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Department of Pathology

The Role of Epigenetic Regulators in Melanoma

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Bachelors Programm
"Medical and Pharmaceutical Biotechnology"

Submitted by Sebastian Stefan

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Date: 26.02.2016 Sebastian Stefan

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Abberivations

Ultra Violet _____ UV
Histone protein # _____ H#
Chromatin Regulators _____ CR
Lysine _____ K
Plant Homeo Domain Finger Protein 8 _____ PHF8
Clustered Regularly Interspaced Palindromic Repeats _____ CRISPR
CRISPR Associated _____ Cas
CRISPR RNA _____ crRNA
trans activating CRISPR RNA _____ tracrRNA
Protospacer Adjacent Motif _____ PAM
CRISPR RNA _____ pre-crRNA
Double Strand Break _____ DSB
single guide RNA _____ sgRNA
double strand DNA _____ dsDNA
zinc finger nucleases _____ ZNFs
transcription activator-like effector nucleases _____ TALENs
Homologous directed repair _____ HDR
Non-homologous end-joining _____ NHEJ
WB _____ WB
Overnight _____ ON
sgRNA PHF8 _____ sgPHF8
sgRNA scramble _____ sgScr

1 Abstract

Cutaneous melanoma is a highly prevalent cancer, with increasing incidence worldwide as well as high metastatic potential. Melanoma is the third most common primary tumor to metastasize.

Thus, there is an urgent need for novel and innovative therapeutic strategies to expand the panel of available treamtents.

Despite the breadth of knowledge gained through the identification of genetic alterations and the study of aberrant signal transduction pathways driving tumorigenesis, our understanding of how those upstream pathways impact epigenetic and transcriptional deregulation remains limited.

A couple of considerations point out to a possible role of altered epigenetic regulators in melanoma. Since there is yet not so much known about chromatin regulators in melanoma, PHF8, which is upregulated in melanoma metastasis, has been bolt to identify it´s relation to melanoma metastasis.

1. Introduction

1.1 Melanoma

Malignant Melanoma is a highly prevalent cancer with increasing incidence worldwide as well as high metastatic potential. Also Melanoma is the third most common primary tumor to metastatsize to the brain after lung and breast cancer. It develops mostly from melanocytes but it can also develop from nevi. (Bastonini et al., 2014)

Stages of Melanoma

There are 5 different stages (Figure 1) in Melanoma, they differentiate according how deep the melanom has grown into the skin. At stage IV the cancer has reached the subcutaneous layer and is able to spread to other organs in the body. (Amiri et al., 2005)

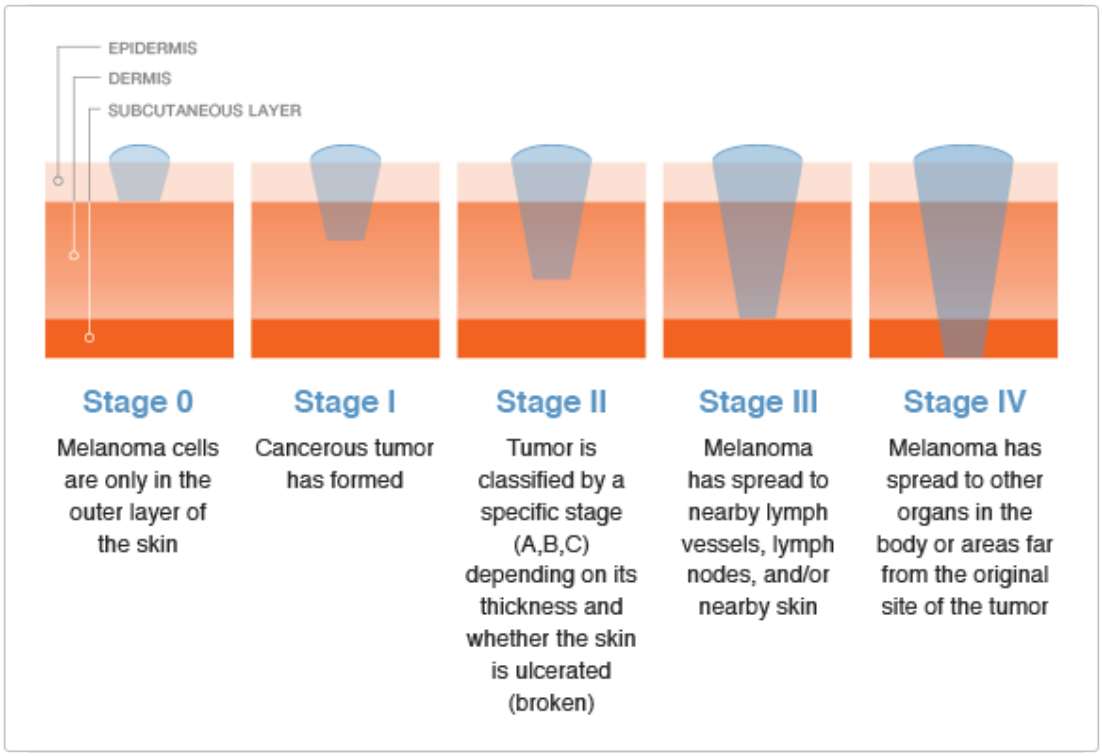

Figure2: Stages of Melanoma progression, Stage 0 - Stage IV

Cause of Melanoma

Mostly melanoma is caused by UV light, but also different mutations can cause melanoma. It is most common on the legs of females and the back of males. (Fiddler et al., 1995)

Ultraviolet radiation which has a wavelength from 400nm to 10nm can damaging the skins cDNA, especially a high sun exposure can increase the possibility to get skin cancer.

UVA

UVA wavelenghts go from 400nm to 315nm and are not visible for the human eye.
Since shortly UVA exposure is known to damage the skin cells DNA indirectly. It can produce chemical intermediates, like hydroxyl and oxygen radicals which then lead to a double strand break. (Boniol et al., 2012)

UVB

UVB wavelengths go from 315nm to 280nm and are more dangerous for the human skin.
UVB can cause damage directly to the DNA by forming cyclobutane pyrimidine dimers like thymine-thymine dimers, cytosine-pyrimidine dimers and cytosine-cytosine dimers. Those dimers are build by adding two adjacent pyrimidine bases within the DNA strand. Mostly the pyrimidine dimers in the DNA will then be removed via nucleotide excision repair. Not all of the dimers will always get ereased by the nucleotide excision repair and those not removed pyrimidine bases will then lead to cell apoptosis or they will cause DNA replication errors which lead then to mutations. (Rünger et al., 2012)

Mutations

Melanoma is mostly caused by UV radiation on the skin but it can also occur through mutations in special genes which can be inherited or occur through spontaneosly mutations, resulting in altered expression of regulating genes. (Haass et al., 2005).

Especially sporadic mutations are found in melanoma are related with oncogenes, which usually in their normal form as proto-oncogenes regulate the cell proliferation or promote the cell cycle contributing to tumor formation.

BRAF mutations are the most common mutations in melanoma. (Davies et al., 2010)

The BRAF mutations, activates the MAPK pathway continously and result in a increased cell proliferation and survival. (Chapman et al., 2011)

Another mutation in the NRAS oncogene will also lead to a constantly activation of the MAPK pathway. (Padua et al., 1985)

Familial melanoma which are approximately 10% of melanomas, is mostly conducted with the mutation in the CDKN2A or CDK4 genes. (Greene et al., 1998)

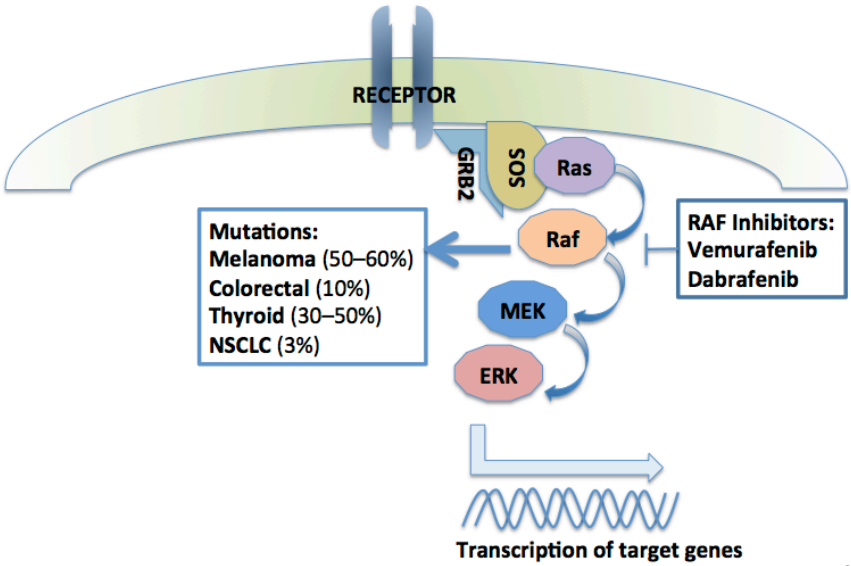

Figure2: BRAF mutation can be treatened wirh RAF inhibitors like Vemurafenib or Darafenib (pLX4720)

2.2 Melanocytes

Melanocytes produce melanin and they are located in the bottom layer of the skins epidermis. They can become malignant and resulting in cancer, their growth is controlled by keratinocytes (Haas et al., 2005). They overcome their control (downregulation of receptors for cell communication or upregulation of receptors for communication between melanoma cells) or changing its properties to loose the conncection between the plasma membrane, to detache from the matrix in order to spread in the neighbour tissue. (Patla et al., 2010)

