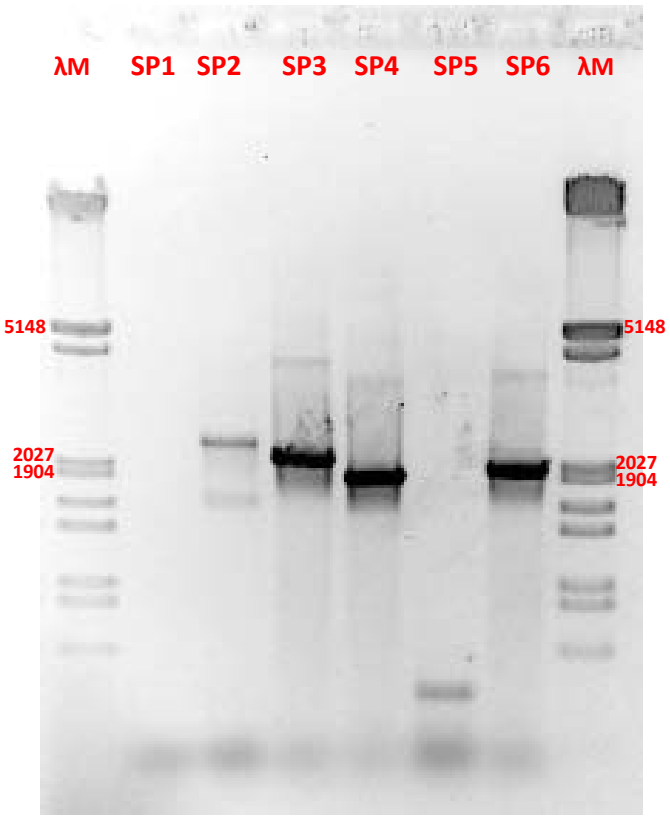


Figure 3.3.4.5 Passage 4 control PCR of pAd/CMV/V5-DEST-ΔN-Tcf4

Subsequently, 4x confluent 15cm Pds with HEK293 cells were reinfected with 15µl HEK293-virus lysate of the 500µl plate and the 250µl plate (passage 3) and a strong cytopathic effect could be observed at day 2. The cells were cracked on day 2 and day3. The medium became acidic on day 3 (yellow color of the indicator). The cracked lysates and the medium supernatants were tested by the PCR according to 5.18.6 and analysed by electrophoresis (see figure 3.3.4.6 and table 3.3.4.6).

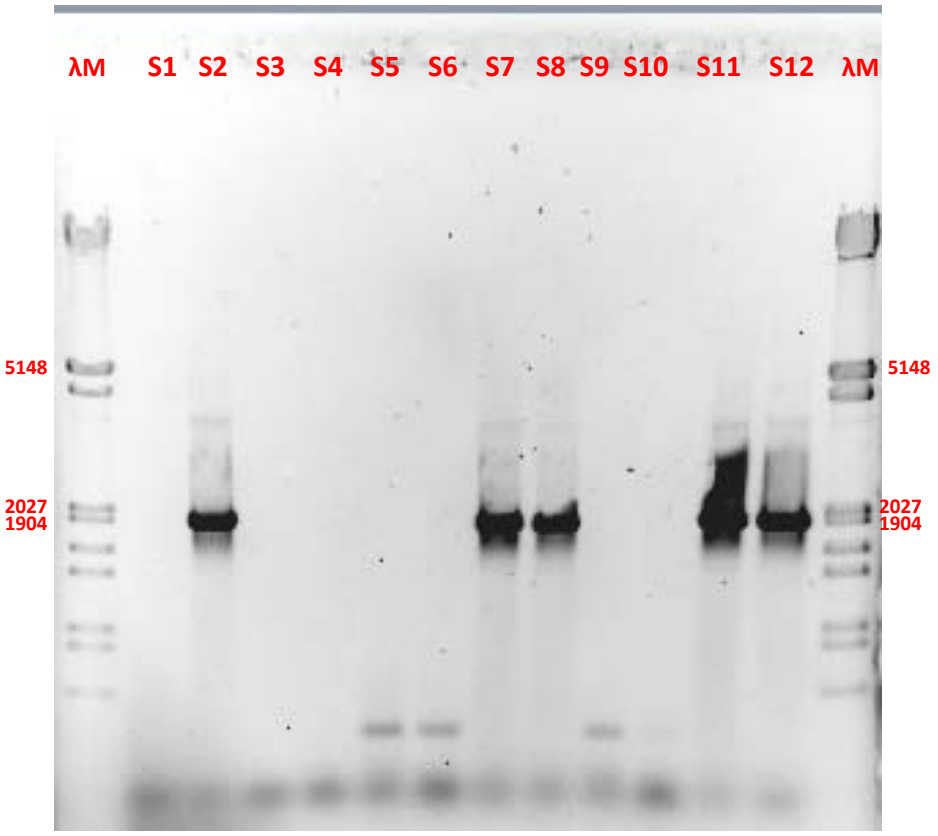


Figure 3.3.4.6 New passage 4 control PCR of pAd/CMV/V5-DEST-ΔN-Tcf4

Table 3.3.4.6 Sample description of figure 3.3.4.6

Sample number	Sample details
S1	NTC
S2	pAd/CMV/V5-DEST- ΔN-Tcf4 plasmid
S3	pAd/CMV/V5-DEST- ΔN-Tcf4 passage 3 500µl plate lysate
S4	pAd/CMV/V5-DEST- ΔN-Tcf4 passage 3 250µl plate lysate
S5	pAd/CMV/V5-DEST- ΔN-Tcf4 virus passage 4 lysate with passage 3 500µl lysate day 2
S6	pAd/CMV/V5-DEST- ΔN-Tcf4 virus passage 4 lysate with passage 3 250µl lysate day 2
S7	pAd/CMV/V5-DEST- ΔN-Tcf4 virus passage 4 medium with passage 3 500µl lysate day 2
S8	pAd/CMV/V5-DEST- ΔN-Tcf4 virus passage 4 medium with passage 3 250µl lysate day 2
S9	pAd/CMV/V5-DEST- ΔN-Tcf4 virus passage 4 lysate with passage 3 500µl lysate day 3
S10	pAd/CMV/V5-DEST- ΔN-Tcf4 virus passage 4 lysate with passage 3 250µl lysate day 3
S11	pAd/CMV/V5-DEST- ΔN-Tcf4 virus passage 4 medium with passage 3 500µl lysate day 3
S12	pAd/CMV/V5-DEST- ΔN-Tcf4 virus passage 4 medium with passage 3 250µl lysate day 3
λM	Figure 5.12.4.1 Lambda 3 marker

Viral particle could not be detected in all lysates but clear bands could be seen at the medium supernatant samples which supported the explanation that most viral particles were delivered into the medium. Passage 3 samples did not show any bands although they were detected in figure 3.3.4.2. This could indicate an error with the PCR. It could not be that passage 4 medium supernatant had bands for the viral particles but passage 3 did not.

After that a new PCR according to 5.18.6 was done with passage 2, passage 3 of the 500µl plate and of the 250µl plate and with all new passage 4 lysates of day 2 and day 3. 20µl of each lysate was mixed with 180µl 1xTE. The PCR products were analysed by electrophoresis but no virus could be detected (data not shown).

Subsequently, 2x10 cm Pds with HEK293 cells were reinfected with 2µl lysate and 100µl medium supernatant of passage 4 lysate of the 500µl plate day 2. The lysate gave a clear and strong cytopathic effect after 2 days. The medium of passage 4 has already shown the correct viral band at the PCR (see figure 3.3.4.6). Thus 15x15 cm Pds with 10^7 HEK293 cells were transfected with 2µl of the concerning passage 4 lysate and after 2 days they were cracked. The virus was isolated by ultracentrifugation according to 5.18.7. There were 4 bands and the lowest of them was the largest (see figure 3.3.4.7).

The highest band, the 2 bands in the middle and the lowest band were collected as 3 different aliquots which were stored at -80°C. They are called from up till down band1, band2 and band3. The upper bands might be premature viral particles. The lowest band seemed to be the mature viral particles

Figure 3.3.4.7 Virus bands after ultracentrifugation

Subsequently, the 3 bands samples were analyzed with virus titration according to 5.18.8.

Then the virus titers were calculated:

Band 1 had 31600 infectious units and band 2 showed 47400. Thus the upper bands included a tiny number of infectious particles which supported the explanation above. Band3 had 15800000/µl infectious units. The controls virus titers were as expected.

In order to prove that band1-band3 had the correct virus a control PCR according to 5.18.6 was performed and analysed by electrophoresis (see figure 3.3.4.8 and table 3.3.4.7)

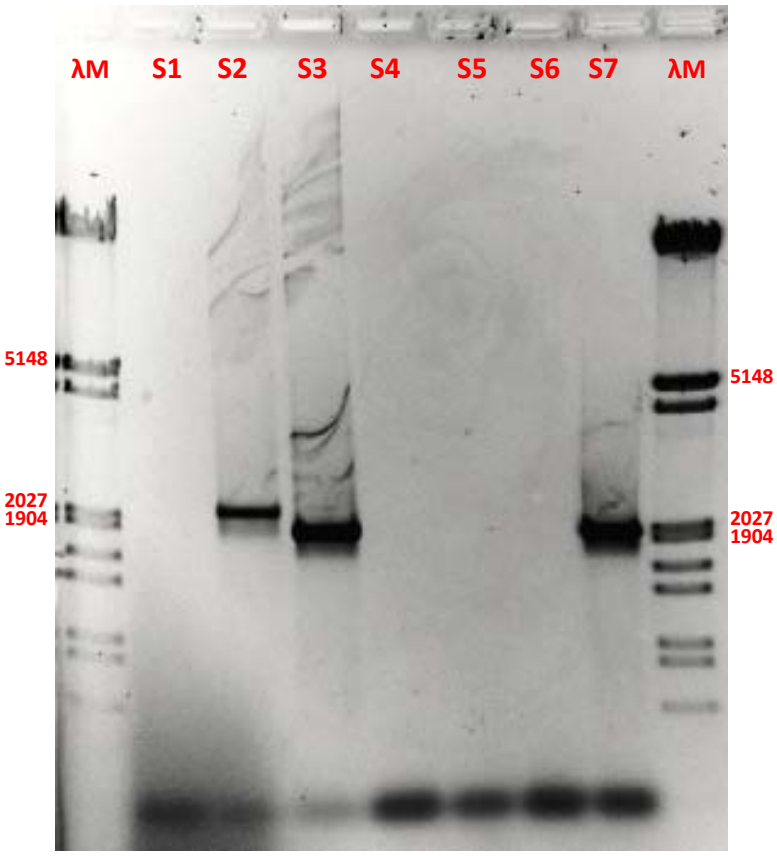


Figure 3.3.4.8 Control PCR with isolated bands

Table 3.3.4.7 Sample description of figure 3.3.4.8

Sample number	Sample details
V1	NTC
V2	pAd/CMV/V5-DEST- VP16-ΔN-Tcf4 plasmid
V3	pAd/CMV/V5-DEST- ΔN-Tcf4 plasmid
V4	pAd/CMV/V5-DEST- ΔN-Tcf4 band3
V5	pAd/CMV/V5-DEST- ΔN-Tcf4 band2
V6	pAd/CMV/V5-DEST- ΔN-Tcf4 band1
V7	pAd/CMV/V5-DEST-Tcf4wt plasmid
λM	Figure 5.12.4.1 Lambda 3 marker

The control PCR showed no bands for all 3 bands samples but clear bands at the controls.

