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Titel der Masterarbeit

„Regulation of DNA- and Chromatin-associated proteins through microRNAs in tumourigenesis“

„Regulation von DNA- und Chromatin-assoziierten Proteinen durch microRNAs in der Tumorigenese“

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1. ABSTRACT

Anaplastic large-cell lymphoma (ALCL) is an aggressive T-cell non-Hodgkin lymphoma, which has its peak incidence in childhood. It was shown that enhancer of zeste homologue-2 (EZH2), the enzymatic subunit of Polycomb repressive complex-2 (PRC2), is overexpressed in human ALCL cell lines and tissue patient samples when compared to peripheral blood mononuclear cells (PBMCs) and normal lymphnode tissue. EZH2 carries histone methyltransferase-(HMT)-activity within its SET domain and as such its major function is the trimethylation of H3K27, which is a repressive histone mark. High levels of EZH2 contribute to epigenetic reprogramming in cancer by indiscriminately silencing a cohort of genes, among them tumour suppressors and transcription factors. Additionally, miRNA-101, which is known to directly target EZH2, was found to be downregulated in ALCL cell lines.

In the current project we aimed to reduce the levels of EZH2 either by direct siRNA knockdown, indirectly through global inhibition of histone methylation using the inhibitor 3-Deazaneplanocin A (DZNep) or via overexpression of the aberrantly downregulated miRNA-101. We hypothesized that all three approaches would reduce not only EZH2 but also the level of repressive histone marks. This, in turn, would enable the reactivation of genes that were abnormally silenced during cancer progression and would cause attenuated cell proliferation, cell cycle arrest and apoptosis of ALCL cell lines.

We used two anaplastic lymphoma kinase positive (ALK^+) cell lines Karpas-299 and SR786 and the ALK^- ALCL cell line Mac-2a for our in vitro studies. When we treated the cell lines with EZH2 small interfering RNA (siRNA) we found both mRNA and protein levels of EZH2 to be highly reduced. Generally, cells did not arrest in the cell cycle or show a significant increase in apoptosis. Only Karpas-299 appeared to be less proliferative. In contrast, DZNep significantly induced apoptosis in all three cell lines and led to a cell cycle arrest in G2/M phase. Overexpression of miR-101 did not result in a reduction of EZH2 mRNA or protein level. Cells appeared to be less proliferative, though.

We conclude that epigenetic silencing is not dependent on EZH2 alone but does involve other HMTs, which can be targeted by the global inhibitor DZNep.

2. ZUSAMMENFASSUNG

Das anaplastische großzellige Lymphom (Englisch: Anaplastic large cell lymphoma, ALCL) ist eine Subform der Non-Hodgkin-Lymphome mit T-lymphatischem Zellursprung und tritt am häufigsten in der Kindheit auf. EZH2 (enhancer of zeste homologue-2), die enzymatische Untereinheit von PRC2 (Polycomb repressive complex-2), ist sowohl in ALCL Zelllinien, als auch in Gewebsproben von ALCL Patienten deutlich überexprimiert. EZH2 hat die Funktion einer Histon-Methyltransferase (HMT) und setzt die repressive Trimethylierung von H3K27. Die erhöhte Expression von EZH2 führt zu einer unkontrollierten Stilllegung essentieller Gene, darunter diverse Tumorsuppressoren und Transkriptionsfaktoren. Weiters wurde gezeigt, dass die Expression von miRNA-101, einem negativen Regulator von EZH2, in ALCL Zelllinien signifikant herabgesetzt ist.

Im vorliegenden Projekt versuchten wir, die EZH2 Expression in den Zelllinien durch direkten siRNA-Knockdown, global über den Inhibitor 3-Deazaneplanocin A (DZNep) oder mittels Überexpression der miRNA-101 zu reduzieren. Durch die fehlende enzymatische Aktivität von EZH2 erwarteten wir, dass die repressiven Histonmodifikationen zurückgehen würden. Zellen würden im Zellzyklus arretieren, die Proliferation würde deutlich zurückgehen und Zellen würden vermehrt in Apoptose gehen.

Für unsere *in vitro* Versuche arbeiteten wir mit zwei ALK^+ ALCL Zelllinien Karpas-299 und SR786, sowie einer ALK^- Zelllinie Mac-2a. Nach EZH2 siRNA Behandlung, konnten wir einen deutlichen Rückgang der EZH2 Expression sowohl auf mRNA- als auch Proteinebene feststellen. Es gab jedoch weder einen signifikanten Anstieg in der Apoptose, noch arretierten die Zellen im Zellzyklus. Ausschließlich Karpas-299 zeigte eine leicht verminderte Zellproliferation. Die Behandlung der Zellen mit DZNep führte zu einem starken Anstieg der Apoptose, Zellproliferation war signifikant reduziert und die Zellen arretierten in der G2/M Phase. Die Überexpression der miR-101 zeigte keinen reduzierte Expression auf mRNA- oder Proteinebene und nur einen schwachen Rückgang der Proliferation.

Wir schlussfolgern daraus, dass die epigenetische Reprogrammierung nicht ausschließlich von EZH2, sondern offensichtlich noch anderen Histon-Methyltransfasen und Faktoren abhängt, welche erst durch die globale Behandlung mit DZNep ausgeschaltet werden konnten.

3. INTRODUCTION - EPIGENETICS AND CANCER

"Epigenetics is the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence" (V.E.A. Russo 1996). While the definition by Arthur Riggs and colleagues in 1996 is probably the most common to define and express our current understanding of epigenetics, Conrad Hal Waddington, who introduced this term in 1939, initially defined it as *"the causal interactions between genes and their products, which bring the phenotype into being"* (Waddington 1939). Waddington used the term epigenetics, which combines the greek word "*epi*" (above, over) with the field of "*genetics*", to describe how genotypes contribute to the emergence of phenotypes during development. Riggs and colleagues incorporated in their definition that epigenetic events are heritable and not due to genetic mutations. In 2007, Adrian Bird proposed a new definition aiming to unify the controversial definitions of epigenetics. He described epigenetic events as the *"structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states"* (Bird 2007).

3.1. Epigenetic modifications

Epigenetic mechanisms include DNA methylation, RNAi, and histone modifications, which are essential systems to initiate but also maintain epigenetic gene silencing (Egger 2004). These silencing mechanisms are essential for the development of eukaryotic organisms as they are required for key developmental processes such as differentiation, imprinting and X-chromosome inactivation in the female organism (P. a. Jones 2007).

DNA methylation describes the covalent modification of DNA where a methyl group is transferred to the C-5 position of cytosine. These cytosines are located 5' to a guanosine in a CpG dinucleotide, resulting in 5-methylcytosines. CpG dinucleotides are enriched in so called "CpG islands", which are frequently found in the 5' promoter region of around 60% of our genes. While genomic DNA is heavily methylated at CpGs, the islands remain usually unmethylated in somatic cells, thus enabling active gene expression (Bird 2002).

In 1983 Andrew Feinberg and Bert Vogelstein were the first who compared the DNA methylation pattern of normal and cancer cells and could identify a global

loss of DNA methylation in cancer (Feinberg and Vogelstein 1983). Hypomethylation in cancer generally leads to gene activation and affects oncogenes such as HOX11 in T-cell lymphoblastic leukemia (Watt 2000); KRAS, BRAF and PIK3C in epithelial ovarian cancer (Kwon and Shin 2011); c-Myc and H-RAS in gastric and colon cancer cells (Luo 2010) but also DNA repeat sequences, including retrotransposons, retroviral DNA and heterochromatic regions (Ehrlich 2002). However, oncogenesis does not only involve loss but also gain of methylation. Promoter hypermethylation of tumour suppressor genes such as PTEN in endometrial cancer (Salvesen 2001) and colorectal cancer (Goel 2004) or CDKN2A in colorectal cancer (Shima 2011), to name only two examples, is frequently observed in a variety of sporadic cancers (Jones and Baylin 2002). In addition to genetic mutations and deletions, hypermethylation is a mechanism of aberrant gene silencing.

Besides DNA methylation, which is probably the best-known epigenetic marker, I would like to discuss histone modifications, since this mechanism is also relevant for the present study. In order to fit the long stretches of DNA into the eukaryotic nucleus, the DNA has to be compacted. One step of this packaging process involves the complexation of DNA with histone proteins. These form nucleosomes, which are the basic repeat unit of chromatin. Each nucleosome consists of an octameric, globular structure of the core histone proteins H2A, H2B, H3 and H4, and a short stretch of DNA comprising about 146 bases (Wolffe 1999), which is wrapped around this core (Annunziato 2008). The evolutionarily highly conserved N-terminal structures of the histones are protruding outward from the nucleosome core. These histone tails are targets for a variety of different posttranslational modifications, including acetylation, phosphorylation and methylation, which lead to activation or repression of the underlying chromatin and thereby either activate or repress transcription of the respective gene (Fischle, Wang and Allis 2003). Chromatin is a highly dynamic structure, where histone marks are set but can also be removed again to meet the requirements for different cellular processes such as DNA replication, DNA repair and to spatially and temporarily coordinate the gene expression machinery (Fischle, Wang and Allis 2003). These modifications are set by different epigenetic histone modifying enzymes or “writers”: acetyl groups are added to lysine residues by histone acetyltransferases (HATs), serine and

threonine residues become phosphorylated by kinases and HMTs can either methylate arginine or lysine residues. The majority of posttranslational modifications occur on the histone tails. However, some modifications can occur in the globular domain as well. These include mainly ubiquitylation, sumoyilation and methylation. Besides the writer-enzymes, which are responsible to set the covalent histone marks, there are also “reader”-enzymes, which are detectors that specifically recognize histone modifications and “erasers”, which are required to remove these marks again. Depending on the type of covalent modification and the residue, which is affected, histone marks can either have activating or repressing function on transcription. Generally, acetylation, arginine methylation but only some lysine methylations are associated with transcriptional activation. Lysine residues can either be mono-, di- or tri-methylated and it was shown that trimethylated residues of histone H3 and H4 appear to be chemically more stable as they can be propagated during cell division (Lachner 2004). While acetylation of H3K4 (lysine residue number 4 on histone H3) and H3K36 are active marks that are especially important for transcription activation and elongation, methylation of H3K9 leads to transcriptional repression and methylation of H3K27 is involved in Polycomb repression, early B-cell development and X-chromosome inactivation (Allis, Jenuwein and Reinberg 2006). The sum of covalent histone modifications on the different residues provides a “histone code”. This code is not likely to be universal such as the genetic code, since chromatin modifications are highly dynamic and can vary from one organism to the next. However, specific modification patterns have been identified, which do appear to contribute to distinct biological functions (Henikoff 2005).

3.2. Polycomb group proteins and the role of EZH2

The adult mammalian organism comprises about 200 distinct cell types. Each cell type is thereby defined by a specific gene expression profile. This means that appropriate sets of genes are either kept active or silenced in order to meet the requirements for the function and identity of a specific cell type. To ensure proper biological function, it is essential that a cell remembers and reproduces its respective gene expression profile after each cell division. This cellular

memory is essential for development and differentiation but also for adult tissue homeostasis and regulated by the families of Polycomb group proteins (PcGs) and Trithorax group proteins (TrGs). Among others, PcGs and TrGs are essential regulators for the expression of the HOX-gene family and thus play an important role during development (Pasini, Bracken and Helin 2004). Both protein families are organized into large multimeric complexes that act on their target genes by modulating the structure of the underlying chromatin. While PcGs function as transcriptional repressors and are involved in the maintenance of embryonic and adult stem cells by repressing a number of developmental regulators that are required for differentiation (Boyer 2006); (Lee 2006), TrGs act as transcriptional activators. Trithorax allows the gene to remember the state “ON” after cell division and enables active transcription. PcGs play an important role in the control of cell fate and they allow the cell to memorize and reproduce the repressed state “OFF” after each cell division (Allis, Jenuwein and Reinberg 2006). Any changes in chromatin structure can result in altered gene expression and genomic instability. Thus, chromatin modifying enzymes, such as the family of Polycomb, are key players in cancer development and have to be tightly controlled. Imbalances can result in the repression of key tumour suppressor pathways and their role in the regulation of stem cell maintenance might contribute to their oncogenic function as well (Sparmann and van Lohuizen 2006).

Mammalian PcGs are organized in two large complexes: Polycomb repressive complex 1 (PRC1) and PRC2. The writer enzyme that subsequently methylates H3K27 is EZH2, which is the catalytic subunit of PRC2 that harbours HMT-activity within its SET domain. As such, the major function of EZH2 is the trimethylation of H3K27 and to a lesser extent also H3K9 (Sparmann and van Lohuizen 2006, Czermin 2002). Through its chromodomain, PRC1 is able to recognize and interact with trimethylated H3K27 and thereby brings neighbouring nucleosomes into the proximity of PRC2, which enables the spread of methylation over distant chromosomal regions and ensures the maintenance of methylation (Schwartz 2006). EZH2 was shown to be upregulated in advanced stages of several human tumours including prostate and breast cancer as well as multiple types of lymphoma (Kleer 2003, Varambally 2002, Visser 2001). This high EZH2 expression was

correlated with poor prognosis due to its consistent repression of a cohort of genes via H3K27me3 and H3K9me3 as well (Sparmann and van Lohuizen 2006). Among the genes, which are repressed by EZH2 are several transcription factors and cell cycle regulators resulting in the enhancement of cell survival and metastasis of cancer cells (Varambally 2002). Varambally et al. showed that knockdown of EZH2 in prostate cancer cells resulted in growth arrest and reduced tumour growth.

