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Table of contents

Abstract	8
Zusammenfassung	10
1. Introduction.....	12
1.1 Histone modifications	12
1.2 DNA-methylation	13
1.3 DNA methyltransferases	16
1.4 Anaplastic Large Cell Lymphoma and ALK signaling	17
1.5 The Cre/loxP system.....	19
1.6 Project description.....	21
2. Materials and Methods.....	22
2.1 Cell culture	22
2.1.1 Thawing cells	22
2.1.2 Subcultivating cells	22
2.1.3 Cryopreservation of cells	22
2.1.4 Isolation of murine NPM-ALK+ T-cell lymphoma cells.....	22
2.1.5 Genetic deletion of <i>Dnmt1</i> in ALCL cells	23
2.1.5.1 Lentiviral transduction of NPM-ALK+ murine lymphoma cells	23
2.1.5.2 Culture conditions and 4-Hydroxytamoxifen - treatment.....	24
2.1.6 5-aza-2'-deoxycytidine treatment for chemical DNMT1 inhibition	24
2.1.7 Generation of viability curves	25
2.2 Molecular biology methods.....	25
2.2.1 DNA isolation	25
2.2.2 PCR for knockout verification of genetic <i>Dnmt1</i> deletion.....	26
2.2.3 RT-qPCR	27
2.2.4 Protein isolation	28
2.2.5 Western Blot analysis	28
2.2.5.1 Protocol	28
2.2.5.2 Antibodies.....	31
2.2.6 RNA isolation	31
2.2.7 Immunofluorescence.....	31
2.2.7.1 Protocol	31
2.2.7.2 Antibodies.....	32
2.2.8 FACS-Analysis.....	32

2.2.9 RNA-sequencing.....	32
3. Results.....	34
3.1 Genetic deletion of <i>Dnmt1</i> in murine T-cell lymphoma cells	34
3.1.1 Lentiviral transduction and 4-Hydroxytamoxifen treatment.....	34
3.1.2 Characterization of the <i>Dnmt1</i> ^{Δ/Δ} phenotype.....	37
3.2 Inhibition of DNMT1 using 5-aza-2-deoxycytidine.....	40
3.3 Differences between genetic <i>Dnmt1</i> deletion and chemical inhibition.....	44
4. Discussion	53
4.1 Genetic deletion of <i>Dnmt1</i> in murine NPM-ALK+ T-cell lymphoma cells.....	53
4.2 Chemical inhibition of DNMT1 in murine NPM-ALK+ T-cell lymphoma cells.....	53
4.3 Comparison of genetic <i>Dnmt1</i> deletion and pharmacological inhibition	54
4.4 Conclusion and outlook.....	57
5. References	59
6. Appendix.....	64
6.1 Acknowledgements	64
6.2 Abstract.....	65
6.3 Zusammenfassung.....	66
6.4 Curriculum vitae	67

Table of figures

Figure 1: The organization of DNA in nucleosomes. DNA is wrapped around histone octamer cores consisting of two of each histone proteins (H2A, H2B, H3, and H4). Protruding N-terminal histone tails provide a versatile platform for histone-modifications like acetylations, methylations and phosphorylations. Writer and eraser enzymes establish the histone code that is read by reader enzymes. [47]	13
Figure 2: Epigenetic regulation of genes in normal and cancer cells. Tumor suppressor genes are unmethylated at CpG-islands in the promoter in normal cells, enabling normal expression. In cancer, CpG-island hypermethylation in tumor suppressor genes leads to epigenetic silencing. Genes that are normally silenced in normal cells, such as transposable or repetitive elements, can become hypomethylated and therefore active in cancer cells. Both events promote tumorigenesis. [20]	15
Figure 3: Reaction of Cytosine to 5-methylcytosine. By transferring a methyl group from S-adenosylmethionine to the C-5 position of cytosines, DNA methyltransferases catalyze the reaction of cytosine to 5-methylcytosine. [39]	16
Figure 4: DNA methylation and demethylation. DNA methyltransferases DNMT3A and DNMT3B perform <i>de novo</i> methylation at unmethylated CpG sites. During replication DNMT1 methylates hemimethylated DNA strands. Demethylation can either be passive by inhibition or loss of DNMT1 or active by enzymatic reactions. [51]	17
Figure 5: Immunohistochemistry of two ALCL subtypes. Both subtypes are positive for the ALCL marker CD30. The ALK+ subtype is shown in the top row, whereas the bottom pictures show an ALK-subtype. [29]	18
Figure 6: Suggested mechanism of how NPM-ALK signaling regulates <i>Dnmt1</i> . The NPM-ALK downstream target STAT3 upregulates transcription of <i>Dnmt1</i> and recruits DNMT1 to promoter regions of genes. This results in their epigenetic silencing by DNA methylation. [figure provided by Gerda Egger adapted from 31]	19
Figure 7: A mouse-model with tissue-specific <i>Dnmt1</i> deletion. A transgenic mouse expressing NPM-ALK under the CD4 promoter with floxed <i>Dnmt1</i> is crossed with a mouse expressing Cre under a tissue specific promoter like Lck. The resulting mouse will have CD4 specific NPM-ALK expression and Lck specific deletion of <i>Dnmt1</i>	20
Figure 8: The vector used for the lentiviral transduction. The targeting vector used for the lentiviral transduction, MSCV CreERT ² contains a fusion between the Cre-recombinase and the estrogen-receptor, making it inducible upon tamoxifen-treatment. Puromycin resistance is used for selection. [66]	24
Figure 9: Genotyped DNA samples of tamoxifen treated B57 cells. DNA was isolated 24, 48, 72 and 96 hours post treatment. “4HT” indicates treatment, whereas “-” represents DNA from control cells. After 24 hours, cells have a nearly complete <i>Dnmt1</i> deletion, as the <i>Dnmt1</i> 1lox signal is stronger than the <i>Dnmt1</i> 2lox signal. Complete knockout is reached after 48 hours post tamoxifen treatment.	34
Figure 10: LoxP sites depicted as open arrow heads flank exons 4 and 5 of <i>Dnmt1</i> . Upon tamoxifen treatment Cre recombinase will exert its function and remove these exons resulting in a <i>Dnmt1</i> 1 lox allele. Black arrows show primer sites for PCR.....	34
Figure 11: Western Blot analysis of tamoxifen treated, Cre expressing B57 cells. DNMT1 is slightly reduced 24 hours post treatment, but no longer detectable after 48 hours. β -Actin served as loading control.	35
Figure 12: IAP retrotransposon expression after <i>Dnmt1</i> deletion. Deletion of <i>Dnmt1</i> in the murine NPM-ALK T-cell lymphoma cell line B57 results in re-expression of the usually silenced retrotransposon IAP. Expression is elevated in cells four and six days after <i>Dnmt1</i> deletion.	35