3.4 Biological activity of the pAd/CMV/V5-DEST-ΔN-Tcf4 virus

12x6cm Pds with $7x10^5$ HT29 cells and LT97 cells were plated and after 2 days they were infected with a 10x multiplicity of infection with pAd/CMV/V5-DEST- ΔN-Tcf4 band3 and the control virus Cox AS virus. The RNA was extracted and analyzed according to 5.3 - 5.6.
Sybr® green I Real Time PCR was used to quantify survivin in the HT29 and LT97 samples. Taqman® probe kits were used to detect Tcf4, FGF18 and IGFBP3 in both cell lines.

pAd/CMV/V5-DEST- ΔN-Tcf4 infection caused a clear up-regulation of Tcf4 in both cell lines (see figure 3.4.1). In HT29 cells the virus infection caused that the RNA levels of Tcf4 were up-regulated to 168 ± 24.21% ($p = 0.0048$). Furthermore RNA levels of Tcf4 virus infected LT97 cells increased to 470 ± 192.4% ($p = 0.0048$). In fact both cell lines expressed stronger Tcf4 if they were infected with the pAd/CMV/V5-DEST- ΔN-Tcf4 virus.

In HT29 cells the virus did not cause a down-regulation of survivin RNA levels but this may be due to the high standard deviation of GAPDH results. The adenovirus had no effect on survivin RNA levels in LT97 cells. The virus also did not impact on FGF18 levels.
IFGBP3 could not be detected in HT29 cells because of too low RNA levels. In LT97 cells the IGBP3 RNA level was elevated (156% of the control level) but this was not statistically significant.

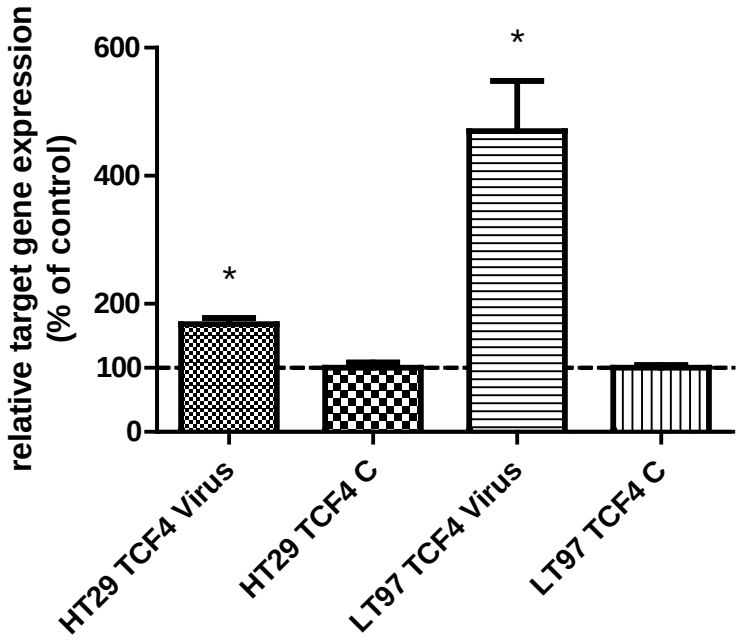


Figure 3.4.1: % in comparison with the control in pAd/CMV/V5-DEST- ΔN-Tcf4 infected HT29 and LT97 cells (Tcf4)

The value of 100 indicates the same value as the control.
* indicates statistically significant data

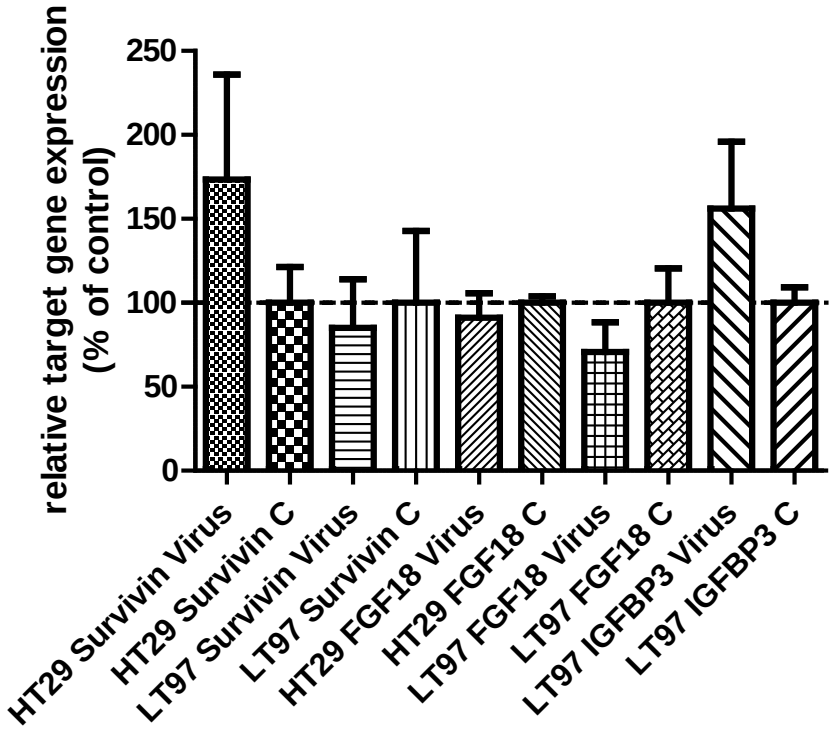


Figure 3.4.2: % in comparison with the control in pAd/CMV/V5-DEST- ΔN-Tcf4 infected HT29 and LT97 samples (Survivin, FGF18, IGFBP3)

The value of 100 indicates the same value as the control.

4. Discussion

4.1 Cell biological effects of sulindac

Sulindac has been introduced as a drug that targets wnt-signalling to induce apoptosis in chemoprevention treatment of high-risk colorectal cancer patients (Piazza et al. 2009). To investigate the underlying cellular mechanisms we have analysed the drug's impact on gene expression in colonic cells. This analysis focused on survivin and FGF18 because they are wnt-target genes (Altieri 2008, Sonvilla et al. 2008) and on IGFBP3 whose regulation in colorectal tumor cells is not yet understood.

On the RNA level survivin was down-regulated in both HT29 carcinoma and LT97 adenoma cells. In the carcinoma cell line the inter-experimental variation and consequently the standard deviation for the controls was rather high but it stays statistically significant. In LT97 cells the impact of sulindac was less but it was a statistically significant down-regulation. This effect was quite robust with low standard deviations in both the control and the survivin group (sulindac treated samples: 3.6% of the control; control samples: 18% of the control). On the protein level sulindac also caused a decrease in both cell lines. Moreover, the impact on survivin protein levels was much stronger in LT97 cells than in HT29 cells.

Survivin is an anti-apoptotic protein that inhibits caspase activity (Altieri 2010). Down-regulation of such a protein therefore constitutes a pro-apoptotic effect that can be employed for therapeutic purposes (Lladser et al. 2011). The pro-apoptotic effect can explain the concentration-dependent cell loss that had previously been described for sulindac (Piazza et al. 2009).

FGF18 also is a wnt-target gene, but surprisingly sulindac treatment did not impact on the FGF18 RNA levels in HT29. This effect was only seen in LT97 whose FGF18 RNA levels decreased to 66% of the control by 10µM sulindac but the standard deviation of the control was too high (47.8%) to permit a statistically significant difference. For this reason FGF protein secreted into the medium was not determined.

FGF18 is a strong survival factor in colorectal cancer cells that is progressively up-regulated in a wnt-dependent fashion. It is therefore a target of therapy whose down-regulation also constitutes a pro-apoptotic effect. The lack of effect in our experiments may be due to the comparatively low wnt-signalling activity in the cell lines we used that does not allow further inhibition (Sonvilla et al. 2008).

For IGFBP3 no connection with wnt-signalling has previously been preported and the results of your own experiments also do not solidly support a wnt-dependent modulation. The analysis of IGFBP3 RNA levels demonstrated a slight increase in HT29 cells but the intra-experimental variations and consequently the standard deviations were large. In LT97 cells sulindac treatment had little influence on the IGFBP3 level.

4.2 Production and biological activity of adenoviral Tcf4-blocking vector

As chemical compounds are never completely specific, a specific genetic construct is needed to establish the role of any cellular target for therapy. We plan to use a dominant-negative Tcf4-mutant, because it blocks wnt-dependent signalling down-stream of APC whose mutation in colorectal cancer cells causes a constitutive activation of the pathway (Kinzler and Vogelstein 1996). As activation of wnt-signaling activates growth as well as survival signaling (Giles et al. 2003) pathway blockade is expected to result in strong inhibition of growth and survival. Therefore it was decided to produce a vector for strong transient over-expression of a dominant-negative Tcf4-mutant.

As the transfection rate by lipofection is low for HT29 (15.87% fluorescent cells, see table 3.2.1) and extremely low (1-2%; data not shown) for LT97 it was necessary to produce a viral vector for efficient transient expression. The choice to produce an adenoviral vector was based on the fact that several other adenoviral constructs have previously been used in the laboratory including several control viruses (Allerstorfer et al. 2007; Sonvilla et al. 2008).

So the ΔN-Tcf4, VP16- ΔN-Tcf4 and Tcf4wt cassettes were excised with BamHI and XhoI from the pAAV-IRES-hrGFP-inserts vectors (Holnthoner et al. 2002) and cloned into a pENTR-1A vector by ligation reaction. Subsequently, the pENTR-1A-inserts are recombined into the adenoviral expression vector pAd/CMV/V5-DEST™ vector by gateway cloning reaction. Only the pAd/CMV/V5-DEST- ΔN-Tcf4 virus was propagated in HEK293 cells and isolated by ultracentrifugation which gives rise to 3 bands. Only the lowest band which is called band 3 had a high virus titer of 15800000/µl infectious units.