3.3. Anaplastic large cell lymphoma

ALCL is an aggressive T-cell non Hodgkin lymphoma (Sandlund 1994). According to the new edition of the WHO classification (Swerdlow 2008), ALK negative (ALK^-) and ALK positive (ALK^+) ALCL are recognized as two types of systemic ALCL. The cutaneous variant of ALCL, which was originally described as systemic ALCL entity by Stein et al. (Stein 1985), has now been recognized as a different disease (Ralfkiaer 2008).

Cell transformation in ALK^+ ALCL is due to the constitutive expression of the oncogenic ALK, a receptor tyrosine kinase, which is mainly involved in neuronal cell differentiation and regeneration, synapse formation and muscle cell migration during mammalian development (Chiarle 2008). The constitutive expression of ALK is caused by chromosomal translocations involving the corresponding ALK gene and a number of partner genes, which result in the formation of oncogenic fusion proteins (Piccaluga 2010). The most common and predominant translocation t(2;5)(p23;q35), which is found in about 85% of cases involves the fusion of ALK with the multifunctional protein nucleophosmin-1 (NPM1) (Stein 2000). Constitutive ALK signalling has clear oncogenic potential as it enhances proliferation and survival by altering several signalling cascades such as PI3K and STAT pathway (Chiarle 2008).

On the basis of their morphology, ALK^- ALCL is not distinguishable from ALK^+ ALCL. However, ALK^- ALCL lacks, as the name already indicates, ALK protein expression. Additionally, ALK^- ALCL patients are generally older with a median age around 60 years while ALK^+ ALCL affects patients within the first three decades of their life (Gascoyne 1999). Due to these differences ALK^- ALCL was accepted as a provisional entity of mature T-cell neoplasms by the WHO in

2008 (Swerdlow 2008). The underlying genetic alterations, which are causative for ALK^- ALCL remain unknown (Falini and Martelli 2009).

Both ALK^+ and ALK^- ALCL patients currently receive the same treatment based on doxorubicin-containing combination chemotherapy (Savage 2008). Cure rates are between 60 and 80% for ALK^+ patients while ALK^- ALCL patients appear to have a poorer prognosis. Savage et al. proposed in 2008 that this difference correlates with the patient's age. They could show that there is no difference in survival between ALK^+ and ALK^- patients under the age of 40 years.

3.4. The role of miR-101 in ALCL

MicroRNAs (miRNAs/miRs) are small, non-coding RNAs of roughly 19-24 nucleotides in length, which are endogenously expressed in animal and plant cells and control gene expression at the posttranscriptional level by causing either translational inhibition or mRNA degradation. Perfect complementarity of the miRNA with the respective mRNA leads to degradation and this mechanism is commonly found in plants. In animals, matches of miRNA and mRNA are mostly imperfect and lead to translational inhibition (Porkka 2007). Within the last years it has been demonstrated that miRNAs are important regulators of various biological processes such as cell proliferation (Cheng 2005), apoptosis (Xu 2004), development (Karp and Ambros 2005), differentiation (Chen 2004) and they play an essential role in the development of cancer. It has been estimated that about 30% of human genes are regulated by miRNAs and thereby each miRNA is thought to target several hundreds of genes (Rajewsky and Socci 2003), which highlights their impact of deregulation of cancer. Many microarray studies have been performed in order to analyze the differential expression profiles of miRNAs in normal versus tumour samples (Calin and Croce 2006) and identify miRNA signatures that enable classification, diagnosis or prediction of cancer patients.

The mechanism, which causes upregulation of EZH2 during cancer progression was long unclear until Varambally and colleagues proposed a role for miRNAs in their study from 2008. They demonstrated that genomic loss of miR-101 leads to the overexpression of EZH2 and specifically predicted two binding sites for the miRNA in the 3'UTR of EZH2. While miR-101 decreases during cancer

progression, EZH2 increases. Varambally et al. suggested that the reintroduction of miR-101 in prostate tumours could have positive impact on the therapeutic outcome by changing the epigenetic program to a more healthy state (Varambally 2008).

In 2010 Merkel and colleagues identified distinct miRNA profiles of ALK^+ and ALK^- ALCL compared to normal $CD3^+$ T-cells using cell lines and primary tumour tissues. Among the miRNAs, which were downregulated in primary patient samples of ALK^+ and ALK^- ALCL tumours they found miR-29a, miR-29b, miR-29c and also miR-101. They overexpressed miR-101 with the help of miRNA mimics and could show a significant reduction in cell number as well as increased apoptosis in ALK^+ ALCL cell lines but not ALK^- ALCL cell lines. Which they correlated to altered mTOR signaling (Merkel 2010).

3.5. Project description and hypotheses

The research group around Dr. Gerda Egger has shown recently that EZH2 is overexpressed in both ALK^+ and ALK^- ALCL cell lines and tissue patient samples when compared to PBMCs and normal lymphnode tissues. Thus, we hypothesized that EZH2 might have an essential role for ALCL generation and/or progression, as it was previously shown for different other tumour types such as prostate cancer. Generally, we aimed to investigate the role of EZH2 in both ALK^+ and ALK^- ALCL cell lines and test its usefulness as therapeutic target for the treatment of ALCL. We planned to inhibit EZH2 by using the following three approaches.

First, we reduced the levels of EZH2 and in turn also the repressive histone mark H3K27me3 using RNA interference (RNAi) directed against EZH2. We hypothesized that the reduction of histone methylation would reactivate regulatory genes, which where abnormally silenced during cancer progression and that this restoration of the gene expression profile would cause cell cycle arrest and apoptosis in cancer cells.

Second, we globally inhibited histone methylation by the usage of the inhibitor DZNep. DZNep is a S-adenosylhomocysteine hydrolase inhibitor that interferes with S-adenosyl-methione-(SAM)-dependant reactions. Originally it was published that DZNep disrupts the PRC2 complex and specifically inhibits H3K27me3 and H4K20me3 and induces apoptosis in cancer cells (Tan 2007). However, SAM is required for a variety of biological processes, thus it was rather surprising that it would only affect EZH2 function. Finally, in 2009 it was shown that DZNep acts as a global inhibitor of histone methylation and affects both repressive and active histone marks (Miranda 2009).

Additionally, Merkel et al. demonstrated that miR-101, which targets EZH2 amongst others, was downregulated in ALK^+ and ALK^- ALCL. Hence, our third experimental set-up was intended to overexpress miR-101 in ALCL cell lines and thereby cause indirect EZH2 reduction through miRNA regulation.

Our hypothesis was that all three experimental designs would result in attenuated cell proliferation and apoptosis of ALCL cells, since the reduction of EZH2 via siRNA knockdown, global inhibition of histone methylation or miRNA regulation would cause a reduction of the repressive histone mark H3K27me3 and thereby cause and imbalance histone methylation in the tumour cells.

4. MATERIALS AND METHODS

4.1. Media, solutions and reagents

4.1.1. Hunt buffer:

20mM Tris pH 8.0

100mM NaCl

1mM EDTA

0.5% NP-40 (10% stock solution)

Proteinase Inhibitor (50X stock; Roche) added shortly before usage.

4.1.2. 2x SDS loading buffer:

100mM Tris-HCL pH 6.8

200mM DTT

4% SDS

20% Glycerin

Bromphenolblue

filled up with Abd to 20ml.

4.1.3. PI-buffer

0,1% Na-citrate dehydrate

0,1% Triton X-100

0,1% RNAse (DNAse-free)

Diluted in 1x PBS. Sterile filtered through 0,2 µM filter. Store at 4°C.

4.1.4. PI stock solution

Dissolve 1 mg/ml PI in PBS, sterile filter through 0,2 µM filter. Store at 4°C.

4.1.5. Nuclear isolation buffer

10 mM Hepes, pH 7.4

150 mM NaCl

1.5 mM $MgCl_2$

0.5% NP-40

Filled up with Abd

4.1.6. Blocking milk

10g commercial milk powder

400 µl 10% NaN_3

Filled up to 200 µl with TBS-T. Store at 4°C.

4.1.7. Electrophoresis buffer

14,4 g Glycine

3g Tris

1g SDS

in 1L Abd.

4.1.8. Transfer buffer

200 ml Methanol

2,5 g Tris

11,2 g Glycine

in 1L Abd.

4.1.9. 10xTBS (0.5 M)

60,5 g Tris-Base

90 g NaCl

Adjust with HC to pH 7.5

(TBS-T: 0,1% Tween-20)

4.1.10. 10x PBS

28,8 g Natriumhydrogenphosphat-dihydrat

5,2 g Natriumdihydrogenphosphat-monohydrat

90 g NaCl

Adjust to pH 7.2-7.6

fill up with Abd to 1L

4.1.11. SDS-polyacrylamide gels

	5%	12%	15%	Stacking gel
H_2O	22 ml	13.2 ml	9.2 ml	13.6 ml
30% acrylamide mix	6,8 ml	16 ml	20 ml	3.4 ml
1.5 M Tris pH 8.8	10 ml	10 ml	10 ml	-
1 M Tris pH 6.8	-	-	-	2.5 ml
10% SDS	0.4 ml	0.4 ml	0.4 ml	0.2 ml
10% ammonium persulfate	0.4 ml	0.4 ml	0.4 ml	0.2 ml
TEMED	2 µl (per gel)	1 µl (per gel)	1 µl (per gel)	20 µl (per 20 ml)

4.2. Cell lines

Both the ALK^+ cell lines Karpas-299 and SR786 and the ALK^- cell line Mac-2a were grown in RPMI (GIBCO) supplemented with 10% FCS (GIBCO) and 1% Penicillin/Streptomycin (GIBCO) at 37°C and 5% CO_2. Cells were seeded at a concentration of $5x10^5$ per ml of culture medium and split every 2-3 days. All three cell lines were kindly provided by Dr. Lukas Kenner.

4.3. Isolation of peripheral blood mononuclear cells

PBMCs were isolated with Lymphoprep Kit (Axis-shield) according to the supplier's protocol.

4.4. Bradford Assay

For the determination of histone and protein concentrations Bradford Assay (Coomassie Plus (Bradford) Protein Assay, Thermo Scientific) was performed according to the supplier's protocol. A standard curve was generated with BSA (Sigma) using the following concentrations: 0.5 mg/ml, 1 mg/ml, 2 mg/ml, 4 mg/ml, 6 mg/ml, 8 mg/ml, 10 mg/ml.

4.4.1. Histone isolation

Cells were harvested and pelleted by centrifugation. Each pellet (1-3 x 10^6 cells) was carefully resuspended in 1 ml of ice-cold nuclear isolation buffer and 20 µl of 50x protease inhibitor (protease inhibitor cocktail tablets, Roche) and centrifuged with 500g, 4°C for 5 min. The supernatant was discarded and the pellet was resuspended in 100 µl 0.4N H_2SO_4 by vortexing followed by incubation on ice for 1 hour. Basic histones were collected by centrifugation at 7500 rpm, 4°C for 5 min. The supernatant was collected and proteins were precipitated by the addition of 1 ml ice-cold acetone and incubation o/n at -20°C. Precipitates were collected via centrifugation for 10 min at 13000 rpm and 4°C. Pellets were washed with 500 µl acetone and again centrifuged for 3 min at 13000 rpm and 4°C. The supernatant was discarded and pellets were dried at RT for a few minutes. Depending on original pellet size, pellets were resuspended in 50-100 µl sterile water. Histone concentration was measured with Bradford-Assay and histones were separated via SDS-PAGE using 12-15% polyacrylamide gels (see also: Western Blot).

4.5. FACS

For cell cycle analysis, 5 x 10^5 cells were harvested and resuspended in 100 µl PBS, 500 µl PI-buffer, 5 µl RNAse I, DNAse-free (Roche) and stained with 5 µl PI (Sigma). Cells were incubated at RT for 1 hour. Cell cycle analysis was performed with BD FACSDiva.

4.6. RNAi

Optimal concentrations for siRNA gene silencing were evaluated with the Hs Cell Death Reagent (Qiagen, RNAi human/mouse starter kit). The day before transfection, cells were seeded at a concentration of 3x10^5 cells per ml of culture medium with antibiotics into a T75 flask and allowed to grow overnight. The following day 6x10^5 cells were seeded per well of a 6-well plate in 600 µl RPMI (10% FCS, 1% Pen/Strep). For each well 75 nmol siRNA (Negative Control, MAPK1, EZH2) and 9 µl HiPerFect reagent were mixed in 100 µl RPMI

without serum and incubated for 10 minutes at RT to allow complex formation. Then complexes were added dropwise to the cells. After five hours incubation at 37°C, 5% CO_2 1,2 ml of RPMI with FCS and antibiotics were added. Cells were fed as required, usually 2 ml of medium were added after 48 hours incubation. For further analysis, cells were harvested 48 and/or 72 hours after treatment. (Protocol was implemented and adapted based on the supplier's recommendations.)