2.3 Histone

Histones are packaging the DNA into nucleosomes (Cox et al., 2005)
They are the main protein components of chramtin and playing a role in gene regulation. (Redon et al., 2002) Five major Histone exists: H1/H5, H2A, H2B, H3 and H4. H1 and H5 are linker histones wherefore H2A, H2B, H3 and H4 are the core histone. They exist as a dimer inside the nucleous. (Marino et al., 2005). The nucleosome core is build out of two H2A-H2B dimers and a H3-H4 tetramer, which are forming two symmetrical halves. The other four histone have a "helix turn helix turn helix" motif to simplify the dimeristaion. Post Translational modification appear at the tails on one end of the amino acid structure. (Alva et al., 2007)

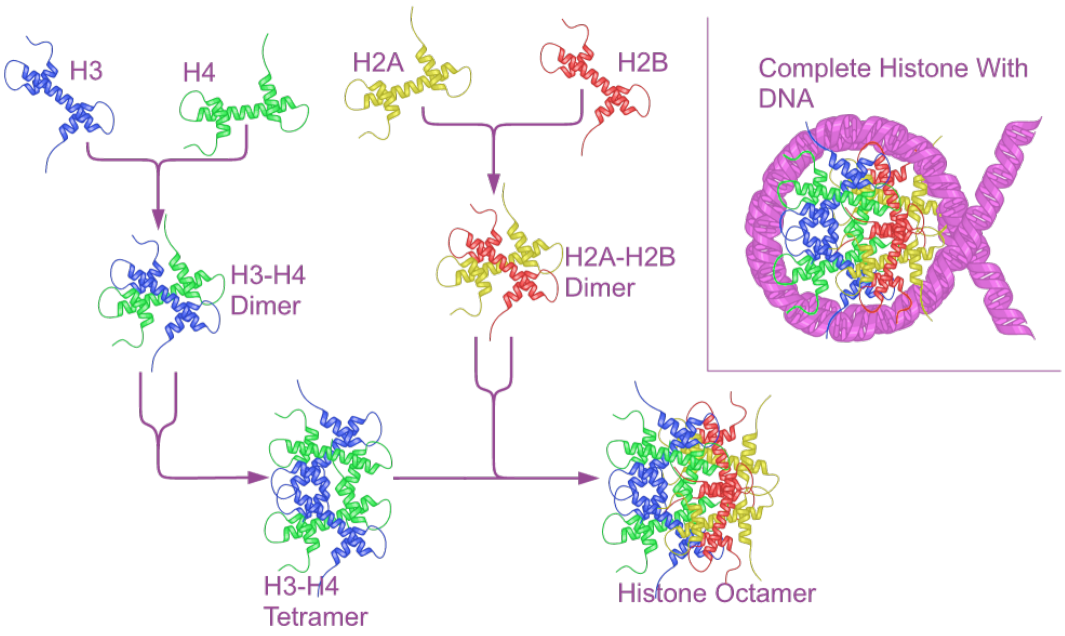

Figure3: Schematic assembly of the core histones into the nucleosome.

2.4 Plant Homeo-Domain Finger protein 8 - PHF8

The Plant Homeo-Domain Finger protein 8 is a histone lysine demethylase that acts on histones in the monomethyl or dimethyl states.
It requires Fe(2+) ion, 2-oxoglutarate and oxygen for its activity. (Liu et al., 2010)

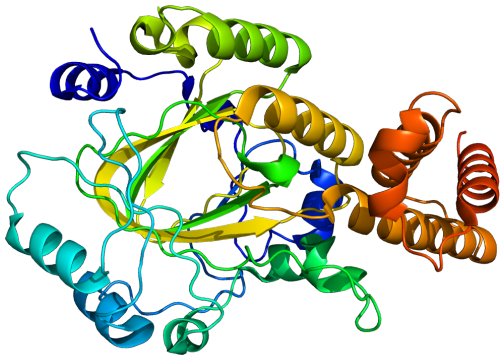

Figure4: Structure of PHF8, based on PyMOL

PHF8 contains of two domains, the JmjC domain and a PHD domain.
The PHD domain binds to methylated peptides and it also is important in the selectivity for methylated histone residues.
The JmjC domain is a binding domain of key cofactors and is connected with a beta-hairpin domain. (Qi et al., 2010)

It demethylates repressive histone marks including mono- and dimethylated histone H3 Lys-9 residue (H3K9Me1 and H3K9Me2), dimethylated H3 Lys-27 (H3K27Me2) and monomethylated histone H4 Lys-20 residue (H4K20Me1) (Qi et al., 2010)

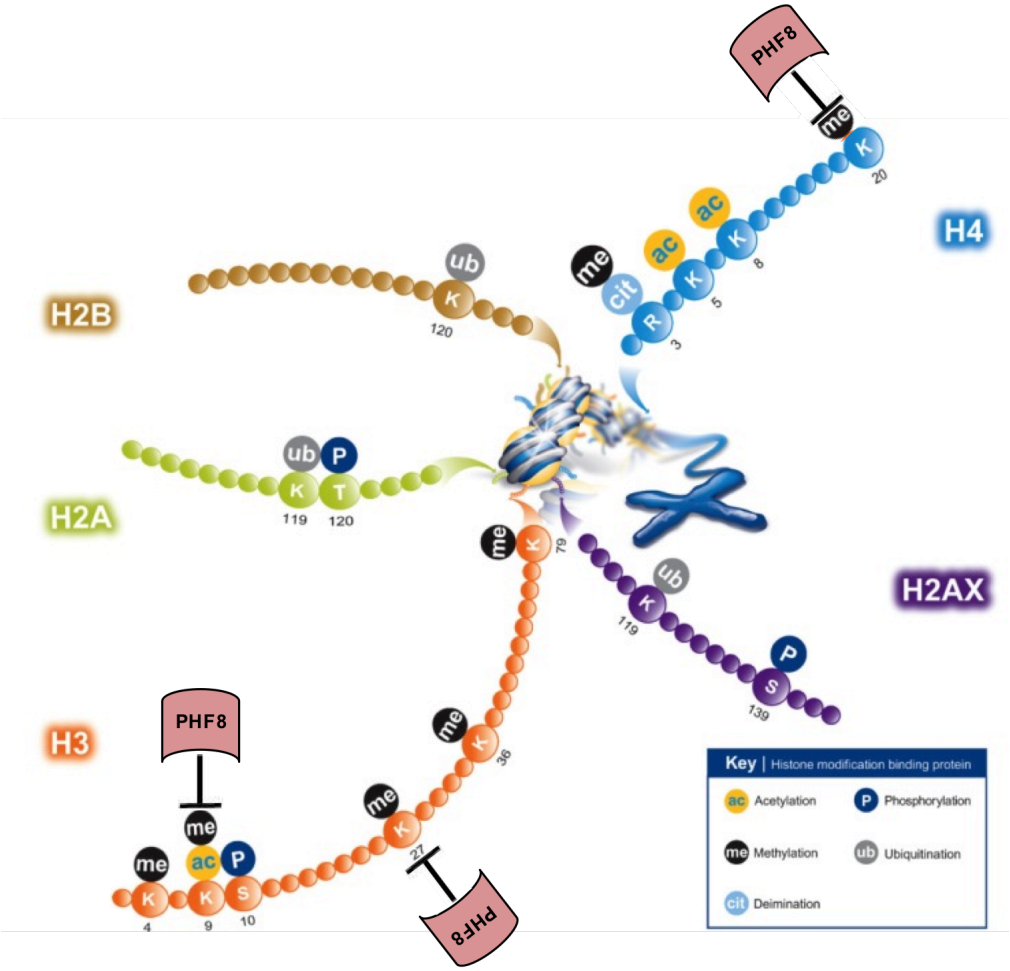

Figure5: demethylation targets of PHF8, H3K9Me1/2, H3K27Me2, H4K

Most of the PHF8 binding sites are at promoters and PHF8 is included in cell cycle regulation by removing H4K20me1 from promoters of certain E2F1-regulated genes.
Also the histone methylation modulated by PHF8 plays also an critical role in neuronal differentiation, brain and craniofacial development
Muatations in PHF8 are associated with X-linked mental retardation and cleft lip/cleft palate. (Kleine-Kohlbrecher et al., 2010)

PHF8 hast been reported in recently published papers that it is involved in several types of cancer like leukemia, prostate cancer squamous cell carcinoma and NSCLC, but a role in melanoma metastasis has not been reported yet.

2.5 CRISPR/Cas9

Genetic engineering means that a genome of an organism directly will be manipulated using biotechnology. The genome can be cleaved on gene regions to either be removed or knocked out by using a nuclease.
Also another modification can be gene targeting by homologous recombination to add a gene, insert point mutations or remove sequences on the genome.

Within the years the techniques for genetic engineering has been improved dramatically. Nowadays the range of targeting genomes is enormous and not imaginable without it in biotechnology.