Infection of HT29 and LT97 cells with 10x moi of the virus resulted in clear statistically significant up-regulation of Tcf4 RNA levels in both virus infected cell lines. In LT97 cells the increase of the RNA level was higher (470%) than in HT29 cells (168%). An explanation could be that the Tcf4 levels of controls in LT97 (CT = 28.11) cells were lower than those in HT29 cells (CT = 26.63).

However, pAd/CMV/V5-DEST- ΔN-Tcf4 infection did not impact statistically significantly on the RNA levels of survivin, FGF18 and IGFBP3.

Further experiments will be necessary using different multiplicity of infection in order to explore the abilities of the virus inhibiting the wnt pathway.

5. Materials and methods

5.1 Cell lines and cell culture

5.1.1 Cell lines:

LT97 is a colorectal adenoma cell line which is very close to primary adenoma cells. It has lost both alleles of APC, one allele of Ki-RAS is mutated but p53 is still functioning (Richter et al. 2002). The cells have a 60% CD44+ subpopulation (Schulenburg et al. 2007). HT29 is a colorectal carcinoma cell line which consists exclusively of CD44+ cells and was obtained from the American Type Culture Collection.

5.1.2 Cell culture materials and methods for HT29 cultivation

5.1.2.1 List of materials

Cell culture dishes: Petri dishes (PD) with 10 cm and 6 cm diameter

PBS/EDTA: 2x 1l powder flasks Dulbecco`s Phosphate Buffered Saline (Sigma) is dissolved in 1500ml aqua bidest; 7.44g EDTA is dissolved in 200ml aqua bidest and adjusted to pH=7.5; both solution are mixed, filled up to 2l and autoclaved;

Trypsin/EDTA in PBS: 45 ml PBS with 5ml Trypsin/EDTA

HT 29 cultivation medium: 10% FCS, 450 ml MEM with 50ml FCS and 2ml Penstrep

5.1.2.2 Splitting of cells

Medium is removed. The cells are washed with 5ml PBS/EDTA if 10 cm PDs are used. Subsequently, 500µl till 800µl Trypsin/EDTA are added and the PD is incubated at 37°C for about 5 minutes till the cells are completely detached. This reaction is stopped with 10 ml HT29 cultivation medium. The cells can be separated by pipetting up and down and split according to needs of the experiments. In order to count the cell number counting chambers are used.

5.1.3 Cell culture materials and methods for LT97 cultivation

5.1.3.1 List of materials

Cell culture dishes: Petri dishes (PD) with 10 cm and 6 cm diameter

PBS/EDTA

Basic medium 10l (400 ml aliquoted): ph 7.0, 2500ml Leibovitz Medium, 100 ml L-Glutamin, filled up to 10l with Aqua bidest, 1 bottle Ham`s F12 Medium (Sigma Nr. N6760), 11.8g $NaHCO_3$, 0.44g CaCl2x2H_2O, sterile filtrated, aliquoted, 250ml HEPES buffer

LT full medium: 400ml Basic medium are added, 8ml FCS, 0.8ml Insulin, 1.6ml Penstrep, 1.6ml V10, 400µl EGF (30µg/ml), 800µl Gentamycin (500x)

5.1.3.2 Splitting of cells

Medium is removed. The cells are washed gently twice with 1ml PBS/EDTA. This is done very gently because the cells detach easily. The cells are detached with 750-1000µl PBS/EDTA and incubated at 37°C for about 10-15 minutes. During that time the PDs are knocked several times in order to detach the cells more efficiently. Then the cells are suspended in twice 5 ml LT full medium, transferred into a falcon tube and centrifuged at 1100rpm for 5 minutes. Supernatant is removed and the cell pellets are dissolved in 4 ml LT full medium. The cells can be split according to the experiment.

5.2 Sulindac treatment

5.2.1 List of materials

HT29 sulindac treatment media: MEM with 2ml Penstrep but serum free, 1:1000 sulindac (stored at -20°C, 10mM stock), 1:100 1% BSA

HT29 treatment control media: HT29 sulindac treatment media without sulindac

LT97 sulindac treatment media: LT full medium without FCS and Insulin, 1:1000 sulindac (stored at -20°C, 10mM stock), 1:100 1% BSA

LT97 treatment control media: LT97 sulindac treatment media without sulindac

5.2.2 Procedures of sulindac treatment for HT29 cells

HT29 cells are split in order to have $5x10^5$ cells in each 6cm Pd. The cells are cultivated for 2 days before changing the medium to HT29 sulindac treatment or HT29 treatment control medium. On day 3 the cells are ready for further analysis.

5.2.3 Procedures of sulindac treatment for LT97 cells:

LT97 cells are split at a ratio of 1:3 and grow till 50-65% confluence. Subsequently, the medium is changed to LT97 sulindac treatment or control medium. On the next day the cells are ready for further analysis.

In order to detect IGFBP3 with Western Blot: LT97 cells are incubated with 3ml LT97 sulindac treatment medium or 3ml LT97 treatment control medium in Pds with 6cm diamter for 2 days.

5.3. RNA isolation

5.3.1 List of materials

0.1M NaOH

DEPC water

70% EtOH in DEPC water

PeqGold Trifast™ (Peqlab Biotechnologie, Erlangen, Germany)

Chloroform

Isopropanol

5.3.2 Procedures of RNA isolation

Scrapers have to be treated 10 minutes with 0.1 NaOH and 10 minutes with 70% EtOH and then dried in the sterile work bench to make them RNAse-free. The medium is removed from the cells, 1000µl PeqGold Trifast™ is added and the cultures incubated 5 minutes on ice. Subsequently, the cells are scraped off and homogenized by a syringe and a 0.6-in-diamter needle and transferred to Eppendorf tubes. 200µl

chloroform is added; the tubes are vortexed for 30 seconds and then left on ice for 10 minutes. After that the tubes are centrifuged at 15000rpm at 5°C for 15 minutes to achieve phase-separation. The upper phase which contains the RNA is pipetted into new Eppendorf tubes. 500µl isopropanol is added and the samples are vortexed for 30sec and incubated on ice for 10 minutes to precipitate of the RNA. Subsequently, the Eppendorf tubes are centrifuged at 12200rpm at 4°C for 10 minutes. Supernatant is removed carefully and the RNA pellet is washed with 70% EtOH and centrifuged at 15000rpm at 4°C for 15 minutes. The supernatant is removed carefully and the pellet is dried in the sterile work bench. After that it is dissolved in 15 till 50µl DEPC water dependent on the size of the pellet. It is denatured at 65-70°C for 10 minutes. The RNA solution is stored at -80°C.

5.4 RNA measurement

The RNA amount is measured with NanoDrop™ PEQLAB spectrophotometer (Peqlab Biotechnologie, Erlangen, Germany) and the ND1000 sofware. 1µl RNA sample are used to quantify the concentration in ng/µl. λ 269/280 ratio

5.5 cDNA synthesis

5.5.1 List of materials

DEPC water

Random Hexamer Primer Mix (1mM stock, used 1:10 diluted; GE Healthcare, Piscataway, NJ, USA)

dNTP Mix

RNase Inhibitor: Riboblock RI

5xfirst strand buffer (MMuLVRT-buffer, Fermentas, Burlington, Canada)

RevertAid™ MMuLV reverse transcriptase (Fermentas, Burlington, Canada)

5.5.2 Procedures of cDNA synthesis

Table 5.5.2.1: Random Hexamer Primer Master Mix (per sample)	
Volume	Substance
1µL	100 µM random hexamer primer mix
1.5µL	DEPC-water

Table 5.5.2.2: Mastermix for cDNA synthesis (per sample)	
Volume	Substance
4 µl	5x first strand buffer
2µl	dNTP Mix
0.5µl	Riboblock RI

The goal of cDNA synthesis is the production of single stranded cDNA by reverse transcription. The advantage to use random hexamer primers is that all RNAs can be converted into cDNA.

All preparations are done on ice. 2µg RNA is pipetted into PCR tube and diluted to final volume of 10µl with DEPC water. 2.5µl Random Hexamer Primer Mastermix is added per sample. The mix is then heated to 70°C for 5 minutes and returned to ice again. 6.5µl Mastermix is added per sample

and the PCR tubes are heated to 25°C for 5 minutes. After that 1µl reverse transcriptase is added. Subsequently, the PCR tubes are incubated at 25°C for 10 minutes, then at 42°C for 60 minutes (DNA elongation) and finally the reaction is stopped at 70°C for 10 minutes. After that the samples are kept on ice and 80µl DEPC water is added. The samples are vortexed and can be stored at -20°C.

5.6 Real Time PCR

The polymerase chain reaction (PCR) is a method to amplify and detect a gene of interest using a DNA template. PCR is characterized by reiterating cycles of denaturation, annealing and extension steps. Forward and reverse primers which are oligonucleotides bind specifically at the annealing step and subsequently the polymerase synthesizes subsequently the complementary strand in the extension phase.

Real Time PCR can be used to quantify the amount of a specific cDNA in a sample during the exponential stages of PCR.

In our experiments 2 different methods of Real time PCR were used: Syber® Green I PCR and Taqman® PCR.

5.6.1 Sybr® Green I PCR:

5.6.1.1 Explanation of Sybr® Green I PCR

Sybr® Green I PCR is characterized by using the Sybr® Green I fluorescent dye which binds preferentially double stranded DNA. If the DNA is amplified by PCR the Sybr® Green I dye binds to the DNA and fluorescence light is emitted. The more DNA is produced the stronger is the fluorescence signal and this can be used to quantify the cDNA template in the exponential stages of PCR.

The Sybr® Green I dye also stains unspecific side-products of the amplification (results of unspecific priming, primer dimers) and thus a melting temperature curve is used to prove the specificity of the PCR product after amplification.