4.7. RNA isolation

RNA, from both cells and tissue samples, was isolated with TRIzol Reagent (Invitrogen) according to the supplied protocol. RNA pellets were resuspended in 30 µl Nuclease-free water and concentrations were determined using the Nanodrop (Peqlab). Purified RNA was stored at -80°C until further use.

4.8. Protein Extraction

Cells were harvested, washed and centrifuged. Pellets of approximately 1-3x10^6 cells were resuspended in 100 µl HUNT buffer and shock-frozen in liquid nitrogen. Then cells were quickly thawed at 37°C and again shock-frozen in liquid nitrogen. Extracts were allowed to thaw on ice for no longer than 15 min and then centrifuged (10 min, 13.000 rpm, 4°C). Supernatants, containing proteins, were collected and protein concentration was determined via Bradford Assay.

4.9. Western Blot

Protein samples (60 µg total protein; 20 µg for histones) in a maximal volume of 40 µl were heated for 5 minutes at 95°C in SDS loading buffer and separated on a gradient gel (5%-15% polyacrylamide gel for separation of normal protein; 12-15% gradient gels for histone separation) at 120V. Separated samples were blotted onto a nitrocellulose membrane (120V, 2 hours) and then initially visualized with Ponceau Red to control for equal loading of samples. Membranes were blocked for 30 min in either blocking milk or 5% BSA/TBS-T

(depending on the antibody used) on the shaker. Primary antibody was added using the recommended dilution and incubated overnight at 4°C on the shaker. The next day membranes were washed thrice with TBS-T and then incubated for 1 hour at RT with the appropriate secondary antibody. Again the membrane was washed thrice with TBS-T. Proteins were visualized with AceGlow (Peqlab) and analyzed on LumiAnalyst (Roche).

4.10. Immunofluorescence stains

For immunofluroescence staining of cells, approximately 100 µl of cell suspension (depending on cell density; approximately 80% of microscope section should be covered) was centrifuged onto a microscope slide in the Cytospin (300 rpm, 5 min). Cells were shortly dried at RT and then fixed in ice-cold acetone for 10 min. After a quick wash in 1xPBS, cells were blocked with 10% goat serum/PBS (2nd Molecular Probes) for 20 min at RT. Slides were covered with appropriate dilutions (according to supplier's recommendations) of the primary antibody in PBS/2% BSA (Sigma) and incubated at 4°C o/n. Primary antibody was removed by washing thrice with PBS for 5 min each. After 1 hour incubation with appropriate dilutions of the secondary antibody and quick staining with DAPI (1:50000 dilution, Serva Feinbiochemica, 1 min incubation at RT) sections were covered with geltol mounting medium.

4.11. Antibodies

4.11.1. Primary Antibodies

Anti-(n-terminal)-EZH2, polyclonal, rabbit (Sigma, E7031)
Dilutions: Western Blot 1:1000

Anit-H3K27me3, monoclonal, mouse (Abcam, ab6002)
Dilutions: Western Blot 1:1000, Immunofluorescence 1:200

Anti-H3, polyclonal, rabbit (Millipore, 06-755)
Dilutions: Immunofluorescence 1:200

Anti-ß-actin, monoclonal, rabbit (Cell signaling, 4967S)
Dilutions: Western Blot, 1:1000

4.11.2. Secondary Antibodies

Polyclonal rabbit anti-mouse IgG (HRP) (DakoCytomation, R0270)
Dilutions: Western Blot 1:10000

Fox F(ab')2-fragment goat anti rabbit IgG (Invitrogen, L43000)
Dilutions: Western Blot 1:10000

AlexaFlour 594, goat-anti-mouse-IgG (Invitrogen, A-11032)
Dilutions: Immunofluorescence 1:1000

4.12. cDNA synthesis and Poly-A Tailing

After RNA isolation, reverse transcription and Poly-A Tailing (NCode VILO miRNA cDNA Synthesis Kit, Invitrogen) was performed. The addition of a Poly-A tail to the 5' end of the RNA enabled the usage of an universal qPCR primer. For the reaction the 5X reaction Mix (4 µl), 10X SuperScript Enzyme Mix (2 µl), RNA (1 µg) and DEPC water (up to 20 µl) were mixed together, vortexed and briefly centrifuged to collect the contents. After synthesis of cDNA (60 min, 37°C; 5 min, 95°C) contents were diluted 1:1 with sterile water and 2 µl were used for each PCR reaction. Samples were stored at -20°C.

4.13. Realtime-PCR

Each RT-PCR reaction contained the following components: 10 µl FASTA Kapa Mastermix, 1 µl Primer-Mix (10pmol/µl forward + reverse), 2 µl cDNA, 7 µl H2O.

RT-PCR program:

1. 95°C 3min
2. 95°C 30 sec
3. 60°C 30 sec
4. 72°C 30 sec

+ Plate read

Repeat from step 2. 39x

5. END

4.13.1. RT-PCR primer sequences

EZH2 human (Eurofins MWG Operon)

Forward: 5'-CGA TGA TGA TGA TGG AGA CG-3'

Reverse: 5'-GCT GTC CCC TTA TCT GGA AA-3'

miR-101 human (Invitrogen)

Forward: 5'-CCG GTA CAG TAC TGT GAT AAC TGA A-3'

Reverse: universal primer (Invitrogen)

miR-92 human (Invitrogen)

Forward: 5'-TAT TGC ACT TCT CCC GGC CT-3'

Reverse: universal primer (Invitrogen)

MAPK-1 human (Eurofins MWG Operon)

Forward: 5'-CAC CAA CCT CTC GTA CAT CG-3'

Reverse: 5'-TCT CAT GTC TGA AGC GCA GT-3'

GAPDH human (Eurofins MWG Operon)

Forward: 5'-GGT GGT CTC CTC TGA CTT CAA CA-3'

Reverse: 5'-GTT GCT GTA GCC AAA TTC GTT GT-3'

4.14. DZNep

DZNep (Cayman chemicals) was diluted with DMSO to a stock concentration of 10 mM and aliquots were stored at -20°C. Shortly before use, the stock was further diluted with 1xPBS to give 100 µM working concentration. $1x10^6$ cells were seeded in 2 ml RPMI (10% FCS, 1% Pen/Strep) per well of a 6-well plate. For optimization of treatment the following concentrations and their effects on cell viability and proliferation were initially assessed: 0,3 µM; 0,6 µM; 1,2 µM; 2,4 µM; 4,8 µM. Since 1,2 µM and 2,4 µM showed the strongest effects, these concentrations were chosen for subsequent experiments. Appropriate amounts of inhibitor were added dropwise to the cells. Cells were fed as required, usually 2 ml of DZNep-containing RPMI cell culture medium were added 48 hours after treatment. During optimization, daily trypan blue cell counts were performed (1:1, trypan blue:cell suspension) with a counting chamber.

4.15. PCR-Cloning

In order to amplify the miR-101 genomic sequence, primers amplifying approximately 500 bp surrounding the mature miRNA were designed.

Primer sequences:

Mir-101_noGFP_Jeff_fo: GATAATCTCGAGATGACAGAGGTGCAGGGTAA

Mir-101-GFP-Jeff_fo: GATAATACTAGTATGACAGAGGTGCAGGGTAA

MiR-101-Jeff_re: GTAATAGGATCCGTCTCCAACCAGAAGGTGAT

The sequences were additionally linked to restriction enzyme consensus sites XhoI, SpeI, BamHI, to allow for the direct cloning of the amplicon after restriction into the lentiviral expression vector Len-EF-GFP-CD24.

For PCR 2X Phusion MasterMix (Finnzymes, 10 µl), Primer Mix containing forward and reverse primer (1 µl), DNA (2 µl) and water (7 µl) were mixed together on ice.

4.15.1. PCR-program:

1. 98°C 10 sec
2. 60°C 30 sec
3. 72°C 30 sec
   Repeat 30x
4. 72°C 10 min

4.16. DNA precipitation

DNA phenol-chloroform-precipitation was performed for PCR product purification. Two volumes of water were added to one volume of PCR sample. Then phenol-chloroform-isoamylalcohol was added 1:1 and the mixture was vortexed and centrifuged for 3 minutes at 13.000 rpm. The aqueous phase was transferred to a fresh tube and DNA was precipitated with 1/10 volume of sodium acetate and 2,5 volumes of ethanol at -80°C for 1 hour. Afterwards DNA was pelleted by centrifugation for 15 minutes at 4°C. The pellet was washed with 1 ml of 70% ethanol and air-dried for 10 minutes before it was diluted in 20 µl of distilled water. DNA concentration was measured on the Nanodrop and DNA stored at -20°C until further use.

4.17. Restriction digest

For restriction digest of lentiviral vectors GFP only, miR-101-GFP and miR-101 the following components were mixed together and then incubated at 37°C for at least 1 hour:

5 µl	DNA
2 µl	10X Buffer (Buffer 4, BioLabs)
1 µl	Enzymes Xho I (BioLabs), BamHI (BioLabs), SpeI (BioLabs)
0.5 µl	BSA (BioLabs)
x µl	DEPC water (Invitrogen)
20 µl	

Digests were analysed on a 2% agarose gel (1x TAE buffer).

4.18. Gel extraction

After restriction digest samples of insert and vector were loaded onto a 1% agarose gel and bands were excised with a scalpel. The gel slice was weighed and 3 volumes of Buffer QG (Qiagen QIAquick spin column kit) were added to 1 volume of gel. Samples were incubated at 50°C for 10 minutes and vortexed until the gel slice had completely dissolved. Afterwards one volume of ispropanol was added to the sample and mixed. In order to bind the DNA, samples were applied to a QIAquick spin column and centrifuged for 1 minute at 13.000 rpm. After the flow-through was discarded QIAquick spin column was placed into the same collection tube and 500 µl of QG buffer were added and centrifuged for 1 minute at 13.000 rpm. To wash the column 750 µl of buffer PE were added to the center of the spin column and centrifuged for 1 minute. The flow-through was discarded and spin columns were centrifuged for additional 1 minute in order to remove and residual ethanol. Spin columns were placed into a fresh Eppendorf tube and 30µl of elution buffer were added directly to the center of the membrane. After 1 minute incubation at room temperature, tubes were centrifuged for 1 minute. DNA concentration was analyzed with the Nanodrop and samples could be stored at -20°C until further use.

4.19. Ligation

For ligation, thrice the amount of insert than vector was used. To estimate the DNA concentration, inserts and vector were separated on an agarose gel. According to this, insert and vector were mixed with Ligase (NEB) and 10X Ligase buffer (NEB) and incubated at 16°C overnight. The following day transformation could be performed.

4.20. Transformation

A tube of competent E.coli cells (BioLabs) was thawed on ice for 10 minutes. Plasmid DNA (1-5 µl containing 1pg-100ng DNA) was added to 50µl of competent cells. In order to mix cells and DNA the tube was carefully flicked 4-5 times, then the mixture was placed on ice for 30 minutes. Afterwards a heat shock was performed at exactly 42°C for 30 seconds. Again tubes were placed

on ice for 5 minutes and then pre-warmed SOC medium (200µl per 50µl of competent cells) was added. Then the mixture was incubated at 37°C for 60 minutes in the shaker at 250rpm. Selection plates, containing the appropriate antibiotic, were pre-warmed to 37°C. After one hour incubation, cells were plated onto a selection plate and incubated overnight at 37°C.

4.21. Miniprep

Bacterial cultures (2 ml LB medium) were set up and inoculated with one single colony of transformed (vector GFP only, miR-GFP, miR only) E.coli cells each. Cultures were incubated overnight at 37°C in the shaker at 200 rpm. The next day, cultures were visually inspected for bacterial growth and then 1,5 ml of culture were centrifuged for 2 minutes at 7.000 rpm. Supernatants were discarded by inverting tubes and pellets resuspended in the remaining LB medium by vortexing. For cell lysis 300 µl TENS buffer was added and tubes shaked sharply. Afterwards, 150 µl KAc was added in order to precipitate cell components and genomic DNA. Tubes were centrifuged for 5 minutes at 13.000 rpm and supernatants transferred to fresh tubes. Then EtOH (900 µl) was added for plasmid precipitation and incubated at -80°C for at least 30 min. Again tubes were centrifuged for 5 min at 13.000 rpm in order to collect plasmid DNA. Supernatants were discarded and pellets washed with 70% ethanol (900 µl). After another centrifugation (5 min, 13.000 rpm) ethanol was removed completely and pellets were air-dried at 37°C for approximately 10 minutes. The pellets were dissolved in 30-50 µl TE buffer. Plasmid DNA could be stored at -20°C until further use.