One of the biggest explorations within the last years was the discovery and understanding of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR). Through further research among this topic scientiest found a way to improve genetic engineering by applying the CRISPR system to any

genome sequence that would contain a special sequence of 3 base pairs.(Jinek et al., 2012)

Crispr Loci Structure

The Clustered Regularly Interspaced Short Palindromic Repeats are usually in the size range from 24-48 base pairs.
They are palindromic, so the loci on the DNA strand is the same if you are reading it from 5' to 3' as from 3' to 5'. (Haft et al., 2005)

Moreover is between every Repeat a Spacer which has the same length as the Repeat. Those spacers are being inserted by Cas genes when a plasmid or Viral DNA invade the cell.(Tyson et al., 2008)

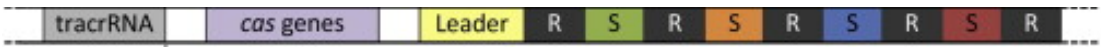

Figure6: CRISPR loci with tracrRNA(only in Type II) and cas gene regions infront of the leader sequence followed by Repeats and Spacers

Stages of CRISPR

The CRISPR/Cas System can be divided into 3 different types. Each of those types has the same 3 different stages:

Adaption Expression and Interference.

Type II

Type II is the most studied Type of CRISPR and can also be used for Genetic Engeneering

Adaption

The invasive DNA or plasmid can be detected by a Prtospacer Adjacent Motif (PAM). This PAM region has the base pair sequence 5'-NGG-3'. Wherefore the N can be any Nucleotide. It targets always a DNA sequence which is 20 bp long and is in front of the PAM region.(Jinek et al., 2012) This short region next to the PAM sequence is then insert into the CRISPR locus as spacer with the help of Cas1 and Cas2 proteins to build pre CRISPR RNA (pre-crRNA). By removing Cas1 or Cas2 the Adaption would not work anymore since the proteins are indispensable.(Swarts et al., 2012)

Expression

During the Stage of *Expression* the CRISPRS associated protein 9 (Cas9) is getting expressed when the tracrRNA hybridizes to the short palindromic repeat. The CRISPR Arrangement getting transcribed with the help of RNAse III.(Deltcheva et al., 2011)
During the reprocess, the transcript will form then CRISPR RNA's (crRNA) through cleavage inside those repeats, consisting a part of the CRISPR and a part of the foreign DNA. Also the Type II system of CRISPR is encrypting another RNA which is complementary to the repeat sequence. It is called a trans-activating RNA (tracrRNA). (Koonin et al., 2013)

Interference

Cas9 is important for the Interference. Cas9 builds a Cas9-RNA complex wherefore tracrRNA binds to the crRNA at the repeat sequence which is also complementary, then it will load into the Cas9, building the Cas9-RNA Complex.

This mentioned Cas9-RNA Complex will then bind to the foreign DNA and will perform a double strand break (DSB) at specific points of the foreign DNA which are complementary to the crRNA's. (Nunez et al., 2014) It cleaves the DNA with its dual HNH and RuvC/RNaseH-like endonuclease domains. Each domain cleaves a strand of the DNA. After this step the crRNA/tracrRNA/Cas9-Complex will unbind from the DNA after the double strand break was performed. (Figure 7) (Haurwitz et al., 2010)

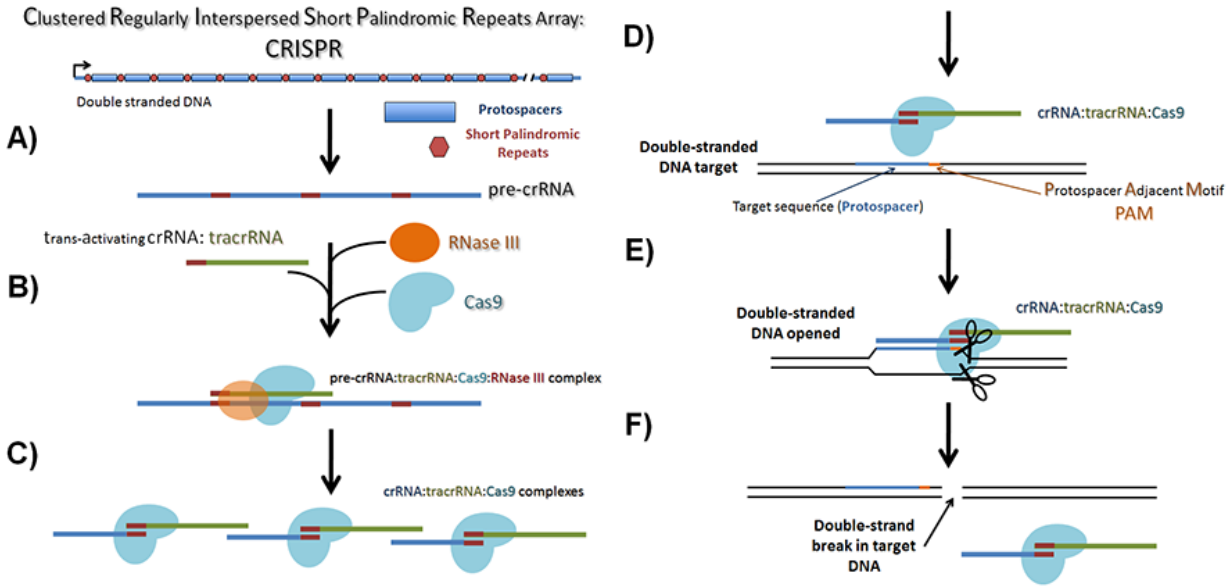

Figure7: CRISPR/Cas system Type II. Process of the DSB of the invading DNA, by generating crRNA and tracrRNA to build a complex with Cas9 and RNAase III to cleave the target sequence next to the PAM region

Type I

In type I systems, the protospacer selection of the invading DNA is only possible by a PAM region, as in Type II.
Cas1 and Cas2 lead to a insert of the proto spacers into the CRISPR loci to frame spacers.

It then gets expressed forming the pre-crRNA with the help of Cas3, which is then being cleaved by Cas6. During that time the Repeats build a hairpin formation next to the protospacers.

The crRNA then build a complex with Cas3 to target DNA, the cleavage is then catalysed by Cas3. It takes part in the HD nuclease domains of Cas3. (Brouns et al., 2008)

Type III

In type III system there is no PAM region necassary. But also Cas1 and Cas2 are inserting the proto spacers into the CRISPR loci.
The loci forms then the pre-crRNA like in all 3 types of CRISPR. Cas6 is responsible that the crRNAs are getting produced and then they transfer to a different Cas complex which called Csm in subtype III-A or Cmr in Subtype III-B. (Hale et al., 2009)

The difference between those two subtypes is that the 3' end of the crRNA is getting trimmed further than the crRNA in subtype III-A. Also subtype III-A cleaves DNA wherefore subtype III-B cleaves RNA.
Since there is no PAM region necessary on invading nucleic acid, it distinguish between pairing the 5' repeat fragment of the crRNA which will lead to no interference or the will be no base pairing which lead to interference. (Marraffini et al., 2008)

Cas9

Cas9, which plays an important role in the CRISPR Typ II, is an RNA-guided DNA endonuclease enzyme.
It can be divided into two main lobes. The alpha helical lobe and the nuclease lobe (Figure 8)

A single bridge helix connects those regions.
The alpha helical lobe consists of a recognition lobe which plays an important role in the immune system of mostly all archaea and some bacteria.

In the nuclease lobe there are three different domains. Two nuclease domains and and the PAM interacting domain. (Jinek et al., 2014)
The nuclease domains consist of the RuvC domaind and the HNH nuclease domain, wherefore RucV cleaves the non-target strand and the HNH domains cleaves the target strand. (Blosser et al., 2015)

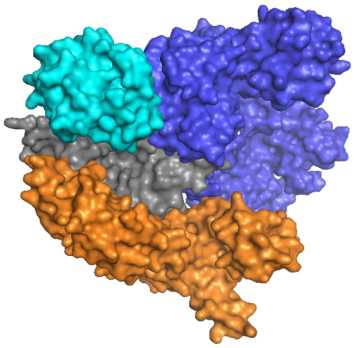

Figure 8: *Cas9 Crystal Structure in the APO form (Blue-alpha helical lobe) (Orange-PAM) (Grey-RuvC) (Cyan-HNH)*

Genomic Engeneering using the CRISPR/Cas9 system

Targeted genome editing using nucleases was already applicable by using zinc finger nucleases (ZNFs) and transcription activator-like effector nucleases (TALENs). (Kim et al., 2014)
Recently a new way was introduced to genome editing. The CRISPR-Cas9 system.
The Clustered Regularly Interspaced Short Palindromic Repeats Type II system ist at this moment the most used RNA Guided Endonuclease technology for Genome Engeneering.
Therefore two important components are necessary: a single guide RNA (sgRNA), which combines the previous mentioned tracrRNA and crRNA (Doudana et al., 2014) and an endonuclease, which is the CRISPR associated nuclease, Cas9, with two catalytic domains, RuvC and HNH. (Ran et al., 2013)

The interactions between sgRNA and Cas9

When Cas9 binds to the sgRNA (same function as in CRISPR Type II) to build the sgRNA-Cas9 complex, all domains, like the recognition lobe the PAM domain and also the nuclease domains, get in contact with the repeat and spacer regions of the foreign DNA. (Figure 4) Each strand thereby bind either to the RucV or HNH nuclease domain. (Wiedenheft et al., 2012)
Cas9 mutants showed a lower activity and stability of the Cas9 protein, if the recognition lobe, both nuclease domains or the PAM domain were mutated. (Nishimasu et al., 2014)

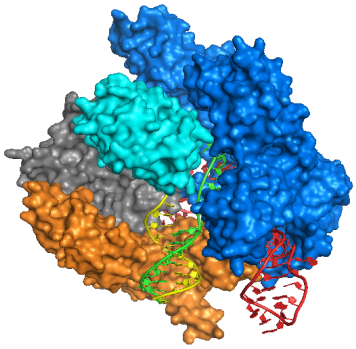

Figure 9: *Cas9:gRNA:DNA complex Crystal Structure in the APO form (Blue-alpha helical lobe) (Orange-PAM) (Grey-RuvC) (Cyan-HNH) (Red-gRNA) (Yellow-non target DNA strand) (Green-target DNA strand)*

Cas9 Nuclease

If there is a PAM region present in the target DNA, sgRNA will direct Cas9 to the DNA by base-paring. Resultint with a DSB. This DSB will be then repaired by either homologous directed repair (HDR) or by non-homologous end-joining (NHEJ) (Cong et al., 2013)

Homologous directed repair (HDR)

HDR will lead to a gene correction or replacement

Non-homologous end-joining (NHEJ)

NHEJ will mostly lead to errors. It is inducing small deletions or small insertions which are happening randomly, affecting mostly the function of the target gene.