5.6.1.2 Procedures of Sybr® Green I PCR

Table 5.6.1.2.1: cDNA mix	
Volume/tube	Substance
1µl-9µl	cDNA sample
8µl-0µl	DEPC water
9µl	**Total**

Table 5.6.1.2.2: Sbr® Green I PCR Mix	
Volume/tube	Substance
1µl	Primers
10µl	**Taqman® Universal PCR Masermix** (Applied Biosystems, Foster City, CA, USA) + 1:50 **Sybr® Green I dye**
11µl	**Total**

11µl of the Sbr® Green I PCR Mix (look at table 5.6.1.2.2) and 9µl of the cDNA mix (look at table 5.6.1.2.1) are pipetted into each well of a 96-well-plate (MicroAmp) which is suitable for Real Time PCR. After pipetting the 96-well-plate has to be covered with an adhesive tape and centrifuged with 1100rpm at room temperature for 5 minutes. The Real time PCR is run in the ABI 7000 thermocycler and analyzed with ABI PRISM 7000 SDS software (Applied Biosystems, Foster City, CA, USA). The Real time PCR is separated in 2 steps: 1repeat at 95°C for 10 minutes and 40 repeats at 95°C for 0:15 minutes and at 60°C for 1 minute.

Additionally, after the Sybr® Green I PCR a melting temperature curve analysis has to be performed in order to test the specificity of the PCR product.

5.6.2 Taqman® PCR

5.6.2.1 Explanation of Taqman® PCR

Taqman® PCR is characterized by the use of specific Taqman® probes which binds within the gene of interest amplicon. Taqman® probes are labelled with a fluorescent dye and a quencher on each side of the probe (look at figure 5.6.2.1.1). If the fluorescent dye is in the vicinity of the quencher no the fluorescence signal can not be detected. The exonuclease activity of the DNA polymerase causes the degradation of the Taqman®

probes which leads to dequenching so that the fluorescence signal can be detected. The more Taqman® probes are degraded the stronger is the fluorescence signal and this fact can be used to quantify the specific cDNA relatively in the exponential stages of PCR.

Taqman® PCR is more specific and more sensitive than Sybr Green I PCR due to the specific Taqman® probes.

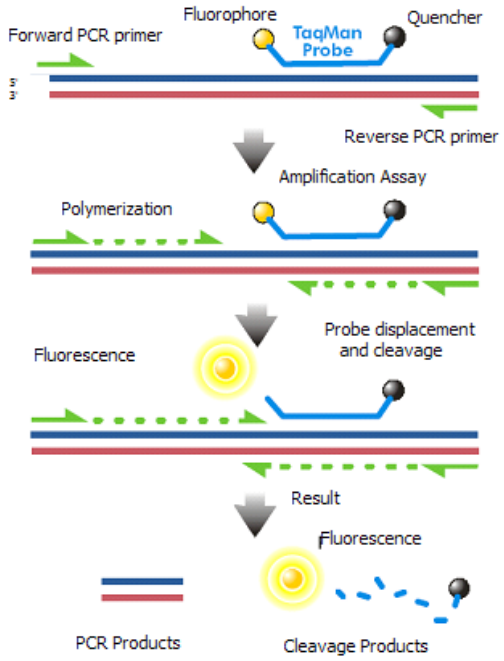


Figure 5.6.2.1.1: Principle of Taqman® probes
http://upload.wikimedia.org/wikipedia/en/0/07/Taqman.png

5.6.2.2 Procedures of Taqman® PCR

Table 5.6.2.2.1: Taqman® PCR Mix

Volum/tube	Substance
1µl	Specific Taqman® probes and primers
10µl	Taqman® Universal PCR Masermix (Applied Biosystems, Foster City, CA, USA)
11µl	**Total**

The same procedures are done as in 5.6.1.2. The Taqman® PCR Mix (look at table 5.6.2.2.1) is used and no melting temperature curve analysis is necessary after PCR run.

5.7 Protein isolation

5.7.1 List of materials:

1xPBS

Acetone

Complete Stock solution: 1 tablet Complete (Roche) dissolved in 2ml aqua bidest

Table 5.7.1.1 Hepes buffer

Amount	Substance
1ml	1M Hepes (PAA
3ml	1M NaCl
2ml	Glycerol
40µl	0.5M EDTA
200µl	1M NaF solution
100µl	1M NaVO4 solution
200µl	Triton X
30µl	1M $MgCl_2$ solution
2 tablets	Complete
13.43ml	Aqua bidest

5.7.1 Procedures of protein isolation:

Pds are washed with cold 1xPBS. Then 100-200µl Hepes buffer are added and the Pds are incubated for a few minutes on ice. The cells are scraped off and homogenized by a syringe and a 0.6-in-diamter needle and transferred in Eppendorf tubes. The samples are kept on ice for about half an hour and they are vortexed three times. Subsequently, the Eppendorf tubes are treated in an ultrasound bath for about 13 minutes. After that they are centrifuged at 15000rpm at 4°C for 5 minutes to remove debris. Then the supernatant is transferred in new Eppendorf tubes and is stored at -20°C.

In order to isolate protein samples for IGFBP3 detection the proteins are precipitated with acetone: The medium is transferred into falcon tubes and complete stock solution is added 20µl complete stock solution is added per 1ml medium. 100µl or 200µl aliquots are transferred into Eppendorf tubes and the tenfold amount of acetone is added. The mixture is

incubated on ice for 30 minutes. After that the vials are centrifuged at 4°C at 15000rpm for 30 minutes. The supernatant is discarded gently and the Eppendorf tubes are dried in the lamina flow. Subsequently, 10µl aqua bidest and 10µl 2x sample buffer (see 5.9.1) are added and the samples are stored at -20°C. Protein measurement is not possible under these conditions.

Alternatively, the 3ml medium are transferred into a 15ml falcon tube and complete stock solution 20µl per 1ml medium is added. After that isopropanol is added at the ratio 2:1 and the sample is incubated at -20°C overnight. Next day the samples are thawed on ice and centrifuged at 3700rpm at 4°C for 45 minutes. The supernatant is discarded. The pellet is dried in the lamina flow and 10µl aqua bidest and 10µl 2xsample buffer is added (see 5.9.1).

5.8 Protein measurement

5.8.1 List of materials:

Coomassie`s Protein Assay Dye Reagent (Bio-Rad Laboratories, Hercules, CA, USA): 1:5 diluted with Aqua bidest, 150µl of the dilution per sample

Hepes buffer

BSA solution (1µg/µl)

5.8.2 Procedures of protein measurement

1µl protein sample is mixed with 9µl Aqua bidest. The protein concentration is determined in duplicates. Protein concentration is calculated from a BSA standard curve determined with each assay. Firstly, all substances are mixed according to table 5.8.2.1 and as the last ingredient 150µl Coomassie`s Protein Assay Dye Reagent dilution is added. The optical density of the samples is measured at 590nm. The standard curve is determined from the absorbance of tubes containing 0-7µg BSA.

Table 5.8.2.1 Pipetting scheme for the BSA standard curve										
BSA conc (1µg/µl)	0	1	2	3	4	5	6	7	8	9
µl Aqua bidest	9	8	7	6	5	4	3	2	1	0
µl Hepes buffer	1	1	1	1	1	1	1	1	1	1
µl BSA (1µg/µl)	0	1	2	3	4	5	6	7	8	9
Total volume in µl	10	10	10	10	10	10	10	10	10	10

5.9 SDS Polyacrylamid Gelelectrophoresis

5.9.1 List of materials:

Table 5.9.1.1: 2x sample buffer	
Amount	Substance
4%	SDS
20%	Glycerol
10%	2-Mercaptoethanol
0.125M ph 6.8	Tris/HCl
traces	Bromphenol blue

Table 5.9.1.2: 10x Electrophoresis buffer (1:10 is used)	
Amount	Substance
72g (14.4%)	Glycin
15g (3%)	Tris
5g (1%)	SDS
500ml	Aqua bidest

10% APS: 0.1g/ml

protein marker (PageRuler™ Prestained Protein Ladder, Fermentas, Burlington, Canada)

40% Acrylamid

10% SDS

TEMED

1.5 Tris ph 8.8

1 Tris ph 6.8

5.9.2 Procedures of SDS Polyacrylamid Electrophoresis

Table 5.9.2.1: Separation gel containing 10% acrylamid (1 gel)	
Volume	Substance
1.25ml	40% Acrylamd
1.25ml	1.5M Tris, ph 8.8
2.45ml	Aqua bidest
0.05ml	10% SDS
0.025ml	10% APS
0.0025ml	TEMED

Table 5.9.2.2 Stacking gel containing 4% acrylamid (1 gel)	
Volume	Substance
0.25ml	40% Acrylamd
0.313ml	1M Tris, ph 6.8
1.9ml	Aqua bidest
25µl	10% SDS
12.5µl	10% APS
2.5µl	TEMED

SDS-PAGE separates the proteins according to their molecular weight. The polyacrylamid gel is composed of two sections: The stacking gel and the separation gel. First of all the separation gel reaction mixture (see table 5.9.2.1) is poured into a sandwich of two glass plates with a spacer of 1mm. In order to have a plane upper edge for the separation gel 70% ethanol are added on the top of the polymerisation mix and removed carefully after the gel has solidified. Subsequently the stacking gel (look at table 5.9.2.2) is poured on top of the separation gel and a comb for the slots is applied. The stacking gel solidifies in about 60 minutes. The comb is removed and the gel is put into the electrophoresis apparatus which is filled with 1x electrophoresis buffer (table 5.9.1.2). 50µg proteins sample is mixed with 2x sample buffer (look at table 5.9.1.1) or 4x sample buffer and the mixture is heated at 70°C for 1 minute. Subsequently, the samples (up to 30µl) and 5µl protein marker (PageRuler™ Prestained Protein Ladder, Fermentas, Burlington, Canada) are loaded into the slots. The gel is run at 60V for 15 minutes and at 125V for 1 hour and 15 minutes.

5.10 Western Blot and immunological detection of proteins

5.10.1 List of materials for Western Blot

Table 5.10.1.1 10x Blotting buffer	
Amount	Substance
72g (14,4%)	Glycin
15g (3%)	Tris
1g (0.2%)	SDS
Filled up to 500ml with	Aqua bidest

Table 5.10.1.2 1x Blotting buffer	
Amount	Substance
50ml (10Vol%)	10x Blotting buffer
100ml (20Vol%)	Methanol
350ml (70Vol%)	Aqua bidest

Methanol

Ponceau S staining solution (Sigma, St. Louis, MO, USA)

Wash buffer: 1x PBS + 0.1% Tween 20 (Sigma Ultra)

5.10.2 Procedures of Western Blot

The goal of Western Blot is to transfer the separated proteins from the polyacrylamid gel onto a BioTrace™ PVDF membrane (Pall Corporation, East Hills, NY, USA). During the transfer the proteins move towards the positive pole.