4.22. Retrograde transfection and virus precipitation

For each sample 6 x 10^6 293FT cells were used. Plasmid DNA (12 µg) was diluted in 1.5 ml of Opti-MEM I Medium without serum and mixed gently. Then, 36 µl of Lipofectamine 2000 were diluted in 1.5 ml of Opti-MEM I Medium without serum. The mixture was incubated for 5 minutes at room temperature and afterwards, the diluted DNA was combined with the diluted Lipofectamine 200 mix and incubated for another 20 minutes at room temperature. In the

meanwhile, 293FT cells were trypsinzed, counted and seeded at a cell density of 1.2 x 10^6 cells/ml in growth medium containing serum and without antibiotics. The DNA-Lipofectamine 2000 complexes were added to a 10 cm tissue culture plate containing 5 ml of growth medium containing serum, without antibiotics. To this plate, 5 ml of 293FT cell suspension, containing a total of 6 x 106 cells, was added, gently mixed and incubated overnight at 37°C in the incubator. The following day, the medium, containing the DNA-Lipofectamine 2000 complexes were replaced with complete culture medium containing sodium pyruvate (D-MEM containing 10% FBS, 2 mM L-glutamine, 0.1 mM MEM, Non-Essential Amino Acids, 1% penicillin/streptomycin, and 1 mM MEM Sodium Pyruvate). Virus containing supernatants were harvested 48 hours post transfection. After centrifugation at 3000 rpm for 15 minutes at 4°C to remove cell debris, virus was precipitated according to supplier's protocol (BioVision, PEG Virus Precipitation Kit). Precipitated virus was aliquoted and stored at -70°C for further use.

4.23. Transduction

The day before transduction, 1 ml of cell suspension (Karpas-299, SR786 and Mac-2a) was seeded to each well of a 12 well plate at a density of either 3.6 x 10^5 (medium containing 5 µg/ml Blasticidin) or 7.2 x 10^5 cells/ml (medium containing 10 µg/ml Blasticidin). To each well 20 µl of the respective SUP (GFP only, miR-GFP, miR only) was added. The following day, the medium was changed. Cells were kept under Blasticidin selection. Transduction showed an efficiency of 80%.

5. RESULTS

The research group around Dr. Gerda Egger has shown recently that EZH2, which is the catalytic subunit of PRC2 and carries HMT-activity within its SET domain, was overexpressed in both ALK^+ and ALK^- ALCL cell lines and patient tumour samples when compared to PBMCs and normal lymphnode tissues (Fig. 1; Melanie Hassler et al., unpublished data). High expression of EZH2 was also shown for a variety of other cancer types including prostate and breast cancer and was correlated with a poor clinical outcome (Sparmann and van Lohuizen 2006).

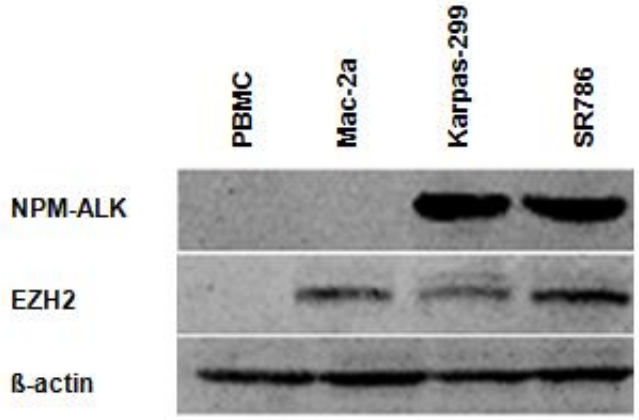


Figure 1. EZH2 protein expression in ALCL cell lines and PBMCs. Western Blot analysis revealed that EZH2 is overexpressed in the ALK^- ALCL cell line Mac-2a as well as in the ALK^+ cell lines Karpas-299 and SR786 when compared to normal PBMCs.

Analysis of the repressive histone marks H3K27me3 and H3K9me3 showed that also here the expression is increased in ALK^- ALCL and highly upregulated in ALK^+ tumours (Fig. 2; Melanie Hassler et al., unpublished data). Thus, the overexpression of EZH2 in ALCL correlated with elevated repressive histone marks in primary tumour tissues.

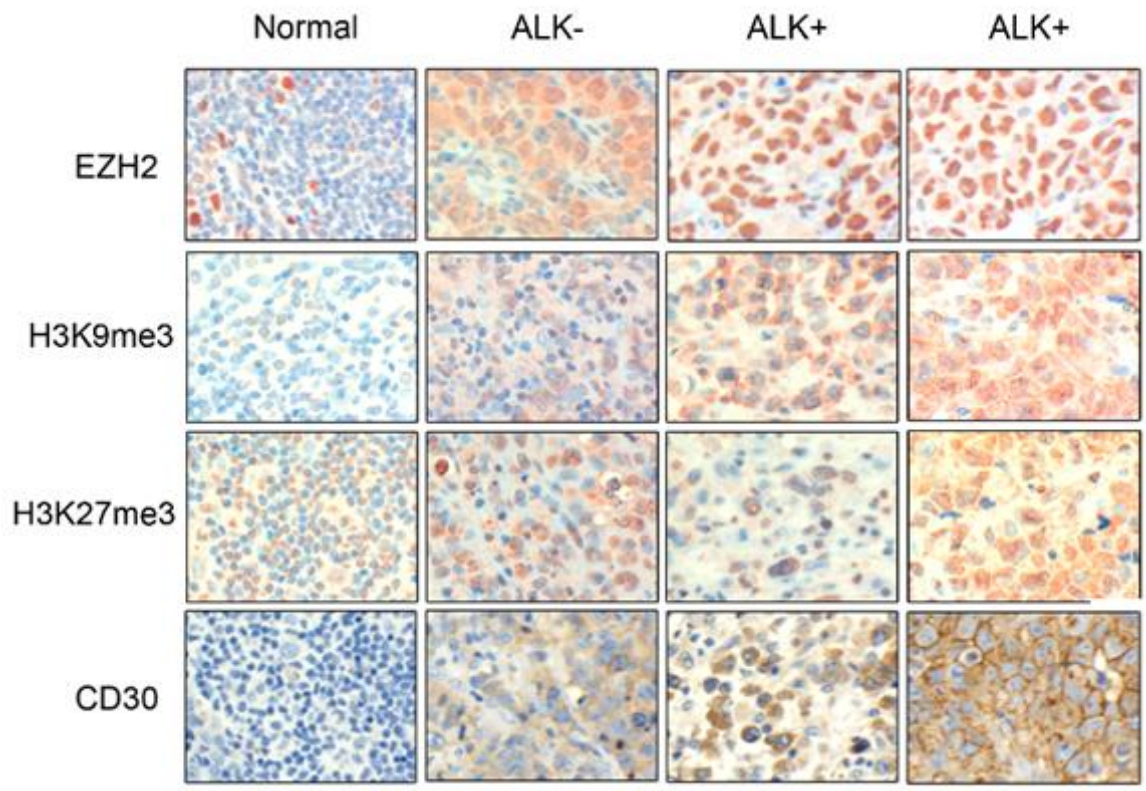


Figure 2. Immunohistochemistry. Immunohistochemical stains of both ALK^+ and ALK^- human ALCL tumours compared to normal lymphnode tissue. CD30 staining was used to assess the degree of ALCL cells in the tumour sections.

5.1. EZH2 knockdown

In our first attempt, we aimed to specifically reduce the elevated levels of EZH2 and in turn also the histone mark H3K27me3. We expected that the reduction of histone methylation would reactivate regulatory genes, which where abnormally silenced during cancer progression and that this restoration of the gene expression profile would cause cell cycle arrest and apoptosis of cancer cells. We treated the three cell lines Mac-2a, Karpas-299 and SR786 with 75 nmol of EZH2 siRNA and HiPerFect reagent (Qiagen) for a time period of 48 to 72 hours. Both mRNA and protein levels where significantly reduced 48 hours after treatment (data not shown) and these effects were further enhanced after 72 hours in Karpas-299 and SR786 (Fig. 3). Interestingly, EZH2 protein levels were less reduced in the ALK^- ALCL cell line Mac-2a after 72h of transfection, although we detected an 3,5-fold decrease of EZH2 mRNA levels. This might be due to increased EZH2 protein stability in this cell line.

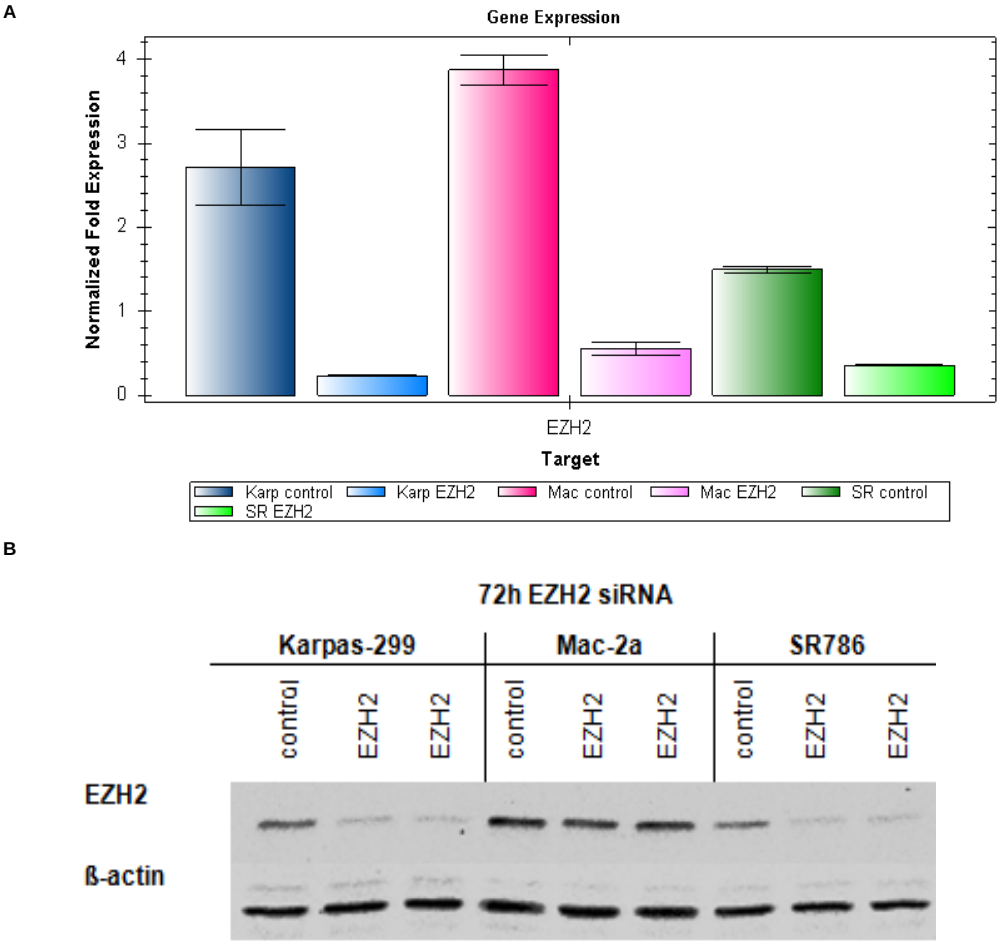
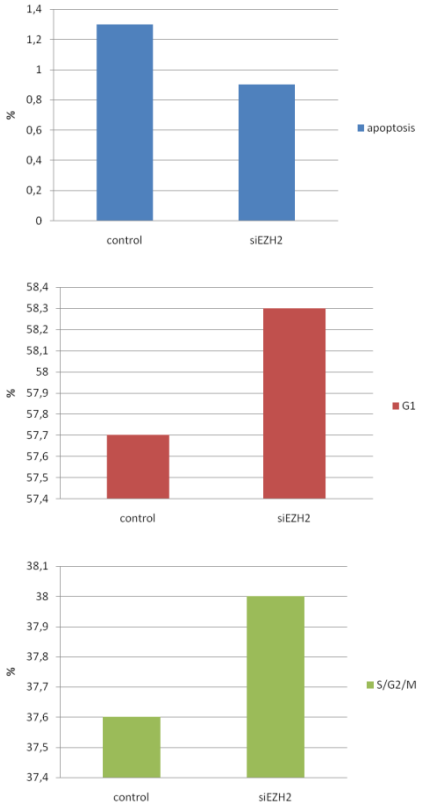


Figure 3. siRNA knockdown of EZH2 in ALK$^+$ and ALK$^-$ ALCL cell lines. (A) 72 hours after treatment, EZH2 mRNA levels were significantly reduced in all three cell lines when compared to negative control. (B) Western Blot analysis of EZH2 revealed significant protein reduction in the ALK$^+$ ALCL cell lines Karpas-299 and SR786 but not in the ALK$^-$ cell line Mac-2a.

When we performed cell cycle analysis 48 hours and 72 hours after treatment for all three cell lines we could not detect a significant increase in apoptotic cells (Fig. 4, data shown for 72 hours only). Overall, cell proliferation was not reduced in case of Mac-2a and SR786. However, Karpas-299 appeared to be less proliferative at both time points as indicated by a reduction of S-phase after siRNA treatment. In contrast to what we had expected, the targeted knockdown of EZH2 did not result in cell cycle arrest in any of the three ALCL cell lines.