The discovery of CRISPR and the comprehension of it, brought huge prospects to researchers among the world. The largest advantages of CRISPR is the CRIPSR/Cas9 system. This system provides now easy and fast genomic engeneering.

It is usefull to a lot of scientists, yet there are over 600 papers published with using the CRISPR/Cas9 system. It is also predicted that this discovery will win a Nobel Price. So hopefully this new method of gene engineering will be helpful for important discoverys since it is still in the early stages.

3 Material & Methods

3.1 Cell Splitting/Cell Preperation

All the work was performed under the fume hood.

All Media are stored at 4°C and should be brought to RT prior to use.

Following Cell Lines were used:

- SK MEL 147
- 501 Mel
- A375
- 451Lu

The cell lines were kept in dishes (10cm) in an incubator (37°C, 5% CO_2).

Provided Media for the cell´s:

SK MEL 147, 501 Mel and A 375:

DMEM 1x (Dulbecco´s Modification of Eagles Medium)

4,5g/L glucose
L-Glutamine
sodium pyruvate
10% FBS
1% Penicillin Streptomycin

451 Lu

WM Media

MCDB153 (with trace elements, L-Glutamine, 28mM Hepes)
20% L-15
4% FBS (heat inactivated)
5µg/ml Insulin
1,68 mM CaCl

About every 72 hours the cell lines needed to be splitted since their continously growing.

Hence the media needed to be aspirate from the dish where the cells are attached and washed with PBS(Dulbeccos Phosphate Buffered Saline without Calcium and Magnesium). After the washing step the PBS got aspirated and Trypsin (0.05% Trypsin-EDTA) was pippeted on the dish (2ml) in order to detache the cells from the dish surface and placed for 3 minutes in the incubator to optimize the trypsin condititions.

The detached cell´s then were mixed with 4ml of Media, to deactivate the trypsin (lower ratio of trypsin) and spinned down for 4 minutes at 16,1 rcf in a centrifuge.

The supernatant is aspirated and the cell pellet can be used now for different methods. It can be either stored at -80°C for a later lysis or resuspended in Media to count the cell´s and perform experiments with them.

For passaging the cell´s, the pellet is resuspended with Media (10ml) and 1 ml of the cell media solution is mixed with 9 ml of new media and pipetted in a new 10 cm dish, so the cell´s got split in a ratio of 1/10. The dish is placed in the incubator again to keep the cell´s in culture.

3.2 Lentivirus Production

BL2 conditions should be used at all times when handling the virus. All decontamination steps should be performed using 70% ethanol/1% SDS, or with 100% bleach. Gloves should be worn at all times when handling lentiviral preparations, transfected cells or the combined transfection reagent.

The Lentivirus Production was performed over 4 Days.

At the first day $5x10^6$ HEK 293T cell´s were plated in a 10cm dish with 10ml DMEM (10%FBS) for each virus condition which was made.

The next day a LIPO mix was prepared with a total volume of 1,03ml/dish and waited 5 minutes.

LIPO Mix

30µl Lipofectamin 2000
1000µl Optimem (Reduced Serum Medium)

During that time 5ml of a Helpers Master Mix was prepared containg envelope and packaging plasmids and also the vector plamid which can be gained from a minipreperation of bacteria described later.

Helpers Master Mix

4µg envelope plasmid (pMD2g)
8µg packaging plasmid (psPX2)
12µg vector plasmid
1ml Optimem

The Helper Master and LIPO Mix is then mixed together with 8ml of Optimem and replaced with the media from the 293T cell´s and incubated at 37°C (5%CO_2)
The media is replaced after 6 hours with DMEM.
After 48 hours postransfection the 293T cell´s produced Lentivirus containing rival RNA

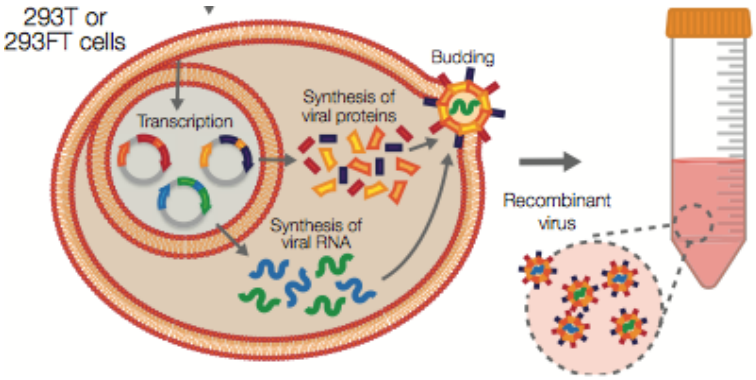

Figure10: Synthesis of viral RNA and proteins for Lentivirus production

The media is harvested and filtered through a 45µm pore size filter.
The lentivirus can now be used for tranfection or stored at -80°C.

3.3 Plasmid DNA Isolation

For isolating plasmid DNA from bacteria for useage for Lentiviral Production a QIAprep® Spin Miniprep Kit was used using the QIAprep® Quick Start Protocol.

3.4 CRISPR/Cas9 induced Knockout of PHF8

For the sgRNA design the genome sequenz of PHF8 was submitted at www.crispr.mit.edu to get a list of sgRNAs generated where it is choosen between the score and the lowest number of off-targets in exonic regions.
Corresponding to the selected regions Forward and Reverse oligos need to be ordered with following sequence:

- Forward oligo: 5' CACC followed by 20nt of guide RNA (if gRNA doesn't start with G, you need to add it between CACC sequence and gRNA)
- Reverse oligo: 5' AAAC followed by the reverse complement sequence to gRNA

Cloning:

The sgRNAs designed as indicated above can be cloned into following vector:
pLKO-GFP-sgRNA: pEHL958 (sgRNA only vector)
cells should be previously infected with pEHL794-LentiCas9-Blast lentiviruses to stable express Cas9 and checked via Western Blot if Cas9 is present. For selection Blasticidin was used.

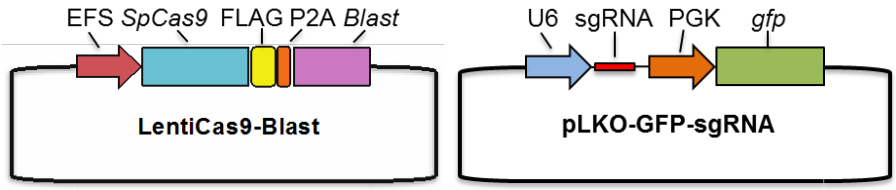

Figure 11: schematic plasmid of LentiCas9-Blast and pLKO-GFP-sgRNA

For cloning the sgRNA into the vector (backbone), the vector needed to be digested. An undigested Backbone was also prepared for negative control.

Digest of Backbone

	DIGEST		UNDIGESTED	
•	DNA (1µg)	1µg	DNA (0.5ug)	µl
•	10x Buffer NEB2.1	5µl		
•	Enzyme BBSI	1µl		
•	ddH2O	µl	ddH2O	µl
	Total	20µl	Total	20µl

Incubated 1 hour at 37°C (shaker)

The digest then got mixed with a 6x Loading Dye und an electrophoresis was performed (1% agarose gel) at 100V for 45 minutes.

The band of interest (7.8kb) then got checked via the negative controll if it got cut and then the band got prepared with the Zymoclean Gel DNA Recovery Kit using the Zymoclean Protocol. The DNA concentration then got maesured using the Nanodrop. The digested backbone could be stored at -20°C.

Oligos annealing:

The oligos got annaeled using following condition

• FW Oligo 2µg	2.75µl	
• RV Oligo 2µg	2.75µl	3-5min at 95°C
• 10x NEB Buffer 2	5 µl	slow cooling to RT
• ddH_2O	39.5µl	
Total	50µl	

Annealed oligos can be stored at -20°C

Ligation and transformation:

Ligation of 1µl vector with 2µl of annealed oligo.

• Vector	1µl	
• annealed Oligos	2µl	
• T4 Ligase Buffer	1µl	16°C Overnight
• T4 Ligase	1µl	
• ddH_2O	6µl	
TOTAL	10ul	

For pLKO-GFP-sgRNA, 30µl of XL1-Blue Bacteria are mixed with 4µl of the Ligate in an eppendorf tube and icubated on ice for 20 minutes. A heatshock at 42°C for 45 second is followed and then again incubated on ice for 5 minutes.

250µl of sterile SOC Media is added and incubated at 37°C for 45 minutes.
It then get plated out on an Agar Plate with Ampicilin and incubated ON at 37°C.