The Western Blot Sandwich is built as shown in figure 5.10.2.1: pointing toward the negative pole of the blotting chamber a sponge and 3 filter papers which are soaked with 1x blotting buffer (look at table 5.10.1.2) are positioned. The gel is put on top and is then covered with the BioTrace PVDF membrane which is activated by methanol. The blotting sandwich is completed with 3 more filter papers and another sponge. The Western Blot is transferred in 1x blotting buffer and it is run at 25V overnight in the cold. Next day the Western Blot Sandwich is disassembled and the membrane is washed in aqua bidest. After that the membrane is stained

with Ponceau S staining solution in order to assess the success of the blotting. Subsequently, the membrane is de-stained with wash buffer and it can be dried after treatment with methanol.

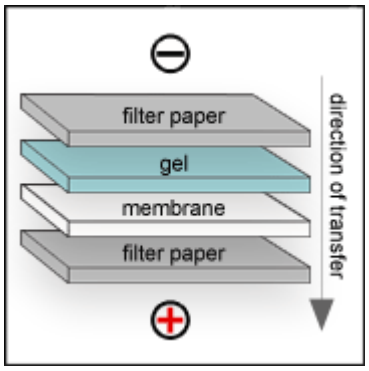


Figure 5.10.2.1: Western Blot Sandwich
http://www.bme.gatech.edu/vcl/WesternBlotting/Background/Assets/nitromembraneSandwich.gif

5.11 Immunological detection of proteins

5.11.1 List of materials for immunological detection of proteins

Wash buffer: 1x PBS + 0.1% Tween 20 (Sigma Ultra)

Blocking solution: 20ml Wash buffer + 1% Skim milk powder (0.2g)

Table 5.10.3.1: List of primary antibodies

Target	Specificity	Dilution	Company	Protein size
Survivin	rabbit	1:1000	abcam	16.5 kDA
IGFBP3	rabbit	1:1000	Sigma	40 kDA
β-Actin	mouse	1:1000	Sigma	42 kDa

Table 5.10.3.2: List of secondary antibodies

Target	Specificity	Dilution	Company	Label
Rabbit IgG	goat	1:20000	Bethyl	HRP
Mouse IgG	goat	1:10000	PIERCE	HRP

5.11.2 Procedures for immunological detection of proteins

The dried BioTrace PVDF membrane is reactivated with methanol. After that the membrane is blocked with blocking solution for about half an hour. Subsequently, the membrane is incubated with the primary antibody solution on a shaker at 4°C overnight. The primary antibody binds specifically to the protein of interest. Next day the membrane is washed with wash buffer 3 times for 10 minutes. After that the membrane is

incubated with the secondary antibody solution for about 1 hour. The secondary antibody binds specifically the primary antibody and is labelled with a horseradish peroxidise (HRP). Subsequently the membrane is washed with wash buffer 3 times for 10 minutes and the secondary antibody is detected with a detection reagent (Amersham™ ECL Plus or Prime Western Blotting Detection System, GE Healthcare). The membrane is incubated with the detection reagent for 5 minutes. HRP produces luminol from the detection reagent and causes chemiluminescence which can be detected by x-ray film. The strength of the resulting bands is proportional to the amount of protein on the membrane. β-Actin is used for the standardization of the measured proteins.

5.11.3 Quantitative Analysis with ImageQuant 5.0

The X-ray films are scanned and the bands are analyzed by ImageQuant5.0.

5.12 Plasmid Amplification

5.12.1 Production of competent TOP10 E.coli

5.12.1.1 List of materials for SEM competent TOP10 E.coli

Table 5.12.1.1.1 300 ml 10x TB-buffer (autoclaved)

Amount	Substance
100mM (3.0237g/100ml)	Pipes, Sigma P1851
150mM (2.2053g/100ml)	$CaCl_2*2H_2O$
2.5M (18.6375g/100ml)	KCl
Adjusted to ph 6.7	With KOH

550mM $MnCl_2$-solution: (5.44 $MnCl_2$-tetrahydrat/50ml), sterile filtrated, stored at 4°C in the dark

1x SEM TB-buffer: 320ml autoclaved aqua bidest + 40ml 10x SEM TB-buffer + 40ml 550mM $MnCl_2$; stored in 30ml portions at -20°C

5.12.1.2 Production of SEM competent TOP10 E.coli

1 colony of a streak is incubated in 7ml LB medium at 200rpm at 37°C for 5 hours on the shaker. Subsequently 10-50µl of the culture are pipetted in 2x200ml LB in large flasks and they are incubated at 200rpm at room temperature overnight till the bacteria density is at OD600= 0.3-0.6. After that the cultures are stored on ice. Then the TOP10 solution is filled into 8x 50ml falcon tubes and incubated on ice for 10 minutes. Then they are centrifuged at 2500g at 4°C for 15 minutes and the supernatant is discarded. The 8 pellets are resuspended carefully in 40ml cold 1x SEM TB-buffer and then transferred into 2 falcon tubes that are then vortexed at 1000rpm 3 times for 15 seconds and kept on ice between. Subsequently, they are incubated on ice for 10 minutes. After that they are centrifuged at 2500g at 4°C for 10 minutes and the supernatant is discarded. Both pellets are diluted in 10ml 1x SEM TB-buffer with the same procedure above and then they are transferred in 1 falcon tube. DMSO is added and mixed till an end concentration of 7Vol% (0.7ml/10ml). The falcon tube is incubated on ice for 10 minutes. The competent TOP10 solution is filled in 700µl portions in 1.5 Eppendorf tubes and they are frozen in liquid N_2 and then they are stored at -80°C.

5.12.1.3 List of materials for Chinese Hyper-Competent Cells (Tu et al. 2005)

Chinese SOB medium (liquid): per liter

20g Tryptone (bacto), 5g yeast extract, 10mM NaCl (584mg);

Autoclaving

Chinese SOC medium:

Cool Chinese SOB medium

Addition of:

2.5 mM $MgCl_2$ (2.5ml of 1M stock 9.5g/100ml),10mM $MgSO_4$ (10ml of 1M $MgSO_4$ stock solution 12,04g/100ml), 20mM glucose (10ml of 2M glucose stock solution 36g/100ml);

Solid SOC antibiotics plates:

15g Select Agar (Sigma) per 1l Chinese SOB medium; addition: as the production of Chinese SOC medium + antibiotics

Chinese 10* TB buffer:

equal to the normal SEM TB buffer with the adaption: 750 mM $CaCl_2$*$2H_2O$ (11,0265g/100ml)

5.12.1.4 Production of Chinese Hyper-competent cells

10µl or 100µl TOP10 glycerol stock is thawed at room temperature. 40 ml liquid Chinese SOC medium is added. The TOP10 culture is incubated at 37°C for 1 hour. Subsequently, the TOP10 culture is shaken at 200rpm at 37°C for 2-3 hours till OD600 of 0.2-0.4. Then the cells are centrifuged at 8000rpm at 4°C for 2 minutes and the supernatant is discarded (centrifuge: Sorvall RC-5B; rotor: F215-8x50). The cells are resuspended in 20ml autoclaved, cold 1xTB (including 75mM CaCl2) and incubated on ice for 25 minutes. Subsequently, the solution is centrifuged at 8000rpm at 4°C for 2 minutes and the supernatant is discarded. 4ml autoclaved, cold 1xTB is mixed with absolute glycerol to a final 20 vol% and the pellet is resuspended with that and stored at -80°C.

5.12.2 Transformation, plating and inocoluation

5.12.2.1 List of materials

Liquid LB: 20g **LB Broth** (Sigma L3022-1KG) in 1l aqua bidest, autoclaved, 100µg/ml ampicillin

LB/agar plates: 15g **Select Agar** (Sigma A5054-250G) in 1L LB, autoclaved, 100µg/ml ampicillin; plates are poured over 60°C;

Table 5.12.2.1.1 SEM SOB-medium (per liter)	
Amount	Substance
20g	Trypton
5g	Yeast extract
0.5g	NaCl
10ml	250mM KCl (1.86g KCl/100ml H_2O)
Adjusted to ph 7	With NaOH

Table 5.12.2.1.2 SEM SOC-medium	
Amount	Substance
Variable	SOB medium
1/100 Vol	Autoclaved 1M $MgCl_2$-solution (9.5g Mg/100ml)
1/100 Vol	Autoclaved 2M glucose-solution (36g/100ml)

For Chinese SOC medium and Chinese SOC antibiotics plates look at 5.12.2.1.

5.12.2.2 Procedures of transformation, platting and inoculation

SEM competent TOP10 E.coli:

SEM competent TOP10 E.coli are thawed on ice. 1µl plasmid is transferred in 1.5ml Eppendorf tubes and kept on ice. 200µl competent TOP10 cells are added and mixed gently. They are incubated on ice for 20 minutes. Subsequently, the samples are heat shocked in a water bath at 42°C for 35 seconds. 800µl SOC-medium is immediately added after the water bath and the samples are inverted twice. Then they are transferred into 14ml tubes and shaken at 200rpm at 37°C for 1 hour. 1/50 of the samples are plated on LB/agar plates with antibiotics. The remaining of sample solution is transferred in 1.5ml Eppendorf tubes and centrifuged at 1000g at room temperature for 3min. Subsequently, most of the supernatant is discarded. This step is used to decrease the volume. The pellet is diluted with the rest of the medium and plated on LB/agar plates with antibiotics. After that the plates are incubated upside down at 37°C overnight. Next day colonies are picked with autoclaved toothpicks and are transferred in 14ml tubes which are filled with 6ml liquid LB with

antibiotics and then they are shaken at 200 rpm at 37°C overnight. Next day the DNA is isolated by miniprep. Aliquots of the bacteria culture can be stored at -80°C if it is 1:1 mixed with 40% glycerol.

Commercial competent TOP10 E.coli:

1µl plasmid is transferred in 1.5ml Eppendorf tubes and kept on ice. 20µl competent TOP10 E.coli cells (invitrogen) are added and mixed gently. They are incubated on ice for 30 minutes. Subsequently, the samples are heat shocked in a water bath at 42°C for 30 seconds and immediately put on ice thereafter. 250µl SOC-medium is added and mixed with the sample. Then the 1.5ml Eppendorf tubes are incubated on the shaker at 900 rpm at 37°C for 1 hour. 1/50 of the samples are plated on LB/agar plates with antibiotics. The rest of the samples are plated on another plate. After that the plates are incubated upside down at 37°C overnight. The next steps are equal with the SEM competent TOP10 E.coli.