Figure 13: Immunofluorescence staining against DNMT1 after tamoxifen treatment. DNMT1 levels are clearly reduced in the NPM-ALK+ T-cell lymphoma cell line B57 after tamoxifen treatment (bottom) when compared to untreated control cells (top). DAPI indicates nuclear staining. Cells were analyzed 96 hours after tamoxifen treatment.	36
Figure 14: Immunofluorescence staining against 5-meC after tamoxifen treatment. 5-methylcytosine levels show a distinct reduction in NPM-ALK+ T-cell lymphoma cells after tamoxifen treatment (bottom) when compared to untreated control cells (top). DAPI indicates nuclear staining. Cells were analyzed 96 hours after tamoxifen treatment.	36
Figure 15: Viability of B57 cells after <i>Dnmt1</i> deletion. Viability starts to decrease after five days post tamoxifen treatment (red), when compared to control cells (blue).	37
Figure 16: Immunofluorescence staining against γ H2AX after tamoxifen treatment. γ H2AX levels are increased in B57 cells after tamoxifen treatment (bottom) when compared to untreated control cells (top). DAPI indicates nuclear staining. Cells were analyzed 96 hours after tamoxifen treatment.	38
Figure 17: Immunofluorescence staining against phospho-H3S10 after tamoxifen treatment. Phospho-H3S10 staining results in a stronger signal in B57 cells after tamoxifen treatment (bottom) when compared to untreated control cells (top). DAPI indicates nuclear staining. Cells were analyzed 96 hours after tamoxifen treatment.	38
Figure 18: Cell-cycle profiling of tamoxifen treated B57 cells. The amount of S-phase cells starts to decrease 4 days after the treatment, while apoptotic Sub-G1 cells increase.	39
Figure 19: Cell cycle analysis after <i>Dnmt1</i> deletion. A cell-cycle profile of B57 cells after <i>Dnmt1</i> deletion shows a clear reduction of S-phase cells (green bars) over 7 days post-treatment, while apoptotic cells (blue bars) increase.	39
Figure 20: Viability of B57 cells after 5-aza-CdR treatment. Different concentrations of 5-aza-CdR were used to treat B57 cells in order to exclude improper concentrations with possible cytotoxic effects. Control cells reach 90 % viability three days after treatment, while 0,01 μ M 5-aza-CdR results in 60% viability. Higher concentrations reach 20 % (0,04 μ M) viability and below.	41
Figure 21: IAP retrotransposon expression of B57 cells after 5-aza-CdR treatment. Inhibition of DNMT1 in the murine NPM-ALK T-cell lymphoma cell line B57 results in re-expression of the usually silenced retrotransposon IAP, two days after 5-aza-CdR treatment. Higher 5-aza-CdR concentrations result in higher IAP expression levels.	41
Figure 22: Immunofluorescence staining against DNMT1 after 5-aza-CdR treatment. DNMT1 levels show no difference in B57 cells after 5-aza-CdR treatment (bottom) when compared to untreated control cells (top). DAPI indicates nuclear staining. Cells were analyzed 52 hours after 5-aza-CdR treatment.	42
Figure 23: Immunofluorescence staining against 5-meC after 5-aza-CdR treatment. 5-methylcytosine levels are decreased in B57 cells after 5-aza-CdR treatment (bottom) when compared to untreated control cells (top). DAPI indicates nuclear staining. Cells were analyzed 52 hours after 5-aza-CdR treatment.	42
Figure 24: Immunofluorescence staining against γ H2AX after 5-aza-CdR treatment. γ H2AX levels are increased in B57 cells after 5-aza-CdR treatment (bottom) when compared to untreated control cells (top). DAPI indicates nuclear staining. Cells were analyzed 52 hours after 5-aza-CdR treatment.	43
Figure 25: Western blot analysis after genetic <i>Dnmt1</i> deletion (left side) and chemical inhibition (right side). DNMT1 is strongly reduced after tamoxifen treatment, but ALK and STAT3 show no differences for both phosphorylated and normal forms. Chemical inhibition of DNMT1 shows slight reduction of DNMT1, ALK and STAT3 and a stronger reduction for phosphorylated forms of ALK and STAT3. β -Actin served as loading control for both blots.	45
Figure 26: Top biological functions of differentially expressed genes after genetic <i>Dnmt1</i> deletion in B57 cells, including antigen presentation, hematopoiesis and cancer.	49

Figure 27: Top biological functions of differentially expressed genes after chemical inhibition of DNMT1 in B57 cells, including cellular growth and proliferation, cancer and inflammatory response.	49
Figure 28: Prediction Legend for IPA core analysis networks of biological function predictions.	50
Figure 29: IPA core analysis of RNA-sequencing data after tamoxifen (left) and 5-aza-CdR (right) treatment of B57 cells. Differentially expressed genes in both treatments lead to predicted activation of apoptosis.	50
Figure 30: IPA core analysis of RNA-sequencing data after tamoxifen (left) and 5-aza-CdR (right) treatment of B57 cells resulted in predicted activation of T-lymphocytes and activation of inflammation.	51
Figure 31: Prediction of Interleukin 6 being an activated upstream regulator for cells with genetic <i>Dnmt1</i> deletion (left), but inhibited upon chemical DNMT1 inhibition (right).....	51
Figure 32: Prediction of Lipopolysaccharide being an activated upstream regulator for cells with genetic <i>Dnmt1</i> deletion (left), but inhibited upon chemical DNMT1 inhibition (right).	52

Abstract

With about 14 million new cases and approximately 8.2 million cancer related deaths in 2012, cancer is among the leading causes of death. One of the most common hematologic cancer types are Non-Hodgkin-Lymphomas which present 90% of lymphoma-type cancers. NPM-ALK-positive Anaplastic Large Cell Lymphoma is a type of Non-Hodgkin's-Lymphoma that is characterized by the presence of the NPM-ALK fusion protein. In these lymphomas, the receptor tyrosine kinase anaplastic lymphoma kinase (ALK) is fused to the nuclear transport protein Nucleophosmin1 (NPM1), which constitutively activates the kinase. The kinase then leads to malignant transformation by activation of multiple downstream signaling pathways. One of its most important downstream targets is the transcription factor STAT3. STAT3 has been described to upregulate the transcription of the DNA methyltransferase 1 gene (*Dnmt1*) in ALK+ ALCL, and to recruit DNMTs to promoters of tumor suppressor genes, therefore silencing them. We have shown that thymic-specific deletion of *Dnmt1* in a transgenic NPM-ALK mouse model leads to inhibition of lymphomagenesis, and thereby sustains survival of the mice. Furthermore, the inhibition of DNMT1 *in vitro* in human ALCL cell lines was shown to result in the loss of STAT3 phosphorylation and activity.

The aim of this study was to investigate how depletion of DNMT1 affects NPM-ALK tumor cells. To address this, we analyzed tumor cells from NPM-ALK mice by inhibiting DNMT1 chemically with 5-aza-2-deoxycytidine. In addition, an inducible Cre recombinase was used to delete *Dnmt1* on the genetic level. Depletion of DNMT1 resulted in the reduction of cellular viability and increased cell death. Furthermore, enriched DNA-damage as well as loss of overall methylation levels was observed. Western Blot analysis showed reduced levels of the phosphorylated, activated forms of ALK and its downstream target STAT3 for chemical DNMT1 inhibition, whereas this reduction was not observed when *Dnmt1* was genetically deleted. RNA-sequencing resulted in divergent findings for chemical inhibition of DNMT1 and genetic *Dnmt1* deletion but confirmed the importance of the DNA methyltransferase for cellular survival for both approaches.