Selection of positive colonies:

To check if the transformation worked a colony PCR is performed using one of the oligos of the sgRNA and a Reverse Primer on the backbone.

Colony PCR Set up

• Fw Primer (10uM)	1.25 µl	
• Rv Primer (10uM)	1.25 µl	
• TaqPolymerase	0.25 µl	On Ice
• 10x PCR Buffer	2.50 µl	
• dNTP's	0.5 µl	
• ddH_2O	19.25µl	
• add a Colony per Tube		
Total	25 µl	

To each Tube a Colony is added which has to be streaked out before on an Agar Amp Plate, to have later a source to gain plasmid DNA is the Colony PCR shows a positive result

PCR Conditions

- 94°C – 5 min
- 95°C – 1 min
- 56°C – 0.5 min 30x Cycles
- 72°C – 0.5 min
- 72°C – 5 min

A electrophoresis was performed (2% agar gel) in order to check for positive clones containing the sgRNA.
Plasmid DNA can be extracted from the clones which were streaked out before the colony PCR, and then used for the production of Lentivirus containg the sgRNA and transfect it into the cell´s. Through its GFP open reading frame, fluoresence will show how good the tranfection worked.

3.5 Western Blot

Lysis for Western Blot

The trypsinized cells in DMEM are filled in an eppendorf tube and centrifuged for 5 min (0.4rcf, 4°C). The medium is then aspirated and 1ml PBS is added and centrifuged again for 5 min (0.4rcf, 4°C).
During that time the lysis buffer was mixed with 1x EDTA free protease inhibitor.

Lysis Buffer

20mM	Tris pH 7.5
140mM	NaCl
10%	Glycerol
2mM	EDTA
1%	NP40

The PBS is then aspirate and 50-100µl of the Lysis Buffer were added (depending on the cell pellet) and the pellet was resuspend and incubated on ice for 20 minutes.

After the lysis, the cell lysis mix was centrifuged (16.1rcf, 4°C) and then the supernatent placed in a new eppendorf, containing the protein of interest.
It need to be kept on ice.

Lowry Protein Assay

To determine the protein concentration a Lowry Protein Assay was done.
A 96 well plate is therefore necessary as well as a the Bio Rad® DC Protein Assay Kit.
The DC protein assay is used for protein concentration following detergent solubilization.
Each lysed sample is measured mixing it with the following condition:

- Per well
  - 25ul A+S Buffer (100:2)
  - 1ul Sample
  - 200ul Reagent B

3 replicates/condition were made. The absorbance was then measured after 15 minutes using a microplate reader at 650-750nm. The absorbance is stable for at least 2 hours.

Prepare of Western Blot Loadings

The results from the Lowry Protein Assay were put in a special prepared excel file, which automatically calculated the concentration needed to mix the sample with laemmli buffer (1x and 5x) to achieve a volume of 20µl with 20µg of the protein in it.
The mixed samples then are heated for 5 minutes at 95°C.

Western Blot

For the Western Blot first a SDS Page was performed according to seperate the proteins according to their size. The samples were load in a NuPage®Bis-Tris Mini Gel (10 pockets) which is placed in a gel chamber after the comb has been removed. The chamber is then filled with a 1X NuPage® MOPS SDS Running Buffer until the gel is completly covered. For each sample on pocket was filled with a total volume of 20µl. Infront of the samples a Bench Marker Prestained Protein Ladder was placed to identify later the proteinsize.
That the proteins will seperate the chamber was connected to a power device set at 100 V for 15 minutes, to let the samples enter the gel and then raised to 180V for about 1:45 hours since the protein of interest (PHF8) is a big protein (124-160 kDa)

Fot the blotting the gel needed to be transfered into a new chamber which is filled with a transfer buffer.

Transfer Buffer

10ml	10x Transfer Buffer
200ml	Methanol
700ml	ddH_2O

First the filter paper and is cut to the size of the gel and placed with fiber pads in 4°C Transfer Buffer.
The nitrocellulose membrane needs to be activated in MeOH for 2 minutes and then washed with ddH_2O before placing it on the gel.
The cassette is then placed with the black side down. Followed by a soaked fiber pad, soaked filter paper, gel from the SDS Page, NC-membrane, soaked filter paper, soaked fiber pad.

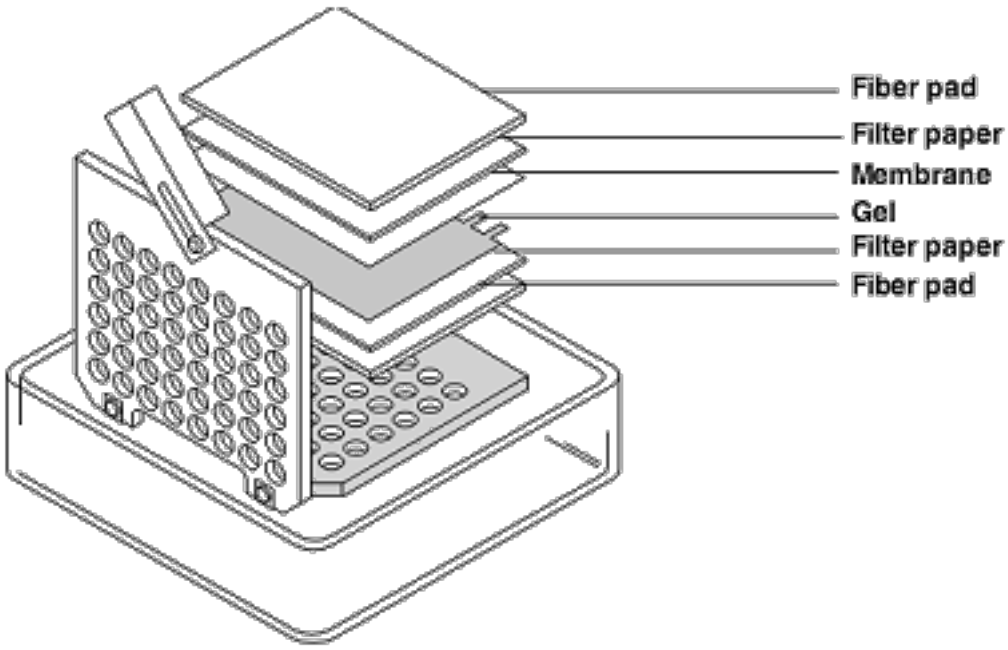

Figure12: General assembly drawing of the gel sandwich

The cassette sandwich is then placed in the Western Blot Chamber (cathode and anode need to be placed in the right way) and filled up with the Transfer Buffer.

Since a lot of heat is generated during the transfer a stirring device should be adapted as well as a cooling device.

The system is then connected to a power supply at 100V for hours.

Blocking

Before a antibody can be used for detection the non specific adsorption capacity has to be saturated by adding a not interferring protein.

Therefore a Milk Powder is used and a 5% Milk Powder Solution with TBST was mixed.

TBST

10% 10xTBS
0.4% 25% Tween

The membrane from the Western Blot is then placed in a clean cassette and filled up with the Milk Powder TBST solution, wraped with parafilm and placed at a shaker at 4°C overnight.

Antibody

After the ON Blocking the membrane is washed 3 times for 5 minutes with TBST.
For detection if PHF8 has been knocked out using CRISPR/Cas9, Rabbit anti-PHF8 Antibody was mixed with TBST in a ratio of 1:5000 and kept at room temperature on a shacker.
Afterwards the membrane was washed again 3 times for 5 minutes with TBST.
For a later detection of PHF8 on the membrane a secondary Antibody will be used which specifically binds to the Anti PHF8 Antibody which is bound to the PHF8 on the membrane if it is present.
The secondary AB is a Rabbit Antibody since the primary AB host is rabbit.
It is mixed in a ratio of 1:20 000 in 5% Milk Powder TBST and incubated for 1 hour at room temperature on a shaker.
Afterwards the membrane was washed 3 times for 10 minutes with TBST.

Developing

On the now prepared membrane the bands are not visible and they need to be developed using a Blot Imageing.
The membrane is first with placed in a small volume of Clarity® Western ECL Subtrate for 2 minutes, which provides sensitivity, verlow low background levels and a long signal durtaion. It is a chemiluminescent western blot detection.
Following the membrane is placed between a transparent film in a light-proof cassette.
For the developing following steps were made in a dark room.
An X-ray film was placed against the blot membrane and the cassette was closed for 2-10 minutes (depending on the outcome of visibliness).
After the exposure for an appropirate time, the film is developed by an auto-processor in the dark room. The film is treated in a serios of chemical baths (fixer, clearing agent) at a specific temperature and treatment time.
The X-Ray now has visible bands which need to be compared to the protein ladder in order to determine if the protein of interest is expressed or not.

The membrane can be reused after washing it with TBST for either detection of different proteins or it can be stained with Naphtal Blue.

3.6 Proliferation Assay

All the work was performed unter the fume hood in order to avoid contamination.

For the proliferation assay 5x 96 well plate were needed. For each cell line the same method was used.