Mac-2a



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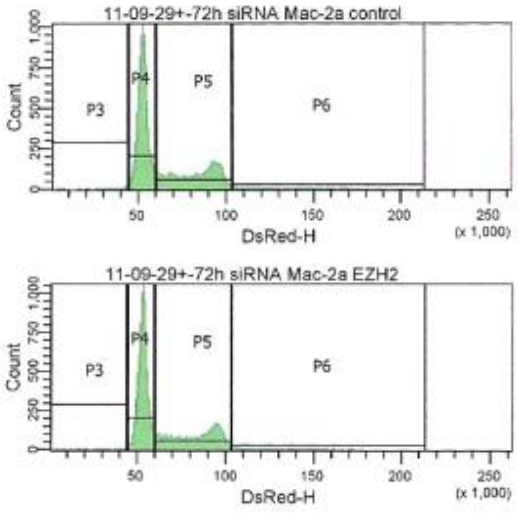
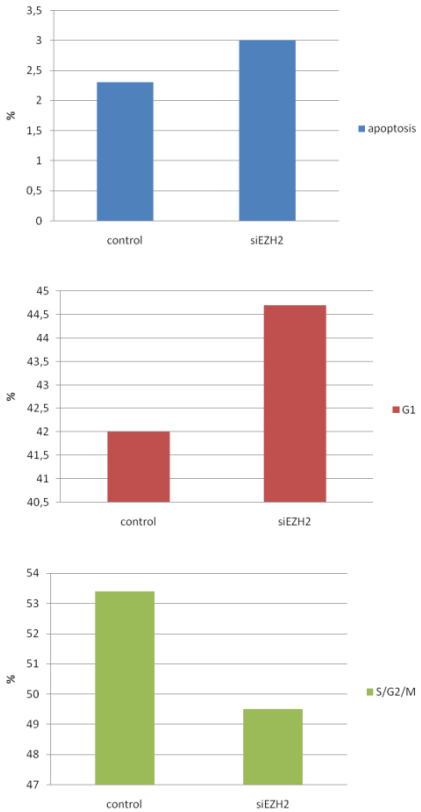
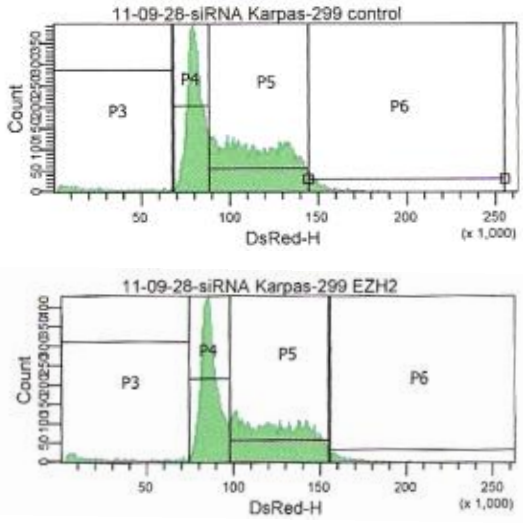


Karpas-299





SR786

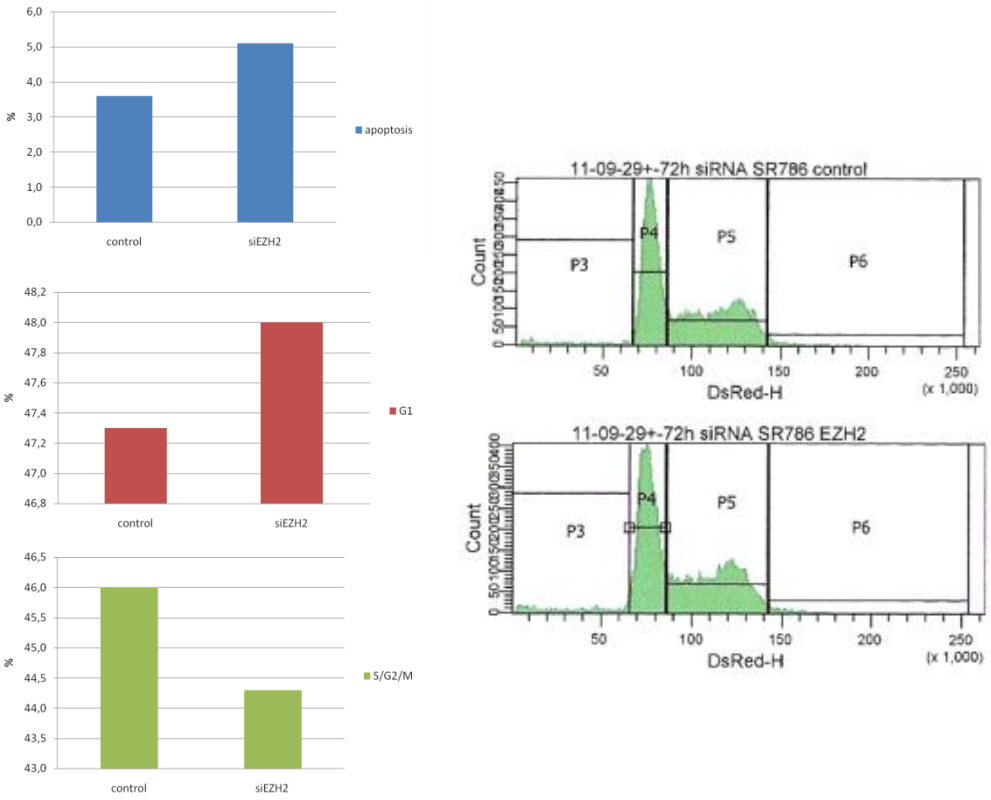


Figure 4. Cell cycle analysis after siEZH2 treatment. FACS data of control siRNA versus EZH2 siRNA treatment after 72 hours for cell lines Mac-2a, Karpas-299 and SR786. Schematic representation of cell cycle distribution 72 hours after treatment on the left. Corresponding histograms on the right P3 = apoptosis; P4 = G1 phase; P5 = G2/M/S phase.

Since FACS analysis revealed a slight reduction in cell proliferation for Karpas-299, we hypothesized that effects may simply require more time to evolve. Thus, we wanted to investigate long-term effects of EZH2 knockdown. Karpas-299 were treated with EZH2 siRNA as usual. After 72 hours of incubation, cells were transfected a second time with siRNA. Daily trypan blue staining and cell counts were performed over a time period of 168 hours for the double-treated Karpas-299 and revealed a decrease in the overall cell number, which was highest 144 hours after treatment (Fig. 5).

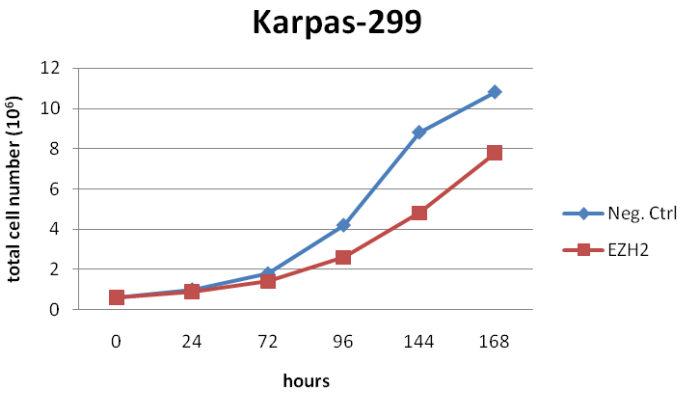


Figure 5. EZH2 siRNA double treatment of Karpas-299. EZH2 knockdown was performed in Karpas-299 with a repetitive treatment after 72 hours. Cell counts were performed over a time-course of 168 hours. Total cell numbers (10^6) are shown for negative control (blue) and EZH2 siRNA treated (red) Karpas-299.

Additionally we wanted to investigate whether siRNA double-knockdown of EZH2 affected H3K27me3 in Karpas-299. After a total of 144 hours incubation, cells were stained against H3K27me3 (Fig. 6). We could observe a significant reduction of the repressive histone mark in double treated cells when compared to negative control cells.

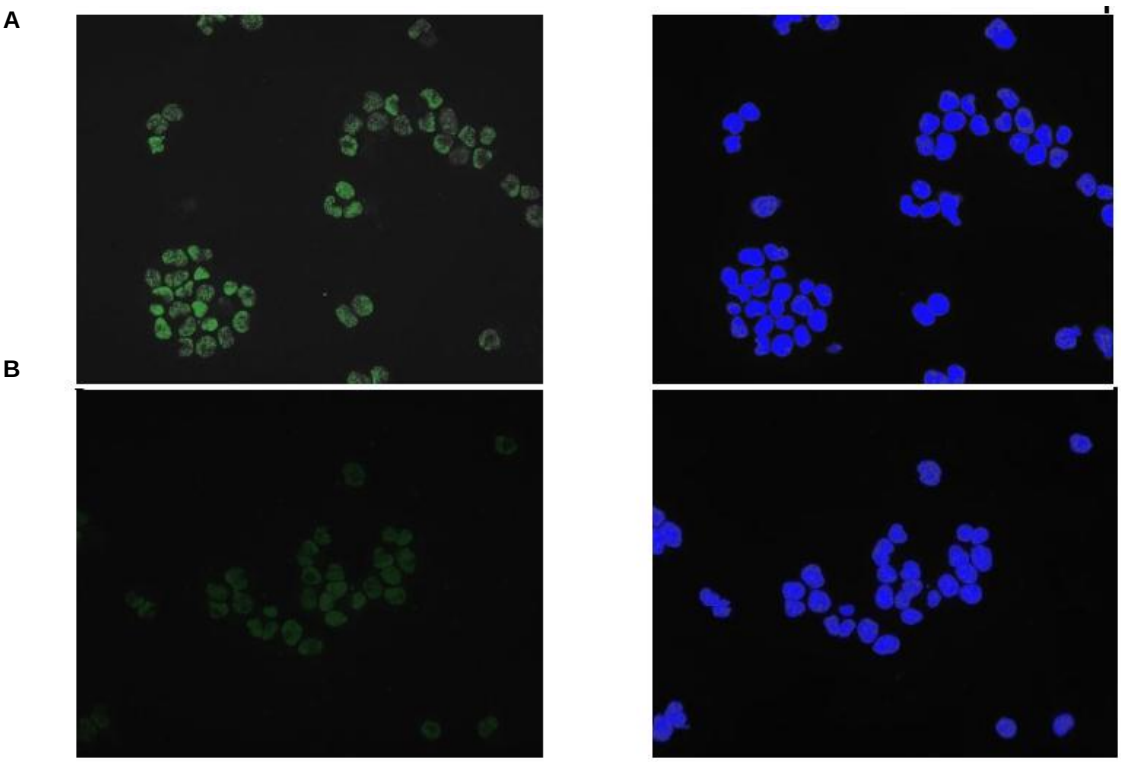


Figure 6. H3K27me3 of Karpas-299 after EZH2 siRNA treatment. (A) Negative control cells stain high against H3K27me3. (B) Double treatment siRNA cells show significant reduction in the intensity of trimethylation. DAPI staining on the right. 25x magnification.

Thus, prolonged EZH2 knockdown led to a reduction of H3K27me3 and reduced proliferation in Karpas-299.

5.2. Global inhibition of histone methylation

Besides the siRNA knockdown of EZH2 we treated the cells with the histone methyltransferase inhibitor DZNep. Cells were treated with two different concentrations of the inhibitor (1,2 µM and 2,4 µM) over a time course of 72 hours. Daily trypan blue staining and cell counts were performed for all three cell lines. Both after 48 and 72 hours we could detect a concentration dependent reduction in the overall cell numbers and cells were less proliferative based on cell population doubling times (Fig. 7).

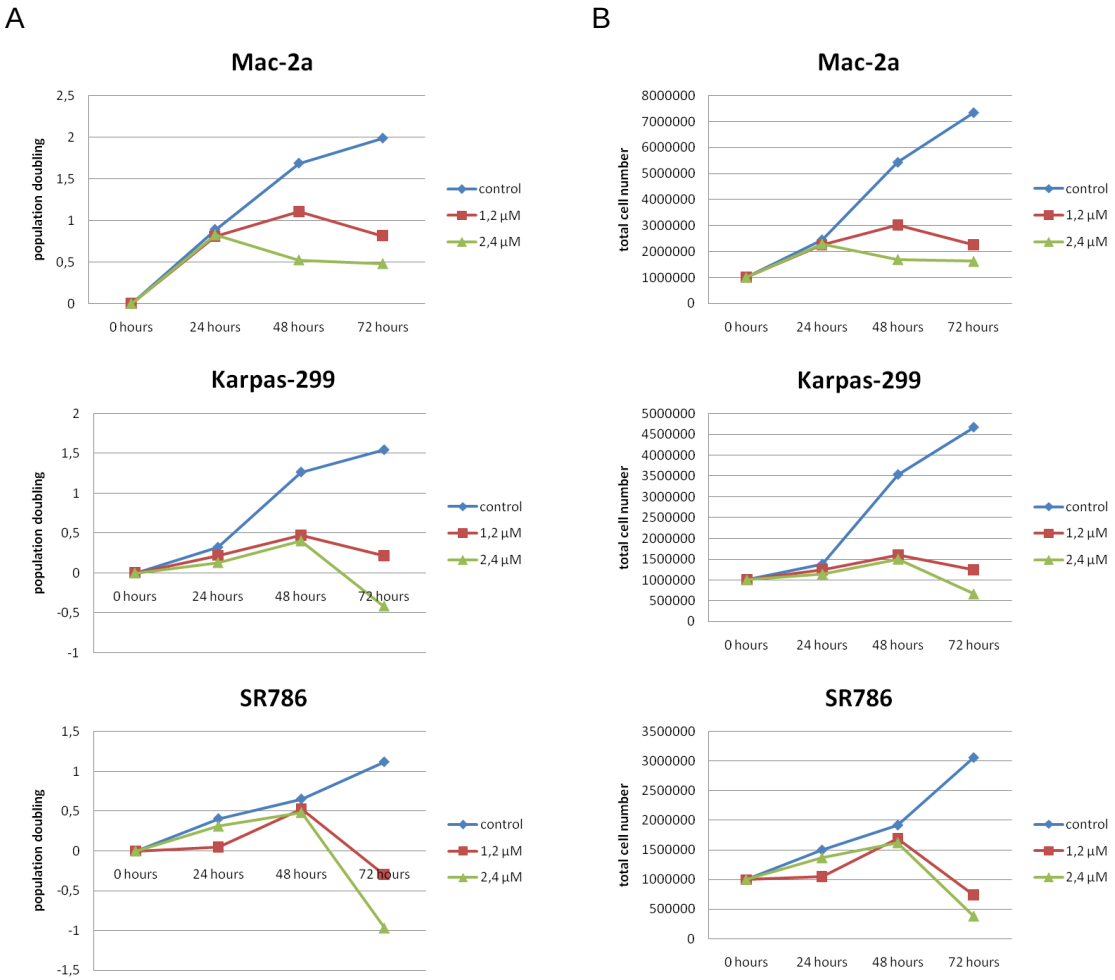


Figure 7. Population doubling times and overall cell numbers after treatment with 1,2µM and 2,4µM DZNep compared to DMSO control. (A) Population doubling times over a time course of 72 hours. (B) Total cell numbers over a time course of 72 hours.