Chinese Hyper-competent TOP10 E.coli:

Solid SOC antibiotics plates are pre-heated at 37°C for 1 hour. 100µl Chinese hyper-competent cells are mixed with 1ng plasmid DNA and 1µl DMSO. Transformation mix is incubated on ice for 30 minutes. Subsequently, the transformation mix is heat-shocked at 42°C for 90 seconds and then it is incubated on ice for 2 minutes. 400µl liquid Chinese SOC is added and the sample is shaken at 200rpm at 37°C for 45 minutes. The sample is spread on the pre-heated plates and they are incubated at 37°C overnight. The next steps are equal with the SEM competent TOP10 E.coli.

5.12.3 Miniprep

Miniprep with column:

Wizard® Plus SV Minipreps DNA Purification System (Promega) is used for isolation of the plasmid from the TOP10 bacteria which have grown

overnight. 1ml of bacterial culture is transferred into 1.5 ml Eppendorf tubes. They are centrifuged at 10000g for 2 minutes and the supernatant is discarded. This procedure is repeated three times. The final pellet is resuspended completely in 250ml Cell Resuspension Solution. Then 250µl Cell Lysis Solution is added, mixed by inverting and incubated 3 minutes. Subsequently, 10µl Alkaline Protease Solution is added and incubated for 5 minutes. Then 350µl Neutralization Solution is added and mixed completely by invertion. The samples are centrifuged at 14000g for 10 minutes. The Spin Column is inserted into the Collection Tube and the supernatant is transferred. Subsequently, it is centrifuged at 14000g for 1 minute. The flowthrough is discarded, 750µl Column Wash Solution is added to the Spin Column and the samples are centrifuged at 14000g for 1 minute. Again the flowthrough is discarded, 250µl Column Wash Solution is added and it is centrifuged at 14000g for 1 minute. Then the flowthrough is discarded and it is centrifuged at 14000g for 2 minutes. The Spin Column is transferred to a new 1.5ml Eppendorf tube, 30µl Nuclease-Free Water is added and incubated for 2 minutes. After that it is centrifuged at 14000g for 1 minute and the Spin Column is discarded. This can also be repeated in order to gain more DNA. The samples are stored at -20°C. The yield is between 100 and 300ng/µl.

STET boiling plasmid miniprep (according to Stratagene):

List of materials:

STET-buffer: 8% Saccharose, 0.5% Triton X-100, 50mM Tris pH8, 50mM EDTA, filled up to 1L with aqua bidest, autoclaved, stored at 4°C;

Lysozyme stock: 5mg/ml in STET buffer, stored at -20°C

STETL buffer: 100µl STET buffer + 10µl lysozyme stock per sample

RNase A: 100mg/ml stock (in 10mM Tris-HCl pH 7.5, 15mM NaCl, heated to 100°C and cooled to room temperature, stored at -20°C

Isopropanol

80% EtOH

Procedures:

1.5ml cell suspension is filled into in 1.5ml Eppendorf tubes and centrifuged at 10000g for 3 minutes. The supernatant is discarded. If 3ml cell suspensions are used the previous step has to be repeated but this is only recommended for bacteria suspensisons of low density. 110µl STETL buffer is added and the pellets are resuspended . The samples are heated to 95°C for 1 minute. After that they are centrifuged at 10000g at 4°C for 10 minutes. The subsequent pellet is removed with a toothpick which has been dipped in RNase A solution. 110µl isopropanol is added and mixed with the sample. Subsequently, the samples are centrifuged at 10000 g at 4°C for 15 minutes. The supernatant is discarded. 1ml 80% EtOH is added and the pellet is resuspended. Then the samples are centrifuged at 10000g at 4°C for 5 minutes. The supernatant is discarded completely and the pellet is dried on the heat block at 37°C for 2 minutes. The dried pellet is resuspended in 50µl 1xTE and is stored at -20°C.

5.12.4 Restriction digest and agarose gel electrophoresis

The plasmid is digested by restriction enzymes and the resulting fragments are analyzed with 1-1.6% agarose gel. The example in table 5.12.4.1-3 is calculated for a digest with 2 restriction enzymes. 3 digests and 1 control are prepared and incubated at 37°C for 1 hour. The substances are mixed and preheated at 37°C before the enzymes are added.

Table 5.12.4.1 plasmid digest with 2 enzymes	
Amount	Substance
1µl	Plasmid sample (app. 200ng)
1µl	10x Buffer X (Roche or Fermentas)
7µl	Aqua bidest
0.5µl	Restriction enzyme 1
0.5µl	Restriction enzyme 2
10µl	Total

Table 5.12.4.2 plasmid digest with 1 enzyme	
Amount	Substance
1µl	Plasmid sample (app. 200ng)
1µl	10x Buffer X (Roche or Fermentas)
7.5µl	Aqua bidest
0.5µl	Restriction enzyme 1 or restriction enzyme 2
10µl	Total

Table 5.12.4.3 control of plasmid digest	
Amount	Substance
1µl	Plasmid sample (app. 200ng)
9µl	Aqua bidest
10µl	Total

SeaKem® LE Agarose (Biozym) is used and dissolved in 1x TAE (table 5.12.4.4 for 50x TAE) is heated and poured to form a 1% agarose gel. The agarose gel solidifies in 20 minutes.

1x TAE is filled into the electrophoresis apparatus and the gel is submerged. The 10µl restriction digest are mixed with 2µl 6xDNA Loading Dye (Fermentas R0611) and loaded. 2µl Lambda 3 marker (see figure 5.12.4.1 and table 5.12.4.5) and 2µl Mass Ruler™ DNA Ladder High Range (Fermentas SM0393, see figure 5.12.4.2) are used. The gel is run at 90V for 1 hour. The gel is dyed with ethidium solution for 10 minutes and after that photos are taken in UV light.

Table 5.12.4.4 50x TAE (autoclaved)	
Amount	Substance
242g	Tris base
57.1ml	Acetic acid
100ml	0.5M EDTA; ph 8
Filled up to 1L	with aqua bidest

Table 5.12.4.5: Lamba 3 marker	
Amount	Substance
200µl	Lambda-DNA (c=0.25µg/µl)
42µl	Buffer B
4µl	HindIII
4µl	EcoRI
167µl	Aqua bidest
417µl	Total

Digested at 37°C; Then 83µl 6x Loading buffer is added; stored at 4°C;

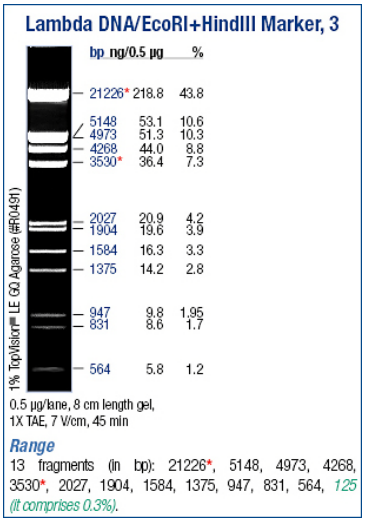


Figure 5.12.4.1: Lambda 3 marker

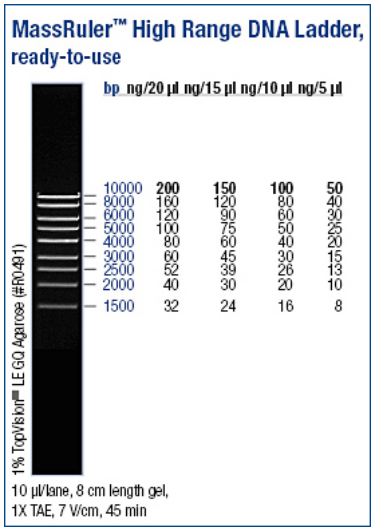


Figure 5.12.4.2: Mass Ruler™ DNA Ladder High Range

5.12.5 Midiprep

In this diploma thesis the PureYield™ Plasmid Midiprep System From Promega is used for midiprep. 200 ml LB medium is poured into a flask and 200µl ampicillin (1:1000 dilution, 100mg/ml) is added. Transformed TOP10 bacteria which are stored at -80°C are scraped off with a pipet tip

from the ice surface. The pipet tip is thrown into the flask and it is incubated at 200rpm at 37°C overnight. Next day the culture is centrifuged at 5000g for 10 minutes and the supernatant is discarded. The pellet is resuspended in 6ml Cell Resuspension Solution. Subsequently, 6ml Cell Lysis Solution is added, mixed properly and incubated for 3min. After that Neutralization Solution is added and incubated for 3 minutes. Then the cell lysate is centrifuged at 15000g for 10 minutes. The Clearing Column is placed onto the Binding Column. The cleared lysate is filled without pellet debris into the Clearing Column, vacuum is applied and it passes through the column combination. Thereby, the plasmid binds on the binding membrane of the Binding Column. After that the Clearing Column is discarded. 5ml Endotoxin Removal Wash is added and pulled through with vacuum. Subsequently, it shall be washed with 20ml Wash Solution. Then the Binding Column tip is cleaned and a 1.5 Eppendorf tube is placed into the Eluator™ Vacuum Elution Device. The Binding Column is inserted into the device. 600µl Nuclease-Free Water is added and vacuum is applied in order to fill the 1.5 Eppendorf tube. This step can be repeated with 400µl Nuclease-Free Water.

5.13 Transfection of HT29 cells

$1x10^6$ HT29 cells are plated on 6-well-plates. Cells are ready for transfection when they grow adherent. 2µg or 4µg plasmid is diluted in 250µl serum free medium (450ml MEM, 2ml Penstrep). 10µl **TransFectin™ Lipid reagent** (Bio-Rad) is diluted in 240µl serum free medium. Finally, the plasmid mix and TransFectin mastermix are combined and incubated at room temperature for 20 minutes. Subsequently, the resulting solution is added to the cells in fresh HT29 cultivation medium **(**see material and methods 5.1.2.1). After 6 hours the medium is changed to HT29 cultivation medium. The expression can be detected within the next 3 days.

5.14 GFP-FACS analysis

For 6-well-plates: The HT29 cells are detached with 100µl Trypsin. After that 3ml HT29 cultivation medium **(**see material and methods 5.1.2.1) are added and transferred into a 15ml falcon tube which is centrifuged at 1100rpm for 5 minutes. The supernatant is removed; the pellet is diluted in 0.5ml 1xPBS and transferred into FACS tubes. After that the samples are analyzed with the FACS machine.