The cell´s where trypsinized as mentioned above and then counted with the help of a cell counter by staining them before with Trypan Blue Stain (0.4%) 1:1 ratio.
For each condition, 8 replicants were made per day. The proliferation was observed over 5 days.
Each well was filled with 2000 cell´s in a volume of 100µl and then placed in the incubator at 37°C.
After 24/48/72/96/120 hours the 96 well plate was analyzed. Therefore the cell´s were fixed on the plate by aspirating the media and replacing it by 4°C 1% glutaraldehyde fot 15 minutes. Then the well was washed with PBS.
Now 100µl of crystal violet (0,5%) was added to the wells to stain the fixed cell´s for 1 hour.
The crystal violet can be washed away with tab water and afterwards it needs to be air dryed until all the wells are completly dry. In order to determine the proliferation the stained cell´s are eluted with 50µl of 15% Acetic Acid and placed on a rocker for 2 hours.
The absorbance at 590nm is then read to compare the different time points the cell´s did proliferate.

3.7 Invasion Assay

All the work was performed under the fume hood in order to avoid contamination.
For the invasion assay a 24 well plate was needed and Corning®FluoroBlok Insersts with 8µm pore size.

First the matrigel aliquot at -20°C had to be thawed at least 1h before usage. It should be thawn slowly therefore the aliquot was put on ice and stored in the cold room at 4°C.
The inserts were put on the 24 well plate, for each sample at least 3 well´s were used and 1 well was filled without the insert for the seeding control (row D).
The matrigel then was mixed with a already prepared coating buffer (0.01M Tris pH8, 0,7% NaCl, ddH_2O) in a ratio of 1:25 for SK MEL 147, 501MEL and A375 and a ratio of 1:20 for 451Lu.

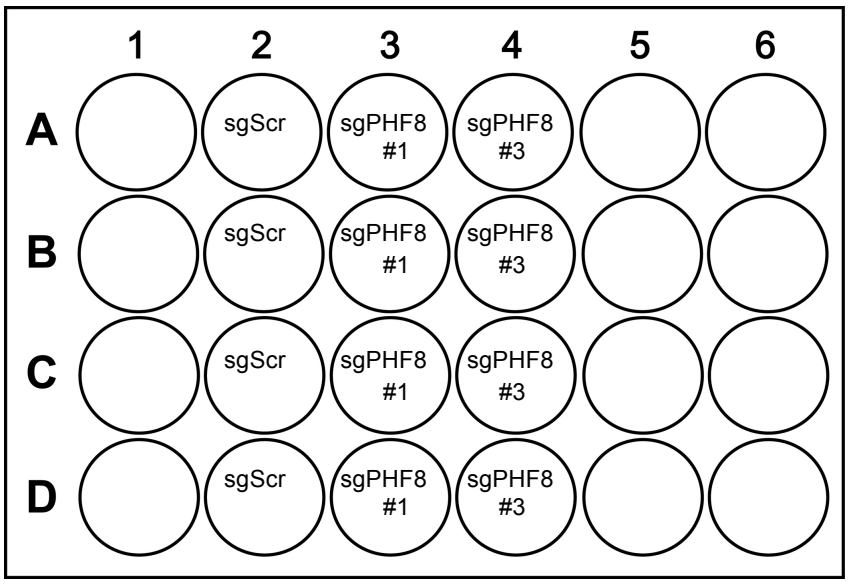

Figure13: 24 well plate, A2-4,B2-4 and C2-4 contain a insert, D2-4 was used for seeding control

In each insert 100µl of the diluted matrigel were placed and then it has to be incubated for 2 hours at 37°C. After 75 minutes the cell´s can be prepared for seeding. They were trypsinized as mentioned above and then resuspended in complete media, spinned down and then diluted in serum-free DMEM.
For each insert, 30 000 cells were needed in a volume of 300µl of serum free media.

After that step the cell´s need 3-5 minutes to settle down to the insert bottom. Also one chamber is filled only with the cell´s in serum free DMEM for the seeding control.

In the lower chamber where the insert is placed, 700µl of 10%FBS DMEM is filled and then incubated for 13 hours (SK MEL 147, 501MEL, A375)and or 18 hours (451Lu).

After the incubation HBSS (Hanks Balanced Salt Solution without calcium, magnesium and phenol red) need to be mixed with Calcein AM at 2µl/ml, which is a fluorescent dye. Then 500µl Calcein AM in HBSS is plated in well´s on a new 24 well plate. One chamber is filled for one insert used.
Now the media from the insert is aspirated and transfered into the 24 well plate containing Calcein in HBSS. Incubate the well for 1 hour at 37°C.

During that time the seeding control can be fixed and stained with Crystal Violet and later eluted acetic acid to determine if the same number of cell´s were used between sgScr. sgPHF8 #1 and sgPHF8#3

After 1 hour pictures were taken of the dyed cell´s under a GFP fluorescence microscope. A whole vertical diameter of an insert at 10x was pictured (8 photos/well).

In order to determine and compare the invasion between sgScr and sgPHF8, the cell´s which did migrate were counted and a statistical analysis was performed. According to the taken pictures and then compared to each other.

4 Results

Data Analysis

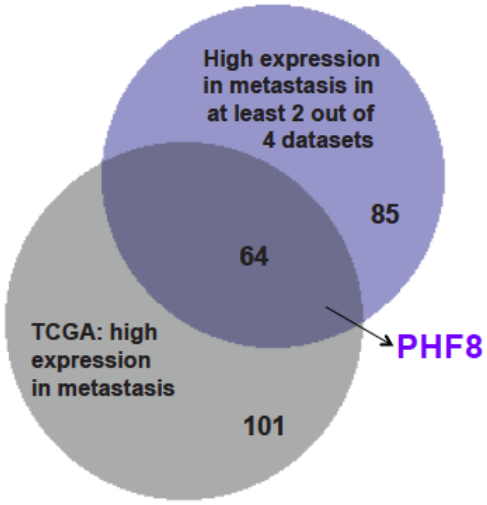

Figure 14: meta-analysis by data mining of The Cancer Genome Atlas(TCGA) for melanoma and four additional public expression profiles.

Figure 14 shows that 64 cromatin regulators are expressed in metastatic melanoma in the TCGA and in at least 2 out of 4 databases. One of the 64 expressed proteins is PHF8, which was selected for this project to be determine how a knockout will affect proliferation and invasion.

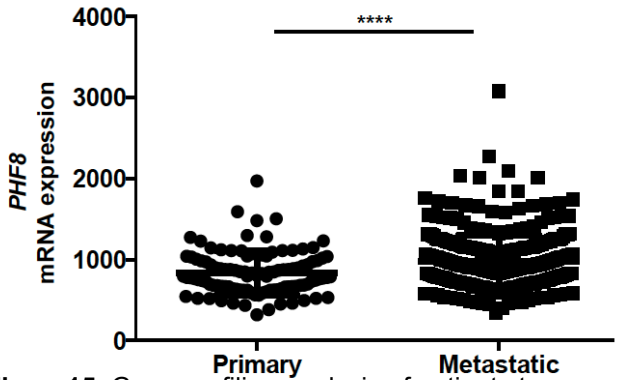

Figure15: Gene profiling analysis of patients tumor samples by TCGA

Figure 15 shows a significant upregulation of PHF8 mRNA expression (****, p<0.0001)in metastatic versus primary patients tumors).

CRISPR/Cas9

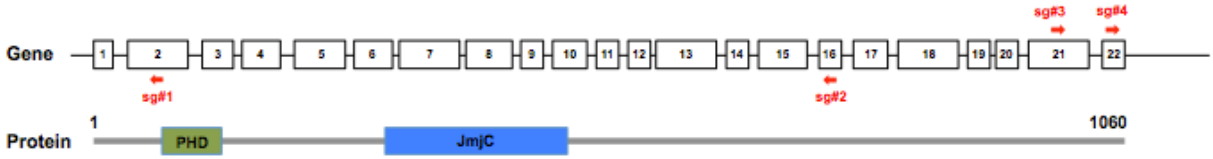

Figure16: sgRNA regions, shown where they are located on the gene regions.

In figure 16, it can be seen that sgPHF8#1 is located on the 2nd gene region close to the PHD domain. sgPHF8#2 is located on the 16th gene region. sgPHF8#3 is located on the 21st gene region and at the end of the protein as well as sgPHF8#4 located on the 22nd gene region.

Following sequences were used:

sgPHF8 #1 F	caccGTCACACTCGATCATGAAGC
sgPHF8 #1 R	aaacGCTTCATGATCGAGTGTGAC
sgPHF8 #2 F	caccgTTCGCACGGGGCAATTTCCG
sgPHF8 #2 R	aaacCGGAAATTGCCCCGTGCGAAc
sgPHF8 #3 F	caccgTCCTCCTGAGCCTAAACAAG
sgPHF8 #3 R	aaacCTTGTTTAGGCTCAGGAGGAc
sgPHF8 #4 F	caccgAAAGCGTCCCAAAAAGGGCC
sgPHF8 #4 R	aaacGGCCCTTTTTGGGACGCTTTc

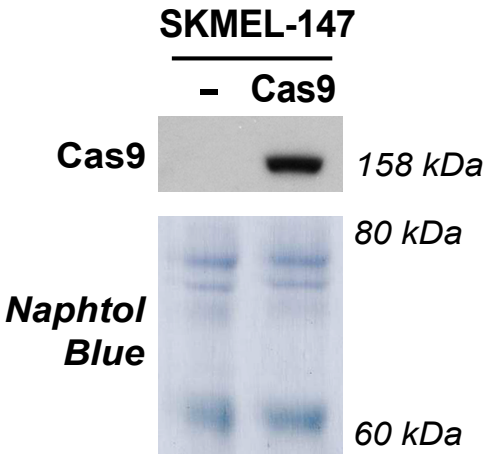

Figure17: SK Mel 147. Left side - negative control, right side - Cas9 expresseing SK Mel 147

Figure 17 shows that in the negative control no band at 158kDA and the Cas9 expressing SK Mel 147 show a thick band at 158kDa. Below is the membrane which was stained with naphtal blue.