Each day, protein lysates were isolated and tested for EZH2 expression via Western Blot analyses (Fig. 8). Mac-2a showed a significant reduction in the protein level of EZH2 at 24 and 48 hours after treatment but not after 72 hours for both concentrations compared to DMSO control. DZNep was added only once, therefore it is likely, that the inhibitor was already degraded after 72 hours and the cells had recovered from the treatment due to the fast turn-over rate of the Mac-2a cell line. For the cell line SR786 we could detect a significant protein reduction at all three time points for both concentrations compared to the DMSO control. Karpas-299 showed the strongest response to treatment after 72 hours and higher concentration of the inhibitor.

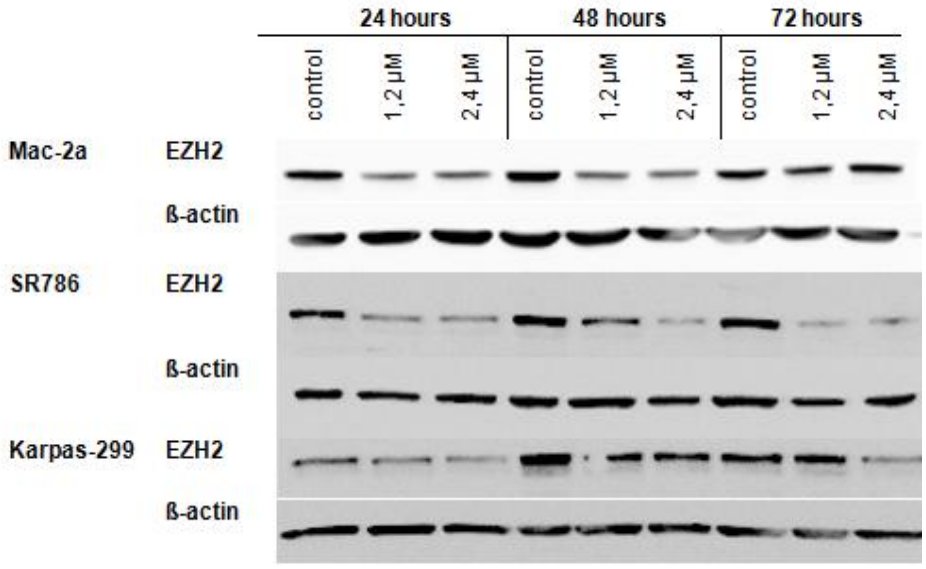


Figure 8. EZH2 protein expression after DZNep treatment over a time course of 72 hours. DZNep was used at 1,2µM and 2,4µM concentrations and compared to protein expression of DMSO control.

We initially expected that DZNep would interfere with enzyme function and thus would only affect protein levels as it has been shown previously (Tan 2007). However, when we analyzed mRNA expression of EZH2 over the time course of 72 hours we could detect a concentration dependent reduction already 24 hours post treatment (Fig. 9).

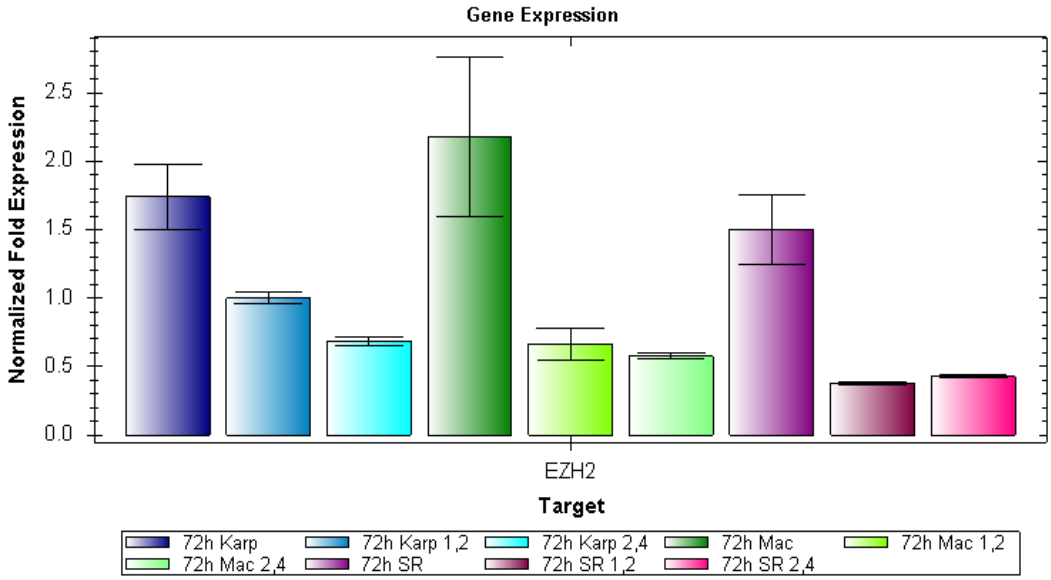
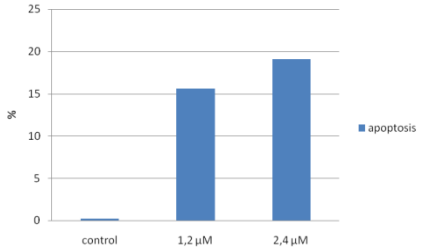
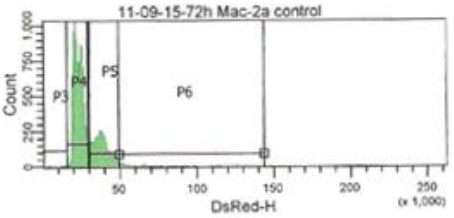


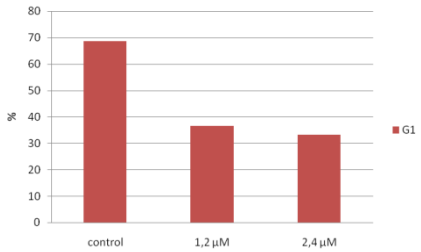
Figure 9. EZH2 mRNA expression levels 72 hours after DZNep treatment. From left to right: DMSO control, 1,2µM DZNep and 2,4µM DZNep for Karpas-299 (blue), Mac-2a (green) and SR786 (violet). EZH2 mRNA levels were significantly reduced for both concentrations compared to the DMSO control.

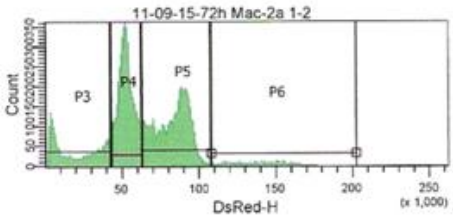
Furthermore, we analyzed the cell cycle over a time period of three days. The apoptotic fraction was continually increasing over time with a peak incidence after 72 hours (Fig. 10; data shown for 72 hours only). For Mac-2a we could observe roughly 20% of apoptosis and even 30-35% for Karpas-299 and SR786. Both ALK^+ and ALK^- ALCL cells showed a G2/M cell cycle arrest, indicated by a low fraction of cells in G1 phase and a high proportion of cells in S, G2 and M phases.

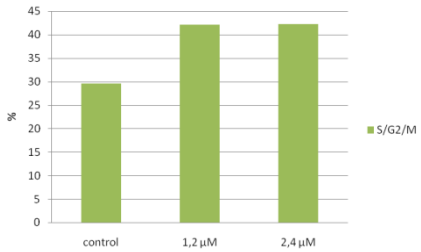
A Mac-2a

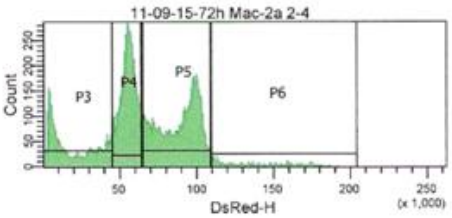




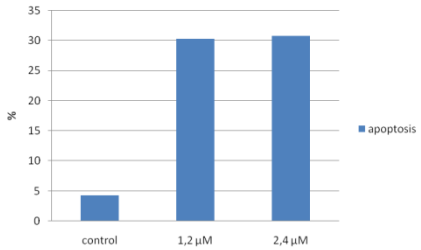


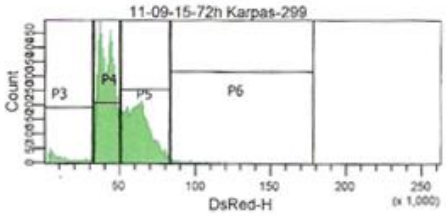


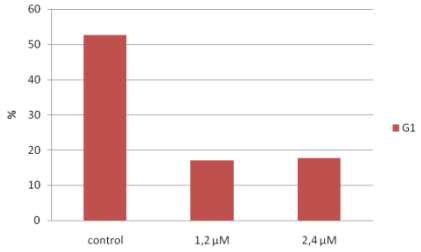


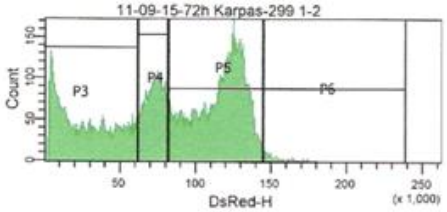


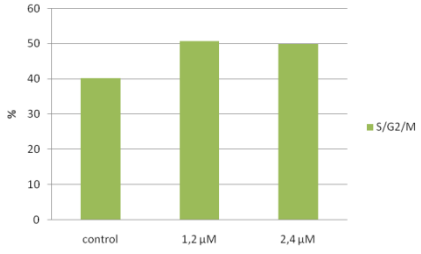
B Karpas-299

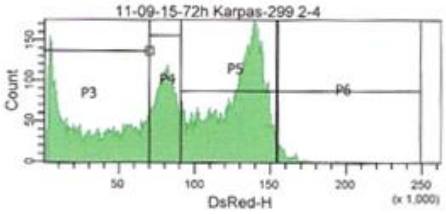












C SR786

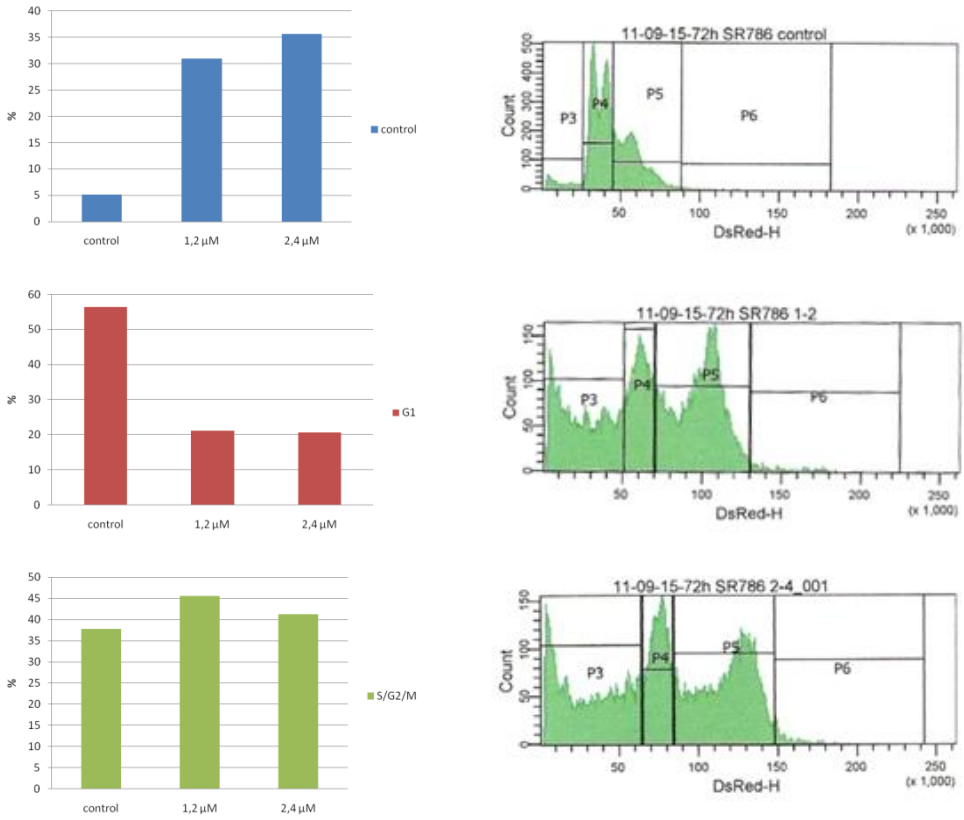


Figure 10. Cell cycle analysis 72 hours after DZNep treatment. Schematic distribution of apoptosis, G1 and S/G2/M phases on the left. Histograms for control; 1,2 µM and 2,4 µM DZNep on the right. (A) Mac-2a, (B) Karpas-299, (C) SR786.