5.15 TOPO® Cloning

5.15.1 Restriction digest with large amount of DNA

Table 5.15.1.1 Restriction digest with large amount of DNA

Amount	Substance
5µg	Plasmid sample (in our samples 25 or 35µl)
5µl	10x Buffer X (Roche or Fermentas)
14µl or 4µl	Aqua bidest (example for our samples)
0.5µl	Restriction enzyme 1
0.5µl	Restriction enzyme 2
50µl	Total

Subsquently, the restriction digest with large amount of DNA is tested with agarose Gel. The sample is prepared: 2µl of the digest is mixed with 8µl aqua bidest and 6xDNA Loading Dye (Fermentas R0611). The remaining digest is stored at -20°C.

5.15.2 Phosphatase treatment

Without gel extraction:

Calf intestinal alkaline phosphatase (CIAP, Fermentas) is used for the phosphatase treatment of 5´overhangs. CIAP stock has 1u/µl. CIAP is diluted with CIAP 1x Reaction Buffer (Fermentas) to a final concentration of 0.01u/µl. Each picomole DNA ends needs 0.01u CIAP. The phosphatase treatment master mix of our experiments is in table 5.15.2.1.

Table 5.15.2.1 Phosphatase treatment master mix	
Amount	Substance
90µl	Nuclease free water
10µl	10x CIAP Buffer
1µl	CIAP stock
101µl	Total

5µl phosphatase treatment master mix is combined with 40µl sample DNA and 5µl 10x Reaction Buffer and then incubated at 37°C for 30 minutes. Subsequently, 5µl phosphatase treatment master mix is added and incubated again at 37°C for 30 minutes. After that the enzyme is inactivated by heat at 85°C for 15 minutes.

With gel extraction:

CIAP stock (1u/µl, Fermentas) is diluted in this manner that 1µl-2µl CIAP dilution is used for the phophatase reaction mix (see table 5.15.2.2). Thereby, 2x0.02u CIAP is used per picomole DNA ends. They are incubated at 37°C for 15 minutes. Subsequently, the samples are incubated at 56°C for 15 minutes. 1µl-2µl of CIAP dilution is added and then the samples are incubated at 37°C for 15 minutes. After that they are incubated at 56°C for 15 minutes. Finally, the samples are heat-shocked at 85°C for 15 minutes and then stored at -20°C.

Table 5.15.2.2 Phosphatase reaction mix	
Amount	Substance
8µl	Sample DNA
1µl	10x Reaction Buffer
1µl-2µl	CIAP dilution
10-11µl	Total

5.15.3 Re-ligation check (sticky-end ligation)

For ligation, T4 Ligase (5u/µl, Fermentas) was used. A re-ligation check master mix is made (see table 5.15.3.1).

Table 5.15.3.1 Re-ligation check master mix (for 6 samples)

Amount	Substance
52.6µl	Nuclease free water
6µl	10x T4 DNA Ligase Buffer
1.4µl	T4 DNA Ligase (1u per sample)
60µl	Total

9µl re-ligation check master mix is added to 1µl sample DNA (approx. 100ng) and is incubated at room temperature (22°C) for 10 minutes. Samples treated with phosphatase and samples without phosphatise treatment are ligated in parallel for comparison. Subsequently, the enzyme is inactivated by heat at 70°C for 5 minutes. The re-ligation check samples are transformed into TOP10 bacteria and analyzed according to 5.12. The phosphatase untreated samples expected to cause colonies but phosphatase treated samples shall not be able to re-ligate.

5.15.4 Recessed 3´end filling with TaqPol and dATPs (blunt ends production)

The ends of the constructs which are treated successfully with phosphatase (see 5.15.2) are converted into blunt ends. The blunt ends mix is prepared on ice (see table 5.15.4.1). Subsequently, the mix is incubated at 72°C for 15 minutes.

Table 5.15.4.1 Blunt ends mix

Amount	Substance
5µl	Phosphatase treated sample
0.7µl	10x PCR Reaction Buffer (Roche)
0.5µl	100mM dATP Solution (Invitrogen)
1µl (0.5u)	Taq DNA Polymerase (Roche)
7.2µl	Total

5.15.5 pENTR/D-TOPO® reaction

pENTR Directional TOPO® Cloning Kit and pENTR/D-TOPO® are used for TOPO® cloning. See the pENTR/D-TOPO® reaction mix in table 5.15.5.1 and is incubated at room temperature for 30 minutes. After that the samples are put on ice and are used for transformation and subsequent analysis according to 5.12. The pENTR/D-TOPO® vector has a kanamycin resistance. The optimal molar insert: vector ratio is 5:1.

Table 5.15.5.1 pENTR/D-TOPO® reaction mix

Amount	Substance
2µl	Phosphatase treated and blunt ends processed sample
0.5µl	SALT solution
0.5µl	pENTR/D-TOPO®
3µl	Total

5.15.6 Gel excision and Gel extraction (Gel elution)

Gel extraction is performed before the procedures of 5.15.2. The restriction digest of 5.15.1 + 6xDNA Loading Dye (Fermentas R0611) is loaded on a 1% agarose gel with large slots. The gel is run at 80V for 2 hours. After that the bands of interest are cut out of the gel with a scalpel under UV light and transferred into new Eppendorf tubes. The QIAEX® II gel extraction kit (QIAGEN) is used for gel extraction. 3 volumes of buffer QX1 is added to 1 volume of the gel slice (1mg gel = 1µl volume). 10µl resuspended QIAEX II (≤ 2µg DNA) is added and the samples are incubated at 50°C for 10 minutes. During that time the samples are vortexed every 2 minutes. The samples are supposed to stay yellow. After that the samples are centrifuged at 10000g for 30 seconds and the supernatant is discarded. 500µl QX1 is added and the pellet is resuspended by vortexing. The samples are centrifuged at 10000g for 30 seconds and the supernatant is removed. 500µl buffer PE is added and the pellet is resuspended by vortexing. The samples are centrifuged at 10000g for 30 seconds and all supernatant is removed again. The pellet is air-dried for 15 minutes till it becomes white. The DNA is eluted by adding 20µl

H_2O and the pellet is resuspended by vortexing. The samples are incubated at room temperature for 5 minutes (DNA ≤ 4kb). Subsequently, the samples are centrifuged at 10000g for 30 seconds. The pellet is resuspended again by vortexing and is incubated 5 minutes. After that the samples are centrifuged at 10000g for 30 seconds again. The supernatant is transferred into new Eppendorf tubes and is stored at -80°C.

5.16 pENTR-1A cloning:

5.16.1 BamHI and XhoI digests

pENTR-1A vector and the concerning pAAV-IRES-hrGFP- Tcf4wt, -ΔN-Tcf4 or -VP16-ΔN-Tcf4 vectors are digested with BamHI and XhoI in separate reactions (see table 5.16.1.1) in the adapted sophisticated attempt. The quick method digests both constructs in one sample.

Table 5.16.1.1 Digest of pENTR-1A or pAAV-IRES-hrGFP-X

Amount	Substance
Xµl	pENTR-1A or pAAV-IRES-hrGFP-X (5µg)
5µl	10x buffer B
Xµl	Aqua bidest
3µl	BamHI
3µl	XhoI
50µl	Total

5.16.2 T4 ligation

Before ligation dephosphorylation could be done according to 5.15.2 phosphatase treatment with gel extraction. For ligation, T4 DNA Ligase (stock concentration: 5u/µl, Fermentas) was used. The “quick method” of pENTR-1A cloning uses the ligation mix in table 5.16.2.1. The sophistacted method of of pENTR-1A cloning uses the ligation master mix in table 5.16.2.2. 5µl of the ligation master mix is mixed with 5µl concerning DNA and nuclease free water.

Table 5.16.2.1 quick ligation mix	
Amount	Substance
5µl	Digested vector and insert
1µl	10x T4 DNA Ligase Buffer
1µl	T4 DNA Ligase (1:10 dilution of stock, 0.5u)
3µl	Nuclease free water
10µl	Total

Table 5.16.2.2 ligation master mix (for 4 samples)	
Amount	Substance
4µl	10x T4 DNA Ligase Buffer
0.8µl	T4 DNA Ligase stock
15,2µl	Nuclease free water
20µl	Total

The successful ligation mix is in table 5.16.2.3.

Table 5.16.2.3 successful ligation mix	
Amount	Substance
Xµl	Vector
Xµl	Insert
1µl	10x T4 DNA Ligase Buffer
1µl	T4 DNA Ligase stock
Xµl	Nuclease free water
10µl	Total

The ligation mix is incubated at room temperature for 10-30 minutes (longer period of time causes higher transformation efficiency). Subsequently, the sample is heat-shocked at 70°C for 5 minutes.

5.16.3 Transformation, platting, inoculation, miniprep and analysis

Transformation, platting, inoculation, miniprep and analysis are according to 5.12.

5.17 LR reaction

The LR reaction is done with Gateway® LR Clonase™ II Enzyme Mix (Invitrogen). At first entryclone, destination vector and 1xTE buffer are mixed in LR reaction mix according to table 5.17.1 or 5.17.2. LR Clonase™ II enzyme mix is thawed on ice for about 2 minutes, vortexed 2 seconds twice and added in the LR reaction mix according to table 5.17.1 or 5.17.2. Then the samples are mixed twice and microcentrifuged briefly. The LR reaction mix is incubated at 25°C for 1 hour. Subsequently, 1µl Proteinase K solution is added (full LR reaction mix) in order to terminate the reaction and the mix is incubated at 37°C for 10 minutes. After that the LR reaction is completed and can be transformed according to 5.12.2.

Table 5.17.1 full LR reaction mix

Amount	Substance
Xµl	Entry vector (150ng)
1µl	Destination vector (150ng/µl)
Xµl	1xTE buffer (pH8.0)
2µl	LR Clonase™ II enzyme mix
10µl	Total

Table 5.17.2 half LR reaction mix

Amount	Substance
Xµl	Entry vector (150ng)
1µl	Destination vector (150ng/µl)
Xµl	1xTE buffer (pH8.0)
1µl	LR Clonase™ II enzyme mix
5µl	Total

5.18 Adenoviral production in HEK293

5.18.1 PacI digest of circular adenoviral constructs

10µg of circular pAd/CMV/V5-DEST-insert vector is digested with 1.5µl PacI (New England Biolabs Inc., #R0547S). The 10x buffer NEBuffer 1 (New England Biolabs Inc., #B70015) is used. The PacI digest mix is in table 5.18.1.1. Additionally, small DNA samples are made which do not have restriction enzyme. The digest is incubated at 37°C at 300rpm for 2 hours or overnight. Subsequently, the digest is heat inactivated at 65°C for 20 minutes. 10µl of the digest are stored for a gel check.