Western Blot

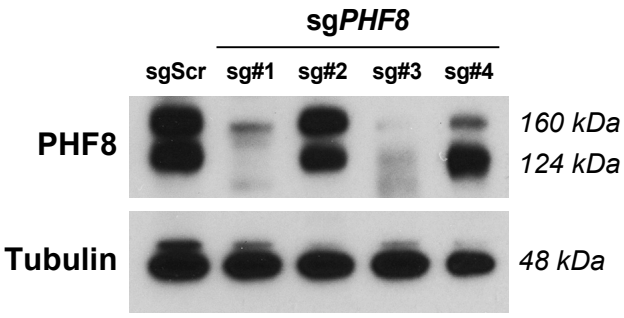

Figure18: Western Blot of sgScr and 4 different sgPHF8

The western blot shown in figure 18 demonstrate for sgScr thick bands at 160 and 124 kDa, sgPHF8#1 only shows a slightly visible band at about 160 kDa and some blurry below it. sgPHF8#2 shows two thick

bands like sgScr. sgPHF8#3 only shows a blurry light visible layer at 124 kDa. sgPHF#4 has a thin band at 160 kDa and a thick one at 124 kDa. All the bands visible at 48 kDa are nearly the same size.

Proliferation Assay with sgScr, sgPHF8#1 and sgPHF8#3

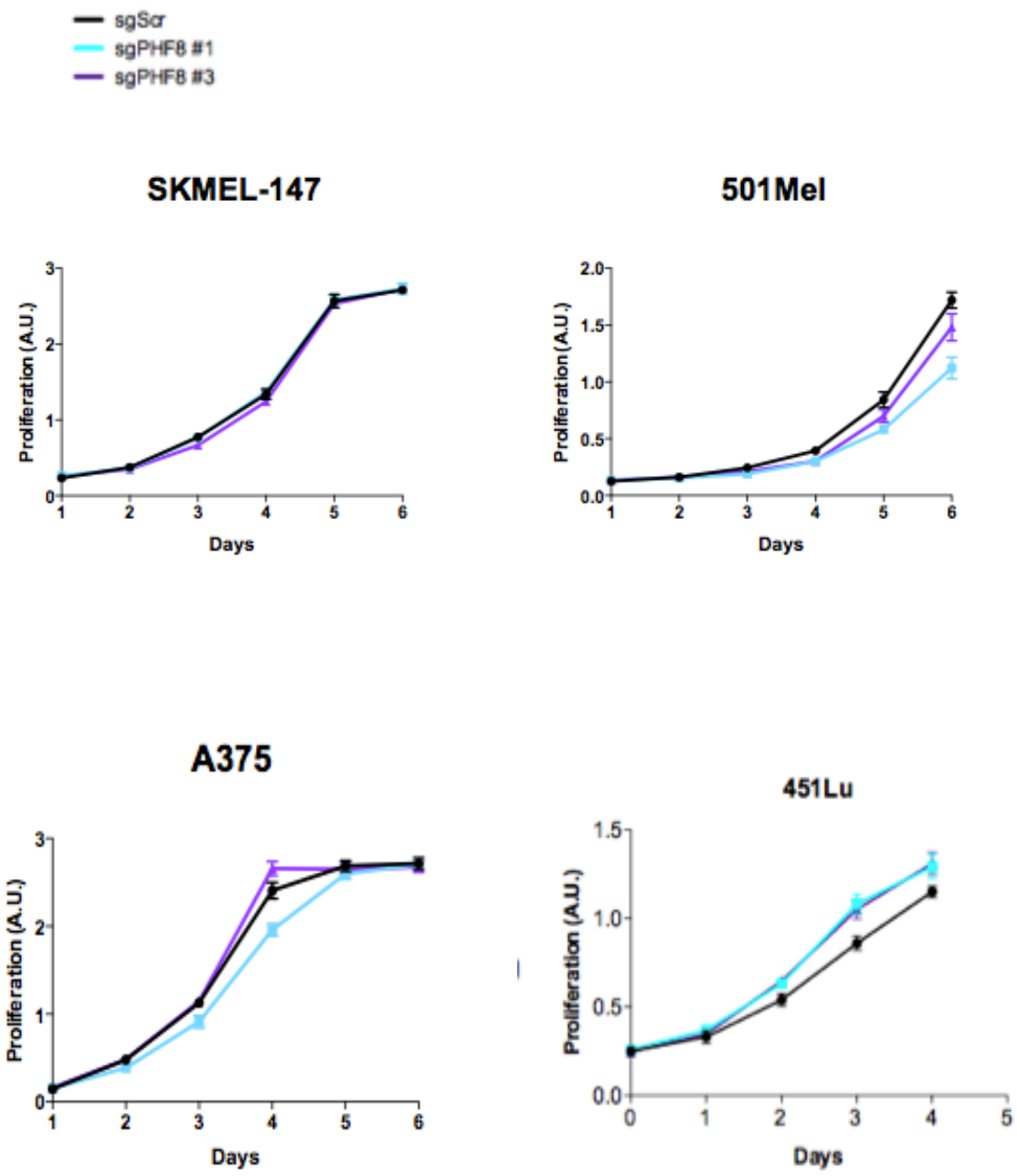

Figure19: Proliferation curves Proliferation (A.U.) x Days of SK Mel 147, 501Mel, A375 and 451Lu

Figure 19 shows black lines for sgScr, cyan lines for sgPHF8#1 and purple lines for sgPHF8#3. SK Mel 147 shows that sgScr, sgPHF8#1 and sgPHF8#3 all have the same dot for proliferation within the same days.

For 501Mel, sgScr shows a value of 1.7 after 6 days. sgPHF8#1 only has a value of 1.1 at day 6. sgPHF8#3 has the value 1,5 after 6 days.

A375 shows after 6 days the same proliferation value for al sg´s but the values are different for day 3 and 4.

451Lu demenstrate the same values for the cyan and the purple line, but lower values for sgScr.

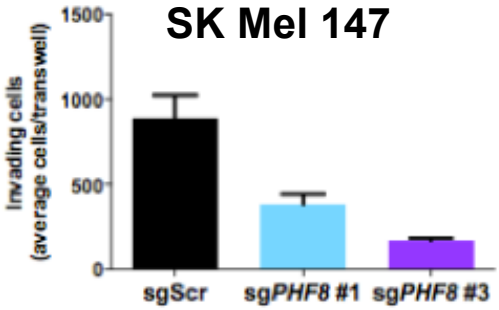

Figure20: Invading cells (average cells/transwell) of SK Mel 147

Figure 20 shows the number on invading cell´s/ transwell with error bars. sgScr reaches a value of about 900, wherefore sgPHF8#1 reaches 400 and sgPHF8#3 about 250.

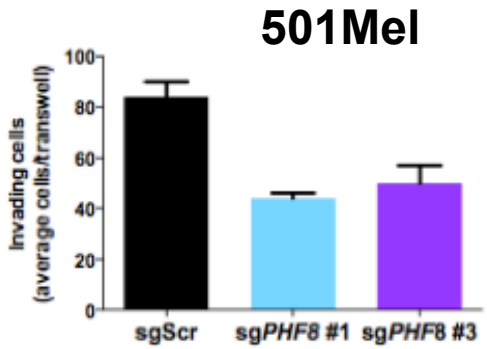

Figure21: Invading cells (average cells/transwell) of 501Mel

Figure 21 shows a value of 85 invading cells for sgScr, 45 for sgPHF8#1 and about 50 for sgPHF8#3. Error bars are higher for sgScr and sgPHF8#1 and sgPHF8#3.

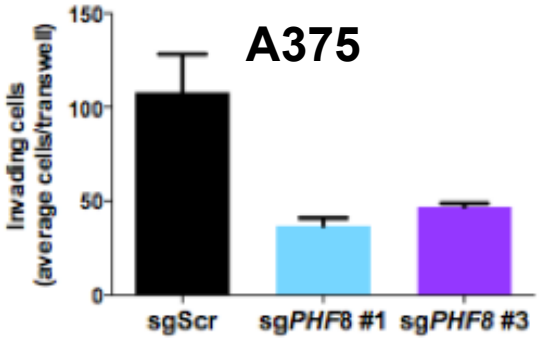

Figure22: Invading cells (average cells/transwell) of A375

Figure 22 shows a value of 110 for sgScr with a high error bar, a value below 50 for sgPHF8#1 and 50 for sgPHF8#3.

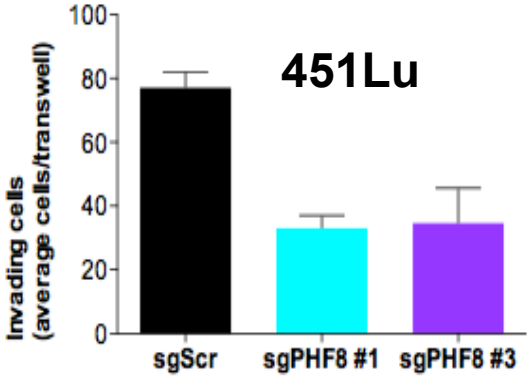

Figure23: Invading cells(average cells/transwell) of 451Lu

Figure 23 shows a double as high value as sgPHF8#1 and sgPHF8#3 which are pretty close together. Also a high error bar is visible for sgPHF8#3.