In general we could observe responses to DZNep treatment already 24 hours after treatment (data not shown) and effects peaked after 72 hours. Thus, we chose this time point to perform immunofluorescence stainings against H3K27me3 (Fig. 11). DMSO control cells showed a high level of H3K27 trimethylation while it was significantly decreased for both concentrations of treated cells, although Mac-2a showed the poorest effects. We could additionally observe a change in morphology after treatment with the inhibitor. Cells became larger and flatter, which was especially true for Mac-2a. However, 72 hours after treatment cells were generally in a poor condition, thus it is impossible to draw a conclusion about H3K27 trimethylation from these immunofluorescence stains only. To ensure the reliability of the data it will be required to additionally perform histone blots against H3K27me3.

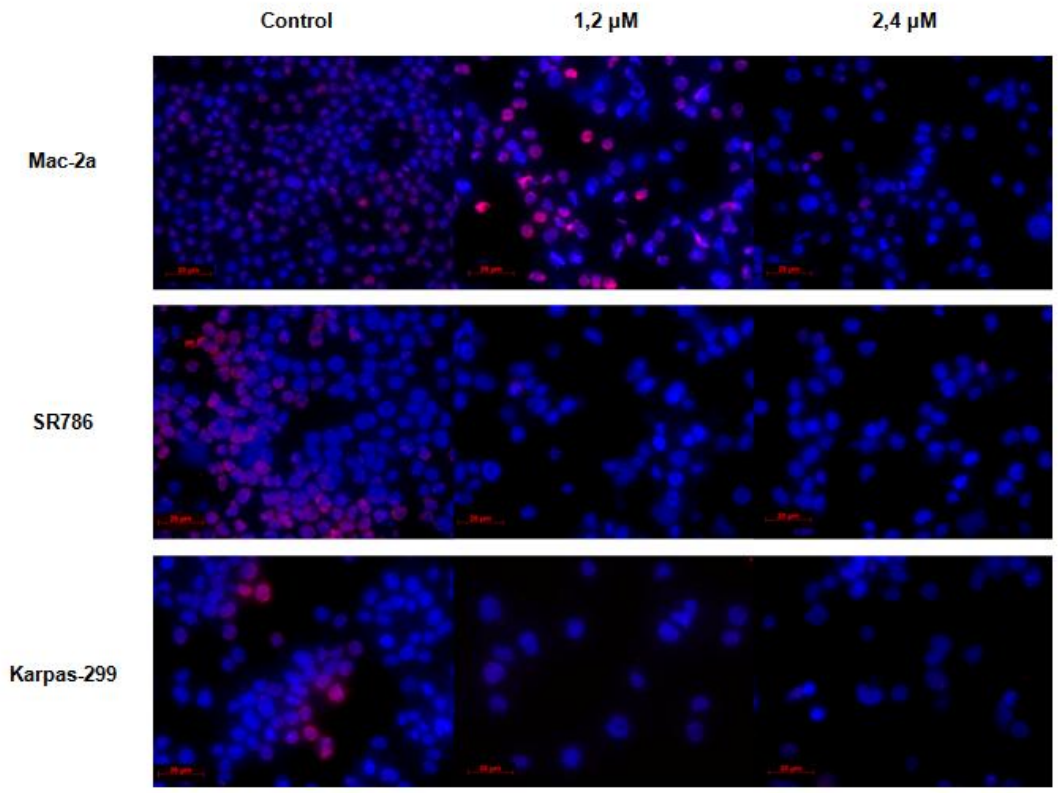


Figure 11. Immunofluorescence stains against H3K27me3 72 hours after treatment with DZNep. Control cells show a high level of H3K27me3 in all three cell lines. The intensity of staining is significantly reduced for both concentrations and all cell lines 72 hours after treatment. 25x magnification.

Thus, we conclude that DZNep treatment was very efficient in inducing apoptosis and cell cycle arrest in different ALCL cell lines. Interestingly, the inhibition was not only affecting enzyme function, but was also reflected by lower EZH2 RNA and protein levels in treated cells.

5.3. Overexpression of miR-101

In 2008 Varambally and colleagues proposed that the genomic loss of miR-101 leads to the overexpression of EZH2. They could show that overexpression of miR-101 in prostate cancer cell lines led to reduced cell growth, attenuated proliferation and decreased invasion capabilities (Varambally 2008). In order to evaluate the effect of miR-101 overexpression in ALCL cell lines on both the cellular behavior and EZH2 expression and to elucidate a putative mechanism of EZH2 deregulation in ALCL, we transduced Karpas-299, SR786 and Mac-2a with lentiviral vector constructs containing the miR-101 sequence. For controls we used vector constructs containing GFP only and a combination of miR-101

and GFP. When we purified RNA from all three cell lines and tested for miR-101 expression we could confirm a more than 10-fold higher expression in miR-101 transduced Karpas-299 and SR786 when compared to GFP only control (Fig. 12). No decrease in EZH2 mRNA levels was detected, which was expected, since miRNAs act on the posttranscriptional level and inhibit protein translation. For Mac-2a we could not confirm an overexpression of the miRNA (data not shown).

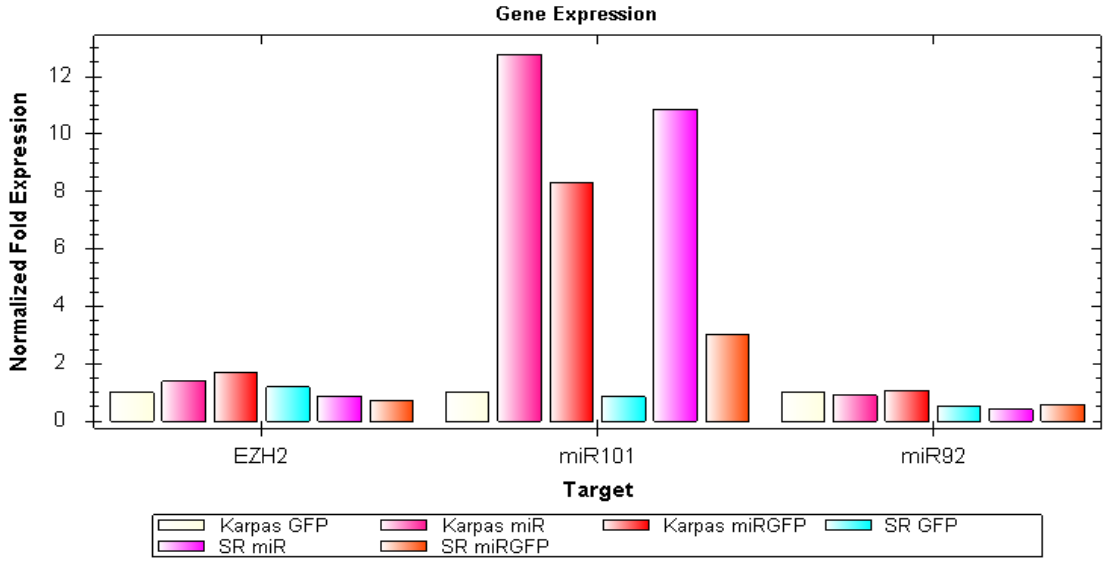


Figure 12. Overexpression of miR-101 and effects on EZH2. RNA was isolated from virally transduced cell lines Karpas-299 and SR786 and tested via qPCR against miR-101 and EZH2 mRNA expression. Expression of miR-92 served as negative control. From left to right for each cell line: GFP only, miR-101, miR-101-GFP. Both cell lines showed a more than 10-fold increase in miR-101. EZH2 levels were not significantly affected by the overexpression.

Since miRNAs act posttranscriptionally we hypothesized that EZH2 protein levels would be reduced after miR-101 overexpression. However, Western Blot analysis of all three cell lines demonstrated that there are no significant changes in the protein levels of EZH2 (Fig. 14). Hence, miR-101 overexpression did neither affect RNA nor protein levels of EZH2.

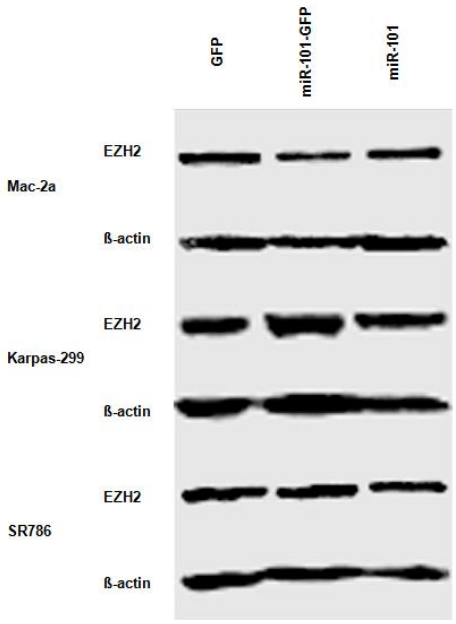
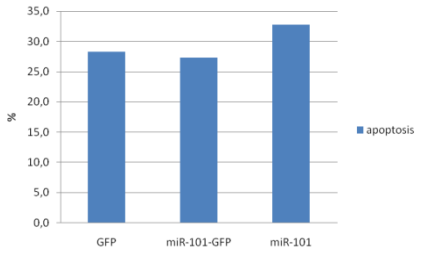


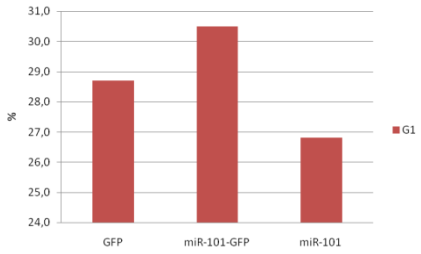
Figure 13. EZH2 protein levels in miR-101 overexpressed cell lines. There are no significant changes in EZH2 protein levels upon overexpression of the miRNA-101 for any of the three cell lines chosen.

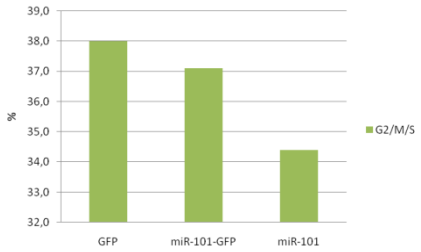
After two weeks under selection pressure, Mac-2a cells grew only poorly. FACS analysis revealed a very high degree of apoptotic cells even in the GFP only control cell fraction (Fig. 15), which might have been due to higher sensitivity to the blasticidin selection. For Karpas-299 we could detect a small increase in the apoptotic fraction in the miR-101 treated cells versus controls. Furthermore we could detect a shift from G1 to G2/M, which may indicate a tendency towards cell cycle arrest. MiR-101 overexpression in SR786 increased the apoptotic fraction and also here cell cycle analysis revealed a tendency to shift towards G2/M.

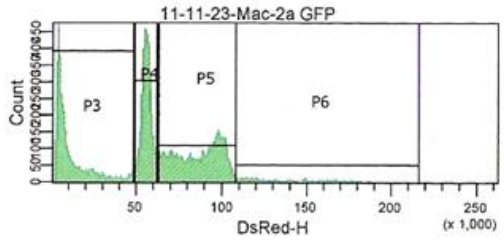
Thus overexpression of miR-101 showed no conclusive effects on EZH2 protein levels but effected proliferation and apoptosis in ALK^+ ALCL cell lines.