Zusammenfassung

Mit etwa 14 Millionen neuen Fällen und ca. 8.2 Millionen Krebs-assoziierten Todesfällen im Jahr 2012, gehört Krebs zu den häufigsten Todesursachen weltweit. Eine der häufigsten hämatologischen Krebsarten sind Non-Hodgkin-Lymphome, die etwa 90% aller Lymphome darstellen. Das NPM-ALK-positive anaplastische großzellige Lymphom ist ein Non-Hodgkins-Lymphom, charakterisiert durch das NPM-ALK Fusionsprotein. Die Rezeptor Tyrosinkinase Anaplastic Lymphoma Kinase (ALK) bildet in diesen Lymphomen ein Fusionsprotein mit dem nuklearen Transportprotein Nucleophosmin1 (NPM1), was die Kinase konstitutiv aktiviert. Daraufhin führt die Kinase zur malignen Transformation durch die Aktivierung mehrerer Signalwege. Der Transkriptionsfaktor STAT3 ist eines der wichtigsten Zielgene von ALK. STAT3 induziert die Transkription der DNA Methyltransferase 1 (DNMT1) in ALK+ ALCL, rekrutiert DNMTs zu Promotoren von Tumorsuppressor-genen und inaktiviert diese dadurch. Wir haben gezeigt, dass spezifische Deletion von *Dnmt1* im Thymus von transgenen NPM-ALK Mausmodellen, zur Hemmung der Lymphomagenese und somit zum Überleben der Mäuse führt. Inhibiert man DNMT1 außerdem *in vitro* in humanen ALCL Zelllinien, so verliert STAT3 an Aktivität und Phosphorylierung.

Das Ziel dieser Studie war es zu analysieren, wie sich der Verlust von DNMT1 auf NPM-ALK Tumorzellen auswirkt. Dafür wurden Tumorzellen von NPM-ALK Mäusen analysiert, in denen DNMT1 chemisch auf Proteinebene mit 5'-aza-2-deoxycytidine inhibiert wurde. Außerdem wurde mit Hilfe einer induzierbaren Cre Rekombinase *Dnmt1* auf genetischer Ebene deletiert. Der Verlust von DNMT1 resultierte in einer Abnahme der zellulären Viabilität und somit in erhöhter Zelltod-Rate. Zudem konnte sowohl ein Anstieg an DNA-Schäden als auch ein globaler Verlust der DNA-Methylierung beobachtet werden. Western Blot Analysen zeigten reduzierten Gehalt der phosphorylierten, aktiven Formen von ALK und dessen downstream target STAT3 nach chemischer DNMT1 Hemmung. Dies konnte für die genetische Deletion von *Dnmt1* nicht beobachtet werden. Genomweite RNA Sequenzierung zeigte zudem unterschiedliche Resultate für beide Ansätze und bestätigte die wichtige Rolle von DNMT1 für das Überleben der behandelten Zellen.

1. Introduction

The field of epigenetics has risen to great importance in biomedical research in the last few years. The term “epigenetics” refers to heritable changes in gene expression that do not involve changes in the underlying DNA sequence. The embryologist Conrad Waddington introduced the concept of the epigenetic landscape and defined epigenetics as “the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being.” [1] This was the understanding of epigenetics in the 1950s, where the term epigenetics was used to describe all genetic events during development. A more precisely, modern definition of “epigenetics” was expressed in 1996 by Riggs and Porter: “the study of mitotically and/or meiotically heritable changes in DNA sequence”. [2] The following chapter explains the basic epigenetic mechanisms and their contribution to gene regulation and linkage to cancer development.

1.1 Histone modifications

DNA is not just a single “naked” molecule in cells, but instead exists as an organized complex with proteins. This complex is called chromatin and DNA is wrapped and organized around repeating nucleosomal units. Every nucleosome is built of a histone octamer core with two of each histone subunits: H2A, H2B, H3 and H4. [3] In addition, 147 base pairs of DNA are wrapped around the octamer core, completing the nucleosome. [4] The N-terminal domain of histones, called “histone tails” protrudes from the octamer core, providing an adaptable platform for post-translational modifications. The most prominent modifications of histone tails are acetylation and methylation, especially on histones H3 and H4. Other modifications identified are phosphorylation, ubiquitination and sumoylation. [5; 6] A special set of chromatin-modifying enzymes, called epigenetic writers, adds modifications to the histone tails, whereas antagonistic enzymes, epigenetic “erasers”, can remove the added post-translational modifications (PTMs). Another subunit of chromatin-related enzymes, called epigenetic “readers”, possesses specific domains that allow them to bind to distinct modifications. Histone acetyl transferases for instance add acetyl groups to lysine-residues on the histone tails. Histone deacetylases, the antagonists of histone acetyl transferases, remove these acetylation-marks and thus act as eraser-enzymes.

The reader enzymes for acetylation-modifications inhabit a domain with specific affinity for acetylations, called the bromodomain. [2]

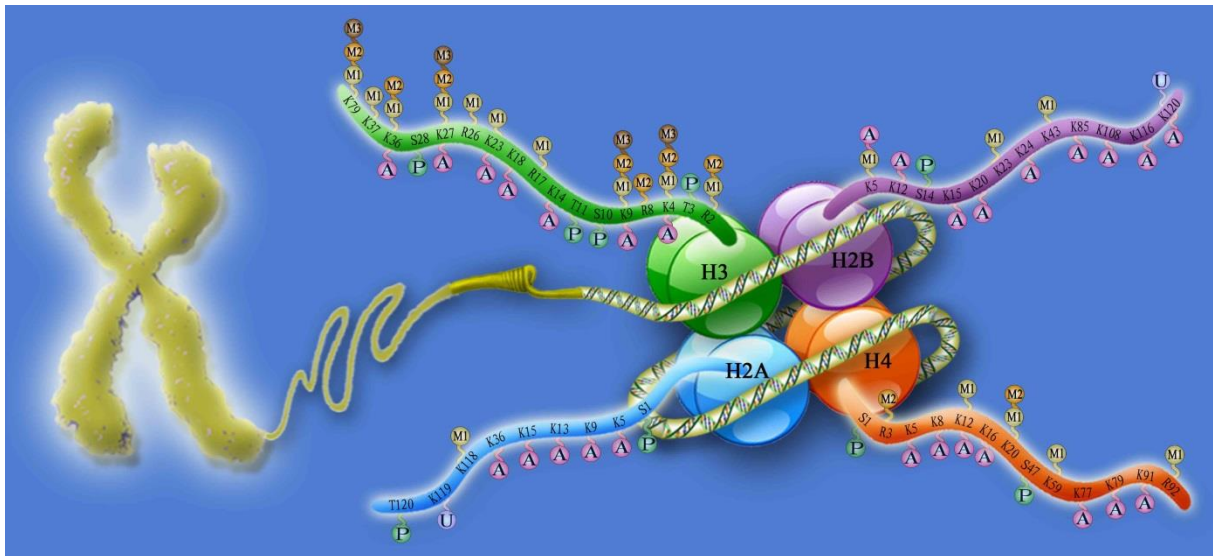


Figure 1: The organization of DNA in nucleosomes. DNA is wrapped around histone octamer cores consisting of two of each histone proteins (H2A, H2B, H3, and H4). Protruding N-terminal histone tails provide a versatile platform for histone-modifications like acetylations, methylations and phosphorylations. Writer and eraser enzymes establish the histone code that is read by reader enzymes. [47]