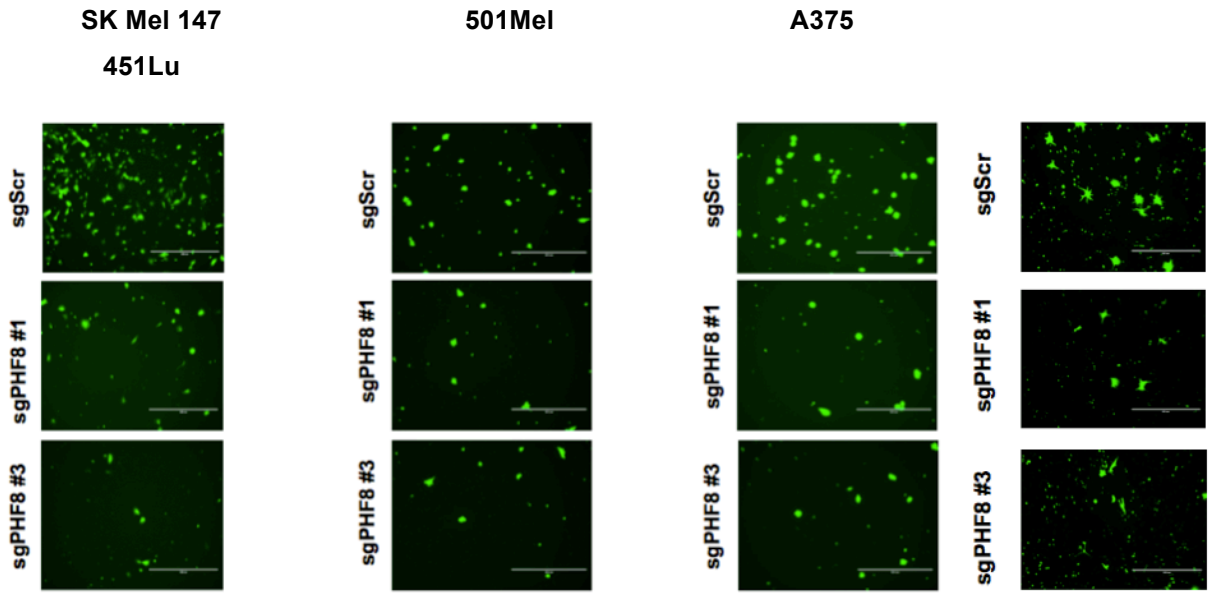

Figure24: Results from the invasion assay. Pictures taken under the fluorescence microscope

Figure 24 shows the pictures taken during the invasion assay. Each cell was stained with Calcein which makes them visible under the fluoroscence microscope. For each cell line more cells are visible at sgScr than at sgPHF8#1 and sgPHF8#3.

5 Discussion

Data Mining

PHF8 was detected as a overexpressed protein in the TCGA as well as in 2 out of following databases:

Talantov et al., (2005)
Xu et al., (2008)
Riker et al., (2008)
Kabbarah et al., (2010)

PHF8 is a chromatin regulators and has been linked with several types of cancer, including leukemia, prostate cancer, squamous cell carcinoma and NSCLC. (Zhu et al., 2015)
Yet there is nothing known about the role of PHF8 in melanoma. For that reason this gene was used for this project.
Also the gene profiling analysis of patients tumor samples by TCGA (Figure 15) shows a significant upregulation of PHF8 mRNA expression (****, $p<0.0001$) in metastatic versus primary patients tumors.

CRISPR/Cas9

The sgRNA regions (Figure 16) were choosen according to their lowest number of off-target effects and tried to have at least on region located at the PHD Domain which is responsible for the demethylation of Lysine at the histone chains. Also prefered regions were at the beginning of the gene in the hope of that the repairing by nonhomologous end joining will insert a aminoacid which will lead to a stop codon.

As can seen in figure 14, sg#1 is located at the beginning of the gene and at the PHD domain as well as sg#3 and sg#4 is located at the end of the gene.

Cas9 expressing Cell´s

The Cas9 expressing cell´s were seperated by Blasticidin.(Figure 11) If the infection with the Lentivirus containing the Cas9 gene region and a Blasticidin resistance region, the cell`s could be seperated by adding blasticidin to the media. To get a clear evidence that Cas9 is present, a Western Blot was performed with the cell line expressing Cas9 and the same cell line without expressing Cas9.
Cas9 was then visible as a band at 158 kDa. A naphtal blue staining was made to see if the same protein size was loaded in each gel pocket. (Figure 17)

Western Blot with sgRNA

To determine if the CRISPR/Cas9 induced knockout of PHF8 worked, a Western Blot was performed.(Figure 18) sgScr shows the cell line without an induced knockout. The result shows that sgRNA #1 (sgPHF8#1) and sgRNA#3(sgPHF8#3). It is most likly that the double strand break induced by Cas9 was resulting in a stop codon and made the protein inactivated. The slightly visible bands could be a few cell´s where the tranfection with the lentivirus did not worked and expressed PHF8 --> a small amount of PHF8 was present.
For sgPHF8#2 and sgPHF8#4 the knockout did not worked, either because the DSB was repaired correctly or the knockout in this region does not affect the expression of PHF8.

Each Western Blot was performed several times (n=3) in order to exclude errors which can occur during the collection of the cell´s, the western blot or the transfer.
Since sgPHF8#1 and sgPHF8#3 worked several times for SK Mel 147 it was decided that both sgRNA´s would be used for 501Mel, A375 and 451Lu since this gene region is not mutated in those cell lines.

Proliferation Assay

SK Mel 147 and 451Lu got nearly the same results for sgScr than for sgPHF8#1 and #3. For 501Mel the proliferation of sgScr was a bit lower than for the two others but the difference was not significant.
A375 reached the highest value of proliferation, wherefore the last 2 days of observation cannot be included since the density reached its maximum in the well´s and the cell´s did not had any more space to proliferate. The values are also pretty close to each other the days before.
Those experiments were performed at least 2 times to exclude off values and confirm the results. (Figure 19)

The total result indicates that the CRISPR/Cas9 induced knockout of PHF8 does not affect the proliferation. So PHF8 should then not be involved in the proliferation of the cell´s. It does not play a significant role.

Invasion Assay

The best result was performed with SK Mel 147(Figure 20), wherefore the lowest number of migrating cell´s through the matrigel resultated. All difference between sgScr and sgPHF8#1, sgPHF#3 are significant. For SK Mel 147 sgPHF8#3 worked better than sgPHF8#1. For the other 3 cell lines sgPHF8#1 had the lowest number of migrating cell´s.
This experiment was performed at least 3 times (n=3), since the implementation of this experiments allows a lot of errors, espacially the most important thing is that the right number of cell´s is filled everywhere in the insert. Even a small difference of cells between the insert can falsify the result.
In addition, always the same area of the insert should be obtained, in order to have the same conditions everywhere.
Besides them, it is important to differenciate migrated cell´s to not completly migrated cell´s during counting.
The CRISPR/Cas9 induced knockout of PHF8 does affect invasivness.

6 Conclussion

The performed experiments show that a CRISPR/Cas9 induced knockout of PHF8 does not affect proliferation but does affect invasivness.
PHF8 is in melanoma cell´s usually upregulated and corresponding from the performed experiments, it can be said that it promotes melanoma invasivness of cancer cell´s. Especially when you try to isolate the important gene domains, which was done with CRISPR/Cas9. sgPHF8#1 was located on the PHD domain and through the knockout, special Lysine residues will then not get demethylated by it.

Further expermients will determine a metastatic role of PHF8 in vivo, with 451Lu, since this cell line does not migrate very fast.
The PHF8 downytream targets will identified which mediate the effect in invasion. Also the molecular events will be observed to gain understanding of the upregulation of PHF8 in melanoma metastasis. Also it should be determined the potential of PHF8 as a marker of clinical outcome.

Figure References

Figure 1 http://www.yervoy.com/understanding-metastatic-melanoma (12.02.2016 9:00)

Figure 2 https://www.roswellpark.org/article/targeting-braf-mutation-positive-cancers-melanoma-lung-and-colorectal (12.02.2016 8:30)

Figure 3 Youngson RM (2006). *Collins Dictionary of Human Biology*.

Figure 4 PHF8 (PDB 2WWU) by PyMOL (14.02.2016 10:00)

Figure 5 Rana Moubarak, PhD, New Yew University, 2015

Figure 6 Megan L Hochstrasser , et al., (November 2014). Cutting it close: CRISPR-associated endoribonuclease structure and function. Trends Biochem Sci. 2015;1(1):58-66

Figure 7 https://www.addgene.org/crispr/reference/history/ (16.02.2015 12:45 Uhr)

Figure 8 Jinek et al. (Feb 2014). Structures of Cas9 endonucleases reveal RNA-mediated conformational activation. *Science 343 (6176)*

Figure 9 Anders et al. (2014). Structural basis of PAM-dependent target DNA recognition by the Cas9 endonuclease. *Nature* 13 (7519): 569–73

Figure 10 https://www.thermofisher.com/content/dam/LifeTech/global/life-sciences/CellCultureandTransfection/pdfs/Lipofectamine3000-LentiVirus-AppNote-Global-FHR.pdf (17.02.2016 15:00)

Figure 12 http://www.radio.cuci.udg.mx/bch/EN/Forschung/GelSandwich.gif (27.02.2016 11:00)

Figure 15 The Cancer Genome Atlas, 2015

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