Table 5.18.1.1 PacI digest mix

Amount	Substance
Xµl	Circular pAd/CMV/V5-DEST-insert vector (10µg)
40µl	10xNEBuffer1
4µl	100xBSA (New England Biolabs Inc.)
1.5µl	PacI
Xµl	Nuclease free water
400µl	Total

5.18.2 Precipitation of linearized adenoviral constructs

The digest of 5.18.1 is precipitated with sodium acetate and ethanol. The centrifuge is pre-cooled. 1/10 Vol (40µl) 3M Sodium acetate solution (pH 5.2) and 2.5 Vol (1ml) iced cool ethanol are added and the sample is vortexed. Then the sample is incubated at -80°C overnight. Subsequently, the sample is centrifuged at 14000rpm at 0°C for 15 minutes. After that the supernatant is discarded. Then 800µl iced cool 70% ethanol is added and the sample is centrifuged at 14000rpm at 0°C for 5 minutes. The supernatant is discarded and the pellet is dried. Subsequently, the dried pellet is diluted in 20µl 1xTE. 1µl of the sample is filled up to 10µl with aqua bidest and this sample is checked together with the control sample of 5.18.1 by electrophoresis. The amount of DNA is estimated in comparison of the λ Marker bands.

5.18.3 Transfection of linearized adenoviral constructs

The transfection takes place in 6-well-plates. $1x10^5$ HEK293 cells are plated on wells of 6-well-plates with MEM or DMEM and at 5% or 7.5% CO2. DMEM and 7.5%CO2 improve the growth of the HEK293 cells. Cells are ready for transfection when they grow adherent and are at least 50% confluent. 1-2µg plasmid is diluted in 250µl with serum free medium (450ml MEM, 2ml Penstrep). 10µl **TransFectin™ Lipid reagent** (Bio-Rad) is added to 240µl serum free medium. Plasmid mastermix and TransFectin mastermix are poured together and incubated at room temperature for 20 minutes. Subsequently, the resulting solution is added to the cells in fresh HT29 cultivation medium **(**see material and methods 5.1.2.1). After 6 hours the medium is changed by HT29 cultivation medium. The cells are cracked according to 5.18.4 if a cytopathic effect can be detected.

5.18.4 Cracking of HEK293 cells

The attached HEK293 cells are rinsed off the plate with medium and are transferred in a falcon tube. It is centrifuged at 1100rpm at room temperature for 5 minutes. All supernatant is discarded. The pellet is resuspended in PBS and centrifuged as above. After that the supernatant is discarded and the pellet is resuspended in new 1ml PBS. Before isolation with caesium chloride gradient and viral production in 15cm Pds 10mM TRIS-Cl, pH=8 is used.

Subsequently, the cells are cracked. These steps are repeated 4 times:

The sample is frozen in liquid N_2 and then it is thawed completely in 37°C water bath. Subsequently, it is vortexed strongly.

After cracking the sample is stored at -80°C.

5.18.5 Reinfection with cracked HEK293

Cracked HEK293-virus samples are added to attached, fresh HEK293 cells. When a cytopathic effect is observed the cells are cracked according to 5.18.4. First of all, HEK293 cells are reinfected in 6-well plates, then in 10cm Pds and at last in 15cm Pds. The fresh cells are splitted one day after reinfection. Thereby, $8x10^6$ - 10^7 HEK293 cells are prepared in 15cm plates. The final virus production takes place in 15x15cm Pds.

5.18.6 Control PCR for pAd/CMV/V5-DEST-insert constructs

2µl cracked HEK293-virus sample is diluted in 198µl 1xTE (1:100 dilution) and heat inactivated at 95°C for 15 minutes. pAd/CMV/V5-DEST-empty and pAd/CMV/V5-DEST-insert are diluted 1:10000 in 1xTE. Water is used as non-template-control. The control PCR reaction mix is listed in table 5.18.6.1. The Taq polymerase synthesizes about 1000 bps per minute. The program of the control PCR for pAd/CMV/V5-DEST-insert is in table 5.18.6.2. The PCR starts directly at 95°C (hot start). The primer sequences are in table 5.18.6.3.

Table 5.18.6.1: Control PCR reaction mix

Volume	Substance
12.5µl	2x Go Taq® Colorless Master Mix (Promega)
1µl	T7 primer and V5 primer mix
1µl	Sample DNA (diluted)
10.5µl	Nuclease free water
25µl	**Total**

Table 5.18.6.2: Program of the control PCR

Step number	Number of cycles	Description
1	0	1 second at 95°C, hold at 95°C PCR samples are inserted then
2	1	2 minutes at 95°C
3	35	Denaturation: 30 seconds at 95°C Annealing: 1 minute at 55°C Synthesis: 4 minutes at 72°C
4	1	7 minutes at 72°C Holding temperature at 5°C

Table 5.18.6.3: Primer sequences	
Name	Sequence
T7 primer	TAATACGACTCACTATAGGGAGAC
V5 primer	ACCGAGGAGAGGGTTAGGGAT

5.18.7 Isolation of viral particles with caesium chloride gradient

The final 15x15cm HEK293 Pds which are infected with virus are harvested and cracked in 6-8ml 10mM Tris-Cl, ph=8.

4g caesium chloride is weighed out in a 50ml falcon tube. Subsequently, the virus lysate is added and mixed well with caesium chloride. Then the sample is filled into an ultracentrifuge tube. A counterweight solution of 4g caesium chloride and 10mM Tris-Cl, ph=8 is prepared in another ultracentrifuge tube and it is tared with the sample tube. The virus sample solution is covered with 4 drops petroleum. Subsequently, the ultracentrifuge tubes are centrifuged at 32000rpm at 10°C under vacuum for 18 hours. The brake is not used in order to let the run terminate gradually. In this diploma thesis the Beckman Optima™ LE-80k ultracentrifuge is used.

After that the plastic tubes are pulled out of the ultracentrifuge tubes with tweezers and are fixed on a rack in the lamina flow. A beaker containing a small amount of proline desinfectant is put under the rack. An adhesive strip is stuck on the side of the plastic tube. The virus band is sucked off with a 2ml syringe and 18G needle. For this purpose the needle is pierced carefully with the needle opening downward through the plastic tube at the adhesive strip. The virus solution is filled in falcon tubes, mixed with an equal amount of 2x Storage Buffer (10mM Tris, pH=8.0; 100mM NaCl; 0.1% BSA; 50% glycerol) and stored at -80°C.

5.18.8 Virus Titration

$2.5x10^5$ HEK293 cells per well are plated on 24-well-plates. The concerning viral band aliquots are diluted 1:10000, 1:100000, 1:1000000 and 50µl of these dilutions are added in duplicates in the wells. The

Adeno-X™ Rapid Titer Kit (Clontech) is used for detection. 1:10000 and 1:100000 dilution of the LacZ virus of the kit and the 1:1000000 dilution of a GFP virus of our lab are used as positive controls. The negative control is 50µl 1xPBS. The 24-well-plates are incubated at 37°C for 48 hours. The medium is discarded and the plates are dried with opened cover in the lamina flow for 5 minutes. Subsequently, 1ml ice-cold methanol is added and the plates are incubated at -20°C for 10 minutes. Methanol is discarded and the wells are washed with 1ml 1xPBS (1% BSA). 200µl 1:1000 dilution of the Mouse Anti Hexon Antibody in 1xPBS/BSA is added per well and the plate is incubated at 37°C for 1 hour. The antibody solution is removed and filled in the wells of the other plate in order to recycle it for one time. Then the first plate is washed with 1xPBS/BSA and 200µl 1:500 dilution of the Rat anti Mouse-HRP conjugate antibody in 1xPBS/BSA is added per well. Again this antibody solution is recycled at the second plate. Then the plates are incubated at 37°C for 1 hour and the wells are washed. After all plates have been incubated with the second antibody 200µl DAB dye solution (Dako Liquid DAB Substrate Chromogen System; 20µl DAB Chromogen/ml Substrate buffer) are added and incubated at room temperature for 10 minutes. The dye solution is removed, 1ml 1xPBS is added and the plates are analyzed. The plates can be stored including 1xPBS at 4°C.

The infectious units are calculated:

[(Infected cells/field)x(fields/well)]/[(volume virus ml)x(dilution factor)]

At a 10x magnification of the lenses there are 79 fields.

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8. Zusammenfassung

Dickdarmkrebs ist der dritthäufigste Krebs weltweit. Er entwickelt sich in einem schrittweisen Prozess dessen erster Schritt häufig eine APC Mutation ist. Die Konsequenz dieser Mutation ist die progressive Hochregulation des wnt Signalweges, weshalb dieser ein bedeutendes Ziel der Therapie ist. Sulindac ist ein Medikament, welches den wnt Signalweg inhibiert und bereits in der Präventionstherapie erfolgreich eingesetzt wird. In der humanen Colon Adenoma Zelllinie LT97 wurde eine CD44+ Subpopulation identifiziert, welche FGF18 und Survivin hochexprimiert und IGF-bindendes Protein IGFBP3 herunterreguliert. Es ist noch nicht bekannt, ob diese Ereignisse kausal mit dem wnt Signalweg in Beziehung stehen.

In meiner Diplomarbeit erforschte ich den Einfluss von Sulindac auf die RNA und Protein Spiegel von Survivin, FGF18 und IGFBP3 in LT97 Zellen und in der Dickdarmkrebs Zelllinie HT29. Sulindac verursachte eine Herunterregulierung des wnt Zielgenes Survivin auf 28% bzw 61% der Kontrolle in HT29 und LT97 Zellen. Das Medikament veränderte den IGFBP3 Spiegel in LT97 Zellen nicht. In HT29 Zellen konnte ein leichter stimulierender Effekt von Sulindac auf den IGFBP3 Spiegel beobachtet werden.

Eine dominant negative Tcf4 Mutante wurde in ein adenovirales Konstrukt kloniert um eine spezifische Positivkontrolle für die wnt Blockade herzustellen. Das ermöglicht die Überexpression der Mutante in allen humanen Zelllinien.
HT29 und LT97 Zellen wurden mit dem Virus transduziert wodurch eine 4,7 fache bzw 1,7 fache Erhöhung der Expression von Tcf4 in LT97 und HT29 Zellen verursacht wurde. Signifikante Effekte auf Survivin, FGF18 und IGFBP3 konnten nicht detektiert werden.

Die Sulindac Ergebnisse zeigten dass Survivin aber nicht FGF18 und IGFBP3 in einer wnt-abhängigen Weise reguliert werden. Mechanistische Experimente mit dem neu produzierten Tcf4 Virus sind notwendig um eine wnt-abhängige Reaktion zu demonstrieren.

9. Lebenslauf

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