The concept of histone modifications is tightly connected to gene regulation. Some histone marks such as acetylation of lysine 9 on histone H3 promote a transcriptional active state, whereby the DNA around the octamer core is loosened by changes in their net positive charge and therefore accessible for the transcription machinery. [2; 7] Other modifications like tri-methylation of lysine 27 on histone H3 lead to a more repressive transcriptional state. [2; 8] Together all histone-modifications establish the so called histone-code, contributing to gene regulation. [9]

1.2 DNA-methylation

Besides post-translational modifications of histone tails, another epigenetic process shows high importance for gene regulation. DNA-methylation is an epigenetic mechanism that plays an important role in governing gene function, differentiation and developmental events like genomic imprinting. [10; 15] This modification of the DNA occurs via methyl groups at the C5 position of cytosines. In mammals, DNA-methylation mostly occurs at CpG dinucleotides. [11; 55] Methylation modifies the

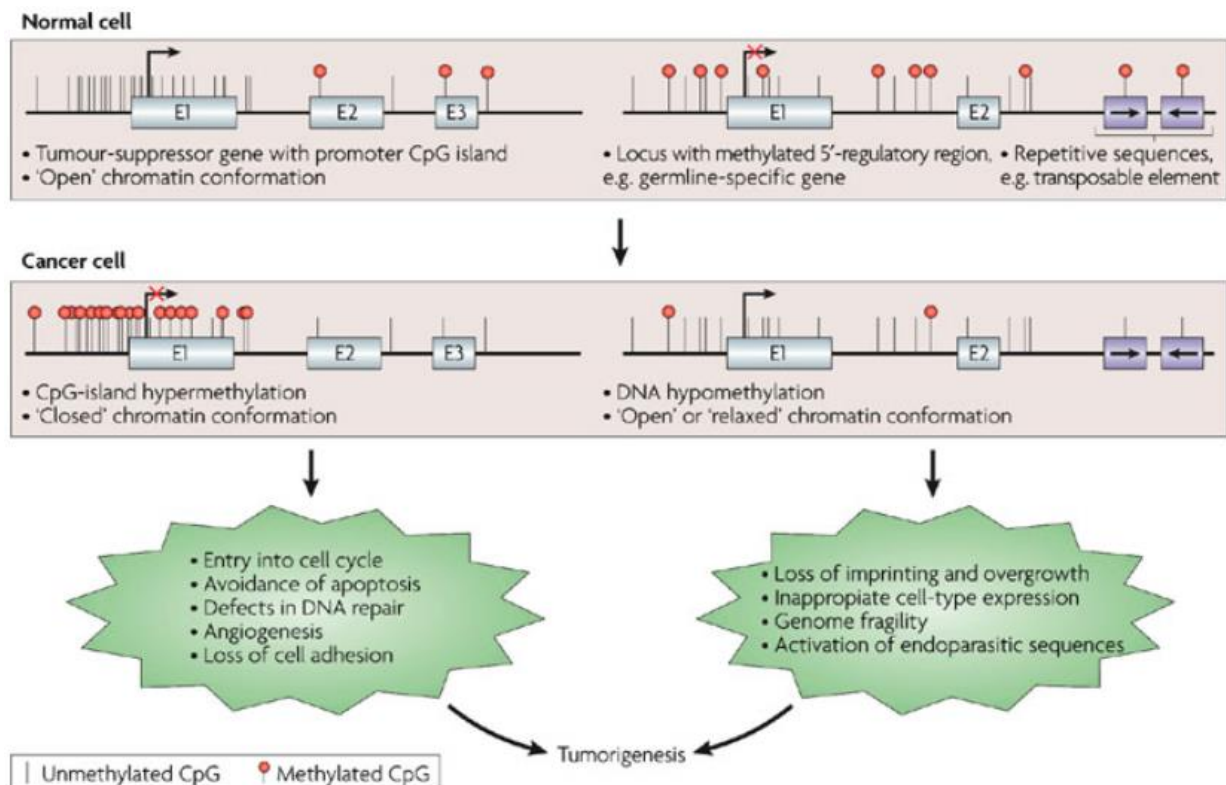
function of the DNA by altering gene expression and is essential for normal gene regulation and during development. [12]

In the somatic tissue of mammals, about 70% of all DNA methylations occur at CpG dinucleotides. [13] Most of these covalent modifications are enriched in transposons and noncoding regions, whereas CpG islands are almost never methylated. These special DNA regions show a GC content of 55% or higher, and have a length of at least 500 basepairs. It is estimated that about 60% of all gene promoters in humans contain CpG islands. [12] Only a very small amount of CpG islands are methylated during genomic imprinting and X chromosome inactivation, but usually these areas are kept free of methylation. [12] The enzymes catalyzing the addition of methyl groups to cytosine-residues are called DNA methyltransferases (DNMTs), which are discussed in chapter 3.1.3.

In the 80s it was suggested that DNA methylation could play an important role in the regulation of gene expression. Especially experiments with the DNA methyltransferase inhibitor 5-azacytidine provided conclusive evidence, that DNA methylation is involved in gene repression. [14] One mechanism of how methylated DNA can repress gene expression is the isolation of recognition sites for transcription factors. The methylated CpGs repel many transcription factors, thereby denying access to the recognition sites. [12] Another known mechanism is based on protein complexes that are attracted to DNA methylation marks, by containing methyl-CpG-binding domains. MeCP2 is an example for such a methyl-CpG-binding protein, which has a repressive effect on gene expression. [12; 16; 17]

The first hint that aberrant DNA methylation might contribute to tumorigenesis, was given when Feinberg et al. discovered hypomethylation in some genes in cancer, when compared to normal cells. [18] A few years later, hypermethylation of tumor suppressor genes in cancer was reported. [19] Usually, reduced DNA methylation levels at promoter regions of genes are associated with transcriptionally active DNA, whereas hypermethylation is associated with gene repression and is present for instance in repetitive sequences like transposable elements, for silencing. [20; 56] If however, hypermethylation occurs in promoters and especially in CpG islands of tumor suppressor genes, these genes will become silenced and therefore favor tumorigenesis. Besides abnormal DNA hypermethylation promoting the development of cancer, a similar effect was shown for aberrant hypomethylation. [18] An overall decrease of DNA methylation leads to genomic instability and to the activation of

genes with oncogenic functions that are normally silenced. [20] Both, activation of oncogenes and genomic instability contribute to tumorigenesis of cells. 5-methylcytosines can be bound by DNA-binding proteins. Unbound methylated cytosines however, are often targets for spontaneous mutations through deamination. The resulting transition of CpG to TpA in the DNA might be another consequence promoting cellular tumorigenesis. [21, p. 41/42]



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Figure 2: Epigenetic regulation of genes in normal and cancer cells. Tumor suppressor genes are unmethylated at CpG-islands in the promoter in normal cells, enabling normal expression. In cancer, CpG-island hypermethylation in tumor suppressor genes leads to epigenetic silencing. Genes that are normally silenced in normal cells, such as transposable or repetitive elements, can become hypomethylated and therefore active in cancer cells. Both events promote tumorigenesis. [20]

During early development, the DNA methylation levels are changing rapidly. At these developmental stages, there is a mechanism to remove DNA methylation marks in a fast manner by active demethylation. In the paternal pronucleus, DNA demethylation for instance occurs within 4-8 hours post fertilization. [22; 37] Another mechanism for the removal of methylation marks on the DNA is passive DNA demethylation. If the maintenance DNA methyltransferase DNMT1 is inhibited or not present during cellular replication, the newly synthesized DNA strands will remain unmethylated. Several replication cycles without DNMT1 will therefore lead to slow and passive

demethylation. [22] Active DNA demethylation however is based on enzymatic reactions, with the key enzymes being described in 2009. [23] These enzymes belong to the Ten-eleven translocation (TET) protein family, which consists of three proteins in mammals and which are Fe^{2+} - and 2-oxoglutarate-dependent dioxygenases. [24; 57] The mechanism of active demethylation seems to be complex and is still poorly understood. However, TET proteins oxidize their substrate 5-methylcytosine to 5-hydroxymethylcytosine (5-hmC), 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-cC). [24] In further reactions 5-fC and 5-caC are thought to become removed by thymine DNA glycosylases (TDG) and finally exchanged with cytosine by base excision repair. Other mechanisms where AID (Activation-Induced Cytidine Deaminase) and APOBEC (Apolipoprotein B mRNA-Editing Enzyme) enzymes deaminate 5-mC and 5-hmC are proposed as well, but less well-founded. [24]

1.3 DNA methyltransferases

DNA methylation is catalyzed by a family of specific enzymes called DNA methyltransferases (DNMTs). Three different types of DNA methylation are known: methylation of adenine bases at their N-6 position, N-4 methylation of cytosine, and the most common modification of cytosine, the C-5 methylation. [34] The latter modification is catalyzed by DNMTs, by transferring a methyl group from the main methyl-donor S-adenosylmethionine to the C-5 position of cytosines as shown in figure 3. [39; 58]

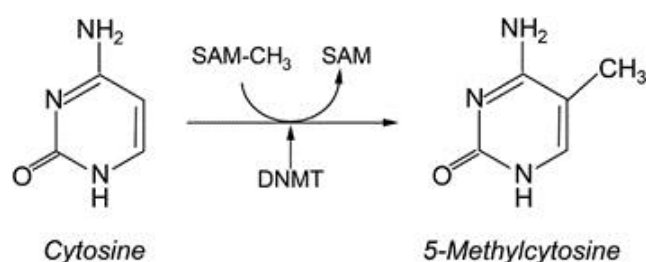


Figure 3: Reaction of Cytosine to 5-methylcytosine. By transferring a methyl group from S-adenosylmethionine to the C-5 position of cytosines, DNA methyltransferases catalyze the reaction of cytosine to 5-methylcytosine. [39]

In mammals, the DNA methyltransferase family consists of four members including DNMT1, DNMT3A, DNMT3B and DNMT3L. Originally there was another DNA methyltransferase called *Dnmt2* until TH Bestor et al. showed in 2006 that it actually methylates aspartic acid transfer RNA, but not DNA. [35] DNMT1 as well as DNMT3A

and 3B have a regulatory domain at their N-terminal region and a catalytic domain at the C-terminal region. [35] Their function however is quite different. DNMT3A and DNMT3B are known as *de novo* methyltransferases because of their important function during development where they add methyl groups to the cytosines of yet unmethylated DNA in order to establish DNA methylation patterns. This *de novo* methylation occurs predominantly in early embryonic stages and gametogenesis. [36] The maintenance methyltransferase DNMT1 however possesses a 5 to 30 fold preference for hemimethylated DNA. This, together with the fact that DNMT1 is present at the replication fork and interacts with proteins like UHRF and PCNA indicate that DNMT1 methylates newly synthesized DNA strands by using the parental strand as a template. Proliferating Cell Nuclear Antigen (PCNA) is a protein present at the replication fork that coordinates the association of replication factors. [64, 65] The multi-structural protein UHRF1 was found to interact with DNMT1 and to target it to replication forks. [65] [38] Maintenance of the DNA methylation patterns is extremely important for genomic stability.

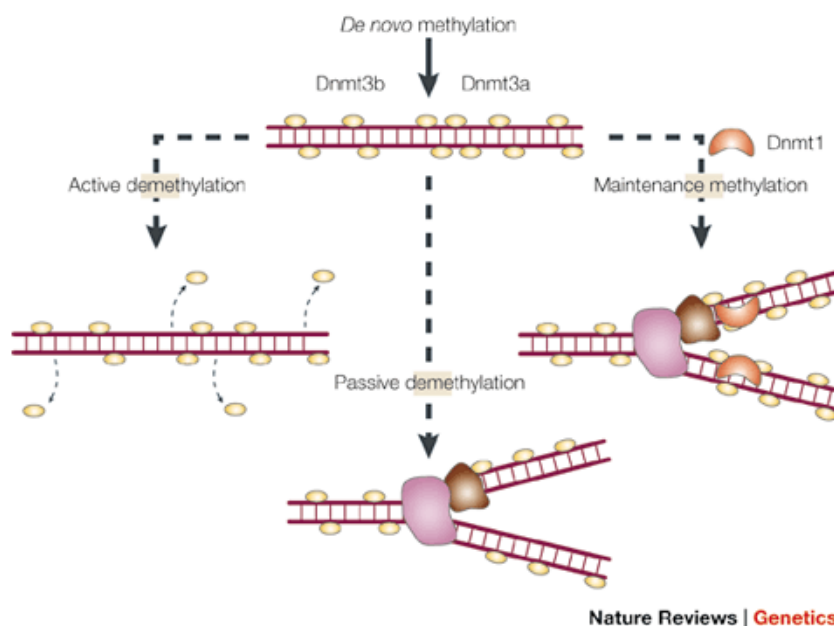


Figure 4: DNA methylation and demethylation. DNA methyltransferases DNMT3A and DNMT3B perform *de novo* methylation at unmethylated CpG sites. During replication DNMT1 methylates hemimethylated DNA strands. Demethylation can either be passive by inhibition or loss of DNMT1 or active by enzymatic reactions. [51]

1.4 Anaplastic Large Cell Lymphoma and ALK signaling

Lymphomas are a group of solid tumors, characterized by affecting immune cells. There are two distinct groups of lymphomas: Hodgkin-Lymphomas which account for

about 10% of all lymphomas and Non-Hodgkin-Lymphomas, representing the remaining 90%. Anaplastic Large Cell Lymphoma (ALCL) is a form of non-Hodgkin-Lymphoma [25], discovered and described by H. Stein in 1985 as a pleomorphic large cell Hodgkin's lymphoma with anaplastic morphology of cells. [26, 27] ALCL is positive for the cell surface marker CD30 and derives from lymphoid cells of the T-cell immunophenotype. [28] Based on morphology there are five subsets of ALCL. [29] About 40 to 60% of ALCLs show a distinct chromosomal translocation between the chromosomes 2 and 5. This translocation results in the generation of the fusion-protein Nucleophosmin1-anaplastic lymphoma kinase (NPM-ALK), defining the ALK-positive (ALK+) ALCL subset, which is responsible for about 3% of all adult non-Hodgkin-lymphomas (figure 5). [30]

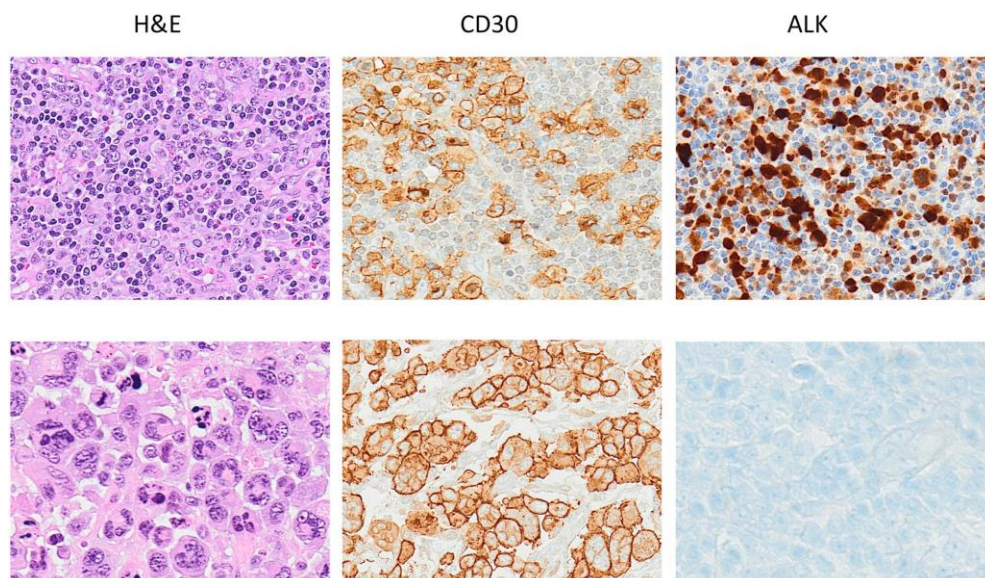


Figure 5: Immunohistochemistry of two ALCL subtypes. Both subtypes are positive for the ALCL marker CD30. The ALK+ subtype is shown in the top row, whereas the bottom pictures show an ALK-subtype. [29]

Besides NPM1, there are other fusion partners of ALK in ALCL, like tropomyosin4, TFG or ATIC. [31; 60] However, the fusion of ALK to NPM is the most common translocation, accounting for 70 to 80% of ALK+ ALCLs. Whereas ALK itself is normally an orphan receptor tyrosine kinase expressed only in cells of neural origin [59], the NPM-ALK fusion protein shows oncogenic activity. [32] The ALK part of NPM-ALK consists of the intracellular tail and kinase domain of ALK, while the NPM part encodes the dimerization and oligomerization region of NPM1, resulting in constitutive activation of NPM-ALK and several downstream signaling pathways. [31]

The most important among these are the JAK/STAT, the MEK/ERK and the PI3K/Akt-signaling pathways, which contribute to enhanced migratory potential, to proliferation and prohibit apoptosis in the lymphocytes. [31]

STAT3 is a transcription factor activated upon growth signals and cytokines, leading to a variety of cellular responses like proliferation and survival. [52] Several studies suggest that STAT3 exerts oncogenic functions in a variety of malignant diseases, including T-cell lymphomas like ALCL. There is evidence for STAT3 regulating the expression of DNMT1 in T-cell lymphomas, linking epigenetics to oncogenic cell signaling. [33] In addition to this finding, Wasik et al. reported that STAT3 also recruits DNMT1 to promoters of tumor suppressor genes, resulting in their epigenetic silencing (figure 6). By this mechanism, STAT3 repressors like SHP-1 (protein tyrosine phosphatase, non-receptor type 6) become inactivated, ensuring continuous STAT3 expression. [33]

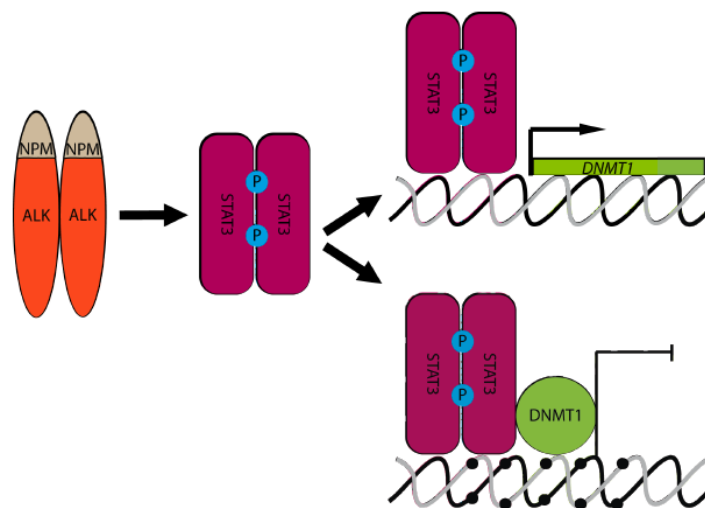


Figure 6: Suggested mechanism of how NPM-ALK signaling regulates *Dnmt1*. The NPM-ALK downstream target STAT3 upregulates transcription of *Dnmt1* and recruits DNMT1 to promoter regions of genes. This results in their epigenetic silencing by DNA methylation. [figure provided by Gerda Egger adapted from 31]

1.5 The Cre/loxP system

One of the most common and established ways to study the function of proteins is to specifically knock out a gene. Since Thomas et al. reported to have successfully conducted a targeted gene disruption in embryonic mouse stem cells [40] in 1987, a large number of genes has been mutated in mice, generating new knockout strains. [41] Using this targeted disruption technique, the function of most genes has been characterized in embryonic development. Postnatal gene functions however could not

be analyzed, since many of the knockout strains carried null mutations leading to developmental defects and embryonic lethality. [41] Therefore scientists were in need for another method to induce targeted gene disruptions, without generating null mutations. The Cre/lox recombination system was discovered in 1978 by Sternberg and offered a great alternative to previous approaches because of its simple mechanism. [43, 44]

Besides conditional gene knockouts, Cre-mediated recombination is used for the activation of genes, by removal of a functional barrier to gene expression by recombination. [42] This system is based on the Cre integrase from bacteriophage P1, which recombines at specifically recognized sequences, the loxP sites. [45] In order to induce a genetic deletion in a specific tissue, the Cre recombinase has to be expressed under the control of a tissue-specific promoter like CD4 for T-cell specificity. An advanced version of the Cre/loxP system, the CreERT², makes use of a fusion protein, consisting of Cre and a mutated form of the estrogen receptor. This mutation in the ligand binding domain of the estrogen receptor inhibits binding of the natural ligand 17 β -estradiol at physiologically normal concentrations. 4-Hydroxy-tamoxifen, however, can bind to the mutated receptor, resulting in the translocation of the fusion protein into the nucleus, where Cre can exert its function and leads to recombination at loxP sites. [42]

We used a transgenic mouse model with loxP flanked exons 4 and 5 of *Dnmt1* that expresses NPM-ALK under the T-cell specific CD4 promoter. Crossing this mouse strain with a strain that expresses Cre under the thymus specific promoter of Lck, a mouse with CD4 specific NPM-ALK expression and Lck-specific *Dnmt1* ^{$\Delta\Delta$} deletion was generated (Figure 7).

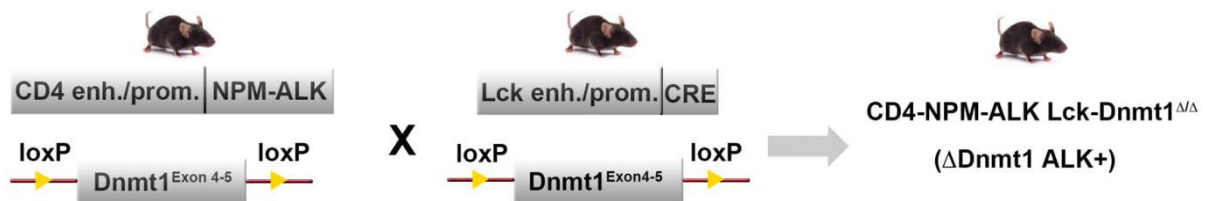


Figure 7: A mouse-model with tissue-specific *Dnmt1* deletion. A transgenic mouse expressing NPM-ALK under the CD4 promoter with floxed *Dnmt1* is crossed with a mouse expressing Cre under a tissue specific promoter like Lck. The resulting mouse will have CD4 specific NPM-ALK expression and Lck specific deletion of *Dnmt1*.

1.6 Project description

Preliminary data have shown that deletion of the DNA methyltransferase *Dnmt1* results in prolonged survival and suppressed lymphomagenesis of a transgenic NPM-ALK⁺ mouse model with T-cell specific *Dnmt1* deletion. Furthermore, *in vitro* studies suggest that aberrant DNA methylation is mediated by the oncogenic activity of ALK via STAT3 signaling. STAT3 upregulates the expression of *Dnmt1* and recruits the DNA methyltransferase to promoters, which causes their hypermethylation and silencing [33]. The aim of this project was to analyze how depletion of DNMT1 effects NPM-ALK positive lymphoma cells.

For this purpose, a tumor from the transgenic NPM-ALK⁺ mouse model harbouring loxP flanked sites between the exons 4 to5 of the *Dnmt1* gene was isolated and cultivated. DNMT1 was then inhibited either chemically with the drug decitabine or deleted genetically by using an inducible Cre recombinase approach. The resulting phenotypes were characterized by immunofluorescence stainings and western blot analysis.

In order to detect differences in gene expression caused by the depletion of DNMT1, RNA-sequencing was performed for both genetic and pharmaceutical approaches.

2. Materials and Methods

2.1 Cell culture

2.1.1 Thawing cells

Cells frozen in liquid nitrogen were thawed in a 37°C incubator for a few minutes and directly pipetted in RPMI-medium (Gibco) containing 10% fetal calf serum (FCS) and 1% penicillin/streptomycin mix (P/S). Cells were then centrifuged for 5 minutes at 300 RCF and washed with sterile PBS. After washing, cells were resuspended in RPMI-medium with 10% FCS and 1% P/S and cultured in 7,5 ml RPMI in T-25 culture flasks.

2.1.2 Subcultivating cells

The cells were cultured in RPMI-medium, containing 10% FCS and 1% P/S. For passaging, cells were grown to about 80% confluency in 15 ml RPMI in T-75 culture flasks. About 5 ml of the cells were centrifuged for 5 minutes at 300 RCF and washed in PBS. Pelleted cells were resuspended in 1ml RPMI-medium and transferred into a new T-75 culture flask with 15 ml RPMI-medium.

2.1.3 Cryopreservation of cells

For cryopreservation, cells were grown to about 80% confluency in T-75 culture flasks. Cells were then centrifuged for 5 minutes at 300 RCF and washed twice in PBS. Pelleted cells were resuspended in 1ml FCS + 10% DMSO and transferred in 3 to 4 cryovials depending on pellet size. Cryovials were put into a Mr. Frosty stored at -80°C for slow freezing. After 24 hours the cryotubes were transferred into liquid nitrogen.

2.1.4 Isolation of murine NPM-ALK+ T-cell lymphoma cells

For the isolation of murine NPM-ALK+ T-cell lymphoma cells, the tumor deriving from the transgenic NPM-ALK+ mouse “B57” with loxP sites flanking *Dnmt1*, was isolated and passed through a 40 µM cell strainer. The passed tumor cells were washed with sterile PBS and cultivated in RPMI-medium with 10% FCS and 1% P/S.

2.1.5 Genetic deletion of *Dnmt1* in ALCL cells

2.1.5.1 Lentiviral transduction of NPM-ALK+ murine lymphoma cells

In order to delete *Dnmt1* in the NPM-ALK+ murine lymphoma cells with floxed *Dnmt1* (figure 8), the Cre recombinase has to be expressed in those cells. Therefore a lentiviral transduction with a vector carrying Cre, the MSCV CreERT² Puro^R vector (figure 8), was performed. MSCV CreERT2 puro was a gift from Tyler Jacks (Addgene plasmid # 22776). [66] The vector was packaged by the helper cell line PHOENIX-Eco. For the transfection of MSCV CreERT² into the helper cell line, PHOENIX-Eco cells were cultivated to about 70% confluency on 10 cm petri dishes in DMEM medium (Gibco). For transfection, the medium was carefully changed to Opti-MEM (Gibco). Lipofectamine 2000 (Invitrogen) was used for the transfection. 60 µl of Lipofectamine were mixed with 500 µl of Opti-MEM in a microcentrifuge tube. In parallel, 10 µg of the MSCV CreERT² vector were mixed with 500 µl Opti-MEM. Both reaction mixes were incubated for five minutes at RT, mixed and incubated for 20 minutes at RT. The reaction mix was finally added dropwise onto PHOENIX-ECO cells. After 24 hours of incubation at 37°C, medium was changed carefully to DMEM and incubated for another 24 hours, before the viral particles containing supernatant was harvested. For the transduction 2,5 ml viral supernatant and 4 µl of a 4 mg/ml polybrene solution (Sigma) were added to 1,5 ml RPMI-medium and 2×10^5 cells in a 6 well plate. The plate was then centrifuged for 30 minutes at 1000 rpm. Next, the cells were incubated at 37°C for 24 hours, before repeating the transduction. After an incubation time of 24 hours, the cells were centrifuged for five minutes at 1300 rpm and carefully resuspended in 1 ml RPMI-medium with 10% FCS, 1% P/S and 0,5 µg/ml puromycin. The cells were then cultivated for two weeks in the above medium for puromycin-selection.

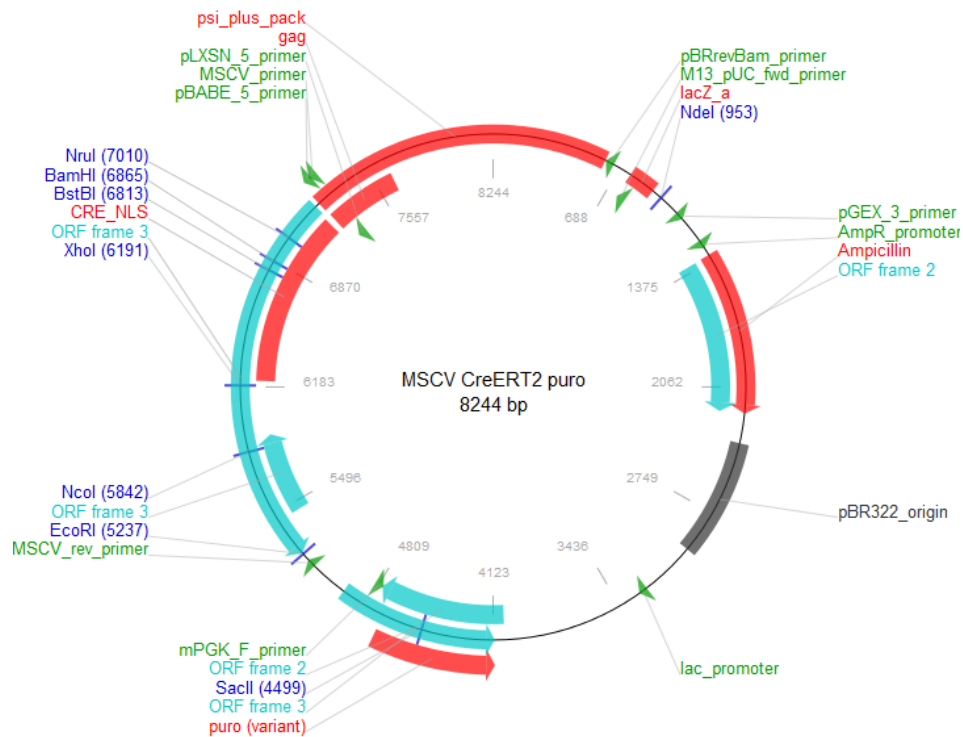


Figure 8: The vector used for the lentiviral transduction. The targeting vector used for the lentiviral transduction, MSCV CreERT² contains a fusion between the Cre-recombinase and the estrogen-receptor, making it inducible upon tamoxifen-treatment. Puromycin resistance is used for selection. [66]

2.1.5.2 Culture conditions and 4-Hydroxytamoxifen - treatment

For the genetic deletion of *Dnmt1* in the B57 cell line, cells were cultured in RPMI-medium with 10% fetal calf serum and 1% P/S in T-75 culture flasks. For the tamoxifen treatment cells were counted, using Cell-Chip PS Neubauer Improved counting chambers and seeded into a new T-75 culture flasks at a density of 2×10^7 cells in 15 ml RPMI with 250 nM 4-hydroxytamoxifen.

Cells were cultured for 96 hours and counted every day. After 4 days the cells were harvested for DNA, RNA and protein isolation, as well as for immunofluorescence and FACS analysis.

2.1.6 5-aza-2'-deoxycytidine treatment for chemical DNMT1 inhibition

For inhibition of DNMT1 on the protein level, the drug 5-aza-2'-deoxycytidine (5AZA-CdR/ decitabine) was used. 5-aza-CdR is a cytidine analogue that inhibits DNMT1 by binding it covalently. The murine NPM-ALK+ T-cell lymphoma cell line B57 was cultured in RPMI-medium with 10% FCS and 1% P/S. For 5AZA-CdR treatment 3×10^7 cells were seeded in a T-75 culture flask and 0,04 μ M 5AZA-CdR was added to

the medium. Cells were then incubated at 37°C for 24 hours before changing the medium. Another incubation time of 28 hours at 37°C followed and cells were finally harvested and pelleted for further procedures.

2.1.7 Generation of viability curves

In order to establish viability curves for the murine NPM-ALK+ T-cell lymphoma cell line B57 after 5-aza-CdR and tamoxifen treatment, cells were stained with 0,4% trypanblue staining solution and counted every day in a Cell-Chip PS Neubauer Improved counting chamber. Therefore 100 µl trypanblue was pipetted onto 900 µl cell suspension and carefully mixed by pipetting. 10 µl of the stained cell suspension were pipetted into a Cell-Chip PS Neubauer Improved counting chamber and living cells as well as dead/dying cells (stained blue) were counted with an optical microscope. Cell viability was calculated by dividing the amount of living cells through total cells.

2.2 Molecular biology methods

2.2.1 DNA isolation

Cells were centrifuged for five minutes at 300 RCF, washed with 1 x PBS once and centrifuged another time. The resulting pellets were dissolved in 500 – 1000 µl of genomic DNA lysis buffer, depending on pellet size, and resuspended by pipetting, whereas vortexing was avoided. 20 µg/ml of RNaseA were added to the samples and incubated at 37°C for 30 to 60 minutes. Next, 500 µg/ml of Proteinase K were added and incubated at 55°C overnight on a thermal shaker. The next day phenol/chloroform extraction was performed: the samples were transferred to special phase lock gel tubes (5 prime) and 1 volume of buffered phenol/chloroform was added, mixed by shaking and centrifuged for 10 minutes at 16000 RCF. The upper phase was transferred into new microcentrifuge tubes by careful pipetting. Now 1 volume of isopropanol was added and the tubes were shaken vigorously for 15 seconds until a white precipitate (DNA) was visible. The samples were centrifuged for 15 minutes at 16000 RCF and the supernatant was discarded by pipetting. The pellets were washed with 1 ml 75% EtOH by carefully pipetting up and down. Another centrifugation for 15 minutes at 16000 RCF was done and the supernatant was removed completely by pipetting. To dry the pellets, the tubes were incubated at 37°C for five to 10 minutes until pellets became colorless. 50 – 100 µl of sterile, ultra-pure H₂O, depending on

pellet size, were used to dissolve the pellets. A last incubation step at 37°C for at least one hour or overnight until the DNA was well dissolved, was performed.

Buffers and solutions:

Genomic DNA lysis buffer:

- 0,4 M NaCl
- 0,2 % SDS
- 0,1 M Tris
- 5 mM EDTA

2.2.2 PCR for knockout verification of genetic *Dnmt1* deletion

After the 4-Hydroxytamoxifen-treatment, DNA was isolated and a polymerase chain reaction (PCR) was performed, in order to verify the successful knockout of *Dnmt1*. Concentrations of the DNA samples were measured using a Nanodrop 2000 spectrophotometer (Thermo scientific).

For PCR the KAPA2G Fast Hot Start Mix (PEQLab) was used. After PCR the products were run on a 2% agarose gel containing 1% midori green at 120V for 20 minutes. Gel electrophoresis was followed by visualization with UV light.

PCR-setup (15µl reactions):

- 7,5 µl KAPA2G Fast Hot Start Mix (PEQLAB)
- 5 µl ddH₂O
- 1,5 µl primer mix (0,5 µl of each primer; 10µM)
- 1µl DNA Template

PCR conditions:

- 95 °C 3 min
- 95 °C 15 sec
- 60 °C 15 sec
- 72 °C 30 sec
- 72 °C 10 min
- Repeat step 2-4 35 times

TAE buffer (1 l):

- 4,48 g Tris
- 2 ml EDTA (500 mM, pH = 8)
- 1,14 ml glacial acetic acid
- Fill up to 1 l with H₂O

Primer:

- Dnmt1_forward: 5'-GGG CCA GTT GTG TGA CTT GG-3'
- Dnmt1_reverse1: 5'-TGA ACC TCT TCG AGG GAC C-3'
- Dnmt1_reverse2: 5'-ATG CAT AGG AAC AGA TGT GTG C-3'

2.2.3 RT-qPCR

For measuring IAP retrotransposon expression levels after tamoxifen and 5-aza-CdR treatment, a RT-qPCR was performed. After RNA isolation, RNA was converted to cDNA using the SuperScript II reverse transcriptase kit with the supplied protocol from Invitrogen. For RT-qPCR the Kapa Sybr Fast qPCR mastermix (Peqlab) was used.

PCR-reaction (15 µl):

- 7,5 µl Kapa Sybr Fast qPCR mastermix
- 2 µl cDNA
- 4,5 µl ddH₂O
- 1 