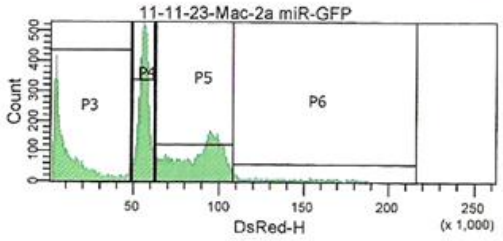
A **Mac-2a**

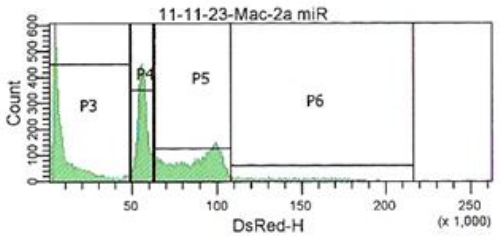




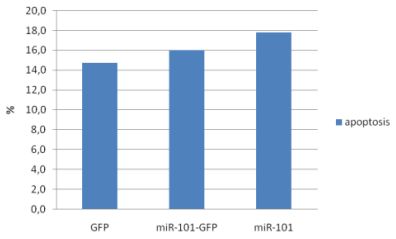


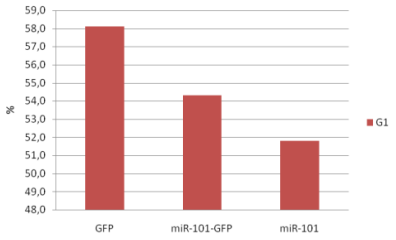


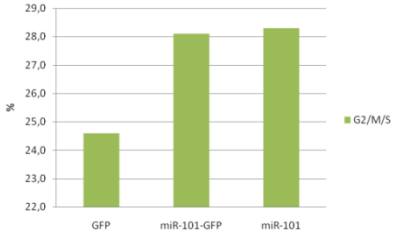


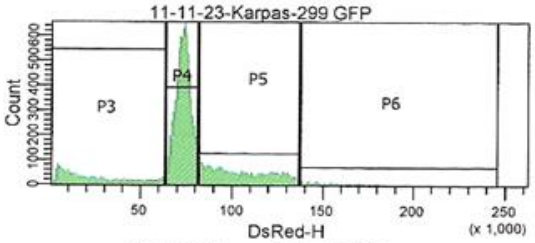


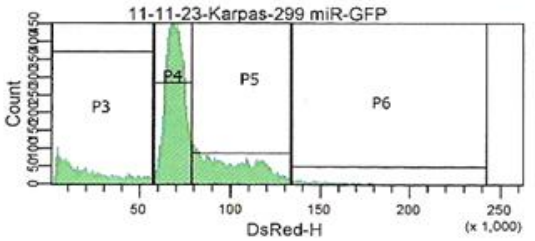
B **Karpas-299**

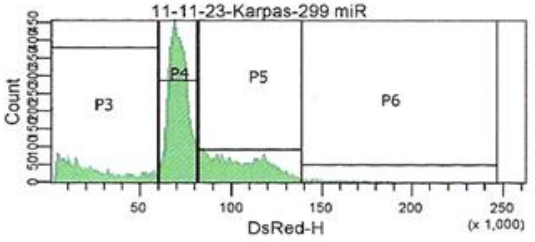












C SR786

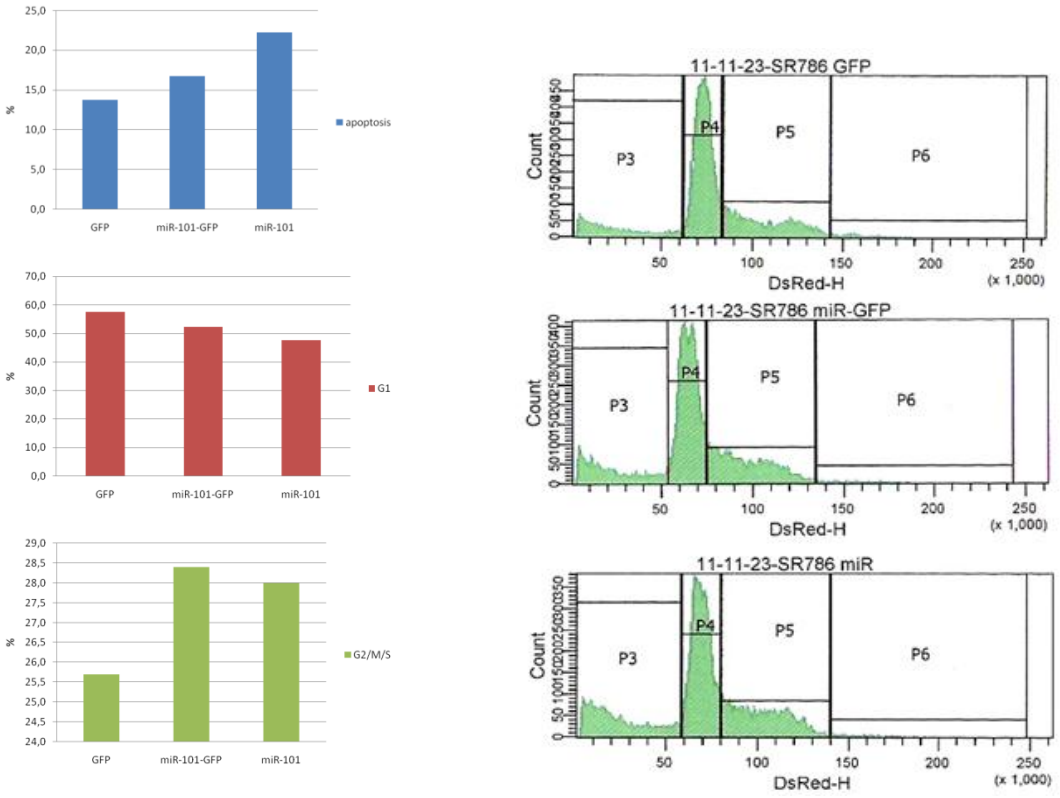


Figure 14. FACS analysis after miR-101 overexpression. FACS data of GFP only, miR-101-GFP and miR-101 transduced cell lines (A) Mac-2a, (B) Karpas-299 and (C) SR786. Distribution of apoptosis, G1 phase and phases S/G2/M on the left. Corresponding histograms on the right.

6. DISCUSSION AND CONCLUSION

Within the last years it has been demonstrated that EZH2 is overexpressed in advanced stages of a variety of different tumours (Sparmann and van Lohuizen 2006). We detected increased EZH2 levels in ALCL cell lines and primary tumours. When we knocked down EZH2 in ALK^+ cell lines Karpas-299 and SR786 and the ALK^- cell line Mac-2a we found protein and RNA levels to be significantly reduced. However, we could neither identify a cell cycle arrest in the G2/M phase nor a significantly increased proportion of apoptosis in any of the three cell lines after short time knockdown of EZH2. However, double transfection with anti-EZH2 siRNA and prolonged knockdown resulted in reduced proliferation and reduced H3K27me3 in Karpas-299. Nonetheless, we will need to confirm these data with additional FACS analyses and Western Blots directed against EZH2 protein and H3K27me3 in long term knockdown cells. Since effects of targeted knockdown of EZH2 were lower than expected, we suggest that at least part of the function of EZH2 may be adopted by its homologue EZH1. In mammals, PRC2 is made up of SUZ12, EED, RbAp46/48 and either EZH1 or EZH2. While EZH2 is present in dividing cells only, EZH1 can be found in dividing and differentiated cells (Margueron and Reinberg 2011). We will therefore analyze both RNA and protein levels of EZH1 after EZH2 siRNA knockdown and eventually include the investigation of EZH1 in future studies.

When we treated cells with the global HMT-inhibitor DZNep, we detected reduced RNA levels in all three cell lines. Protein levels were significantly reduced in Mac-2a and SR786 but only partially in Karpas-299. This is in contrast to previously published data (Chase and Cross 2011). Here it was shown that DZNep specifically reduces EZH2 protein levels but does not affect mRNA levels. Cell counts revealed a significant reduction in the overall cell number and an increased cell population doubling time. FACS analysis further enforced these data as cells from all three cell lines significantly arrested in G2/M phase and showed a high degree of apoptosis. As it has been shown previously (Tan 2007) we detected a reduction of H3K27me3 after DZNep treatment in all three cell lines. Based on our results, DZNep appeared to be a very potent drug to induce cell cycle inhibition and apoptosis in ALCL cancer cells.

We initially attempted to overexpress miR-101 in ALCL cell lines using lipofection, but this technique turned out to be very inefficient. In another approach we transduced cell lines with lentiviral vector constructs, which showed a transduction efficiency of almost 80%. Results from qPCR showed a more than 10-fold higher expression of miR-101 in Karpas-299 and SR786. The ALK^- cell line Mac-2a did not show an overexpression at all, though. This might be due to altered processing of the miRNA into its active form. Based on the current data we could not see a downregulation of EZH2 on the protein level. Mac-2a showed a very high degree of apoptosis, which is more likely a result from the treatment and selection pressure than a consequence from miR-101 overexpression since we could observe the same results in the control cells as well. Thus, we will optimize the experimental approach using miRNA mimics instead of vectors to overexpress miR-101 to readdress this issue in the future. Nonetheless, Karpas-299 and SR786 showed a tendency towards G2/M cell cycle arrest after miR-101 overexpression. Since we could not detect a change in EZH2 protein level, however, we conclude, that miR-101 overexpression most likely affects other targets. Besides EZH2, miR-101 was shown to target cyclooxygenase-2 (COX2), amyloid precursor protein (APP) and myeloid cell leukaemia sequence-1 (MCL-1) (Strillacci 2009, Su 2009, Vilardo 2010). Only recently stathmin-1 (STMN1), Ras-associated protein (RAB5A) and ATG4 autophagy related 4 homolog D (ATG4D) were identified as three additional targets of miR-101 that play an essential role in the regulation of autophagy (Frankel 2011). Furthermore, Zhu et al. showed that miR-101 directly targets MAPK phosphatase-1 (MKP-1) which is required for the deactivation of MAPKs (Zhu 2010). Hence, there is a considerable number of targets, which may be affected by miR-101 overexpression aside from EZH2 that may be investigated in the future.

ALK^+ ALCL affects mostly young males while ALK^- ALCL is common among people aged 50-70 years. The prognosis for ALK^+ ALCL is significantly better with complete remission in 90% of cases after antracyclin-dependant therapy and a cure rate of 60-80%, while the prognosis for ALK^- ALCL patients is rather poor as only 30-50% obtain complete remission after the same treatment (Savage 2008). It has been suggested, that ALK has immunogenic properties and causes the production of antibodies, which may contribute to the rather good prognosis of ALK^+ ALCL (Pulford 2000). We know that overexpression of

CD30, which is typical in ALCL, induces constitutive NF-kB signaling (Horie 2002). Furthermore, constitutive ALK signaling activates many different growth-promoting pathways such as Ras/Erk, Stat3 (Piccaluga 2010), Akt/mTOR, cJUN, JunB and c-myc (Merkel 2010). Although, there have been remarkable advances in the understanding of the molecular mechanisms of ALCL, standard therapy is still based on doxorubicin-containing combination therapy (Savage 2008). It has been suggested that the ALK protein itself would be an interesting candidate for targeted therapy as it is not widely expressed in the adult organism and may therefore cause only few toxic effects (Piccaluga 2010). In general, epigenetic drugs have established as powerful treatment options, such as the DNA methylation inhibitors 5-aza-2-deoxycytidine (Saba 2007) and 5-azacytidine (Kaminskas 2005), which have recently been approved for the treatment of myelodysplastic syndromes. Another epigenetic drug, which has been approved for the treatment of cutaneous T-cell lymphoma is the HADC-inhibitor suberoylanilide hydroxamic acid (Olsen 2007). One of the major problems when considering DZNep as therapeutic drug is the fact that somatic changes induced by DZNep are not stably inherited (Miranda 2009). Hence, it has been suggested to use DZNep in combination therapies with HDAC-inhibitors, which may enhance the re-expression of genes. For acute myeloid leukemia (AML) it could be shown that the combination of DZNep with the HDAC inhibitor panobinostat had synergistic effects causing EZH2 depletion and apoptosis of leukemia cells (Fiskus 2009). Additionally it has been demonstrated, that DZNep leads to decreased EZH2 expression and cell cycle arrest in breast cancer cells and causes synergistic effects when administrated in combination with the HDAC-inhibitor trichostatin A (TSA) (Hayden 2011).

We can conclude that the mechanisms of aberrant histone methylation in ALCL are highly divergent and cannot be decoded by focusing on EZH2 alone. Instead global inhibition of histone methylation with the inhibitor DZNep appeared to be highly effective and its application, perhaps in combination with HDAC inhibitors, may be a powerful option for the treatment of ALCL.

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8. APPENDIX

8.1. CURRICULUM VITAE

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References

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Thanks to all of you,
Martina Diem

8.3. Eidesstattliche Erklärung

„Ich erkläre an Eides statt, dass ich die vorliegende Masterarbeit selbstständig verfasst, und in der Bearbeitung und Abfassung keine anderen als die angegebenen Quellen oder Hilfsmittel benutzt, sowie wörtliche und sinngemäße Zitate als solche gekennzeichnet habe. Die vorliegende Masterarbeit wurde noch nicht anderweitig für Prüfungszwecke vorgelegt.“

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8.4. Statutory declaration

“I declare in lieu of an oath that I have written this master thesis myself and that I have not used any sources or resources other than stated for its preparation. I further declare that I have clearly indicated all direct and indirect quotations. This master thesis has not been submitted elsewhere for examination purposes.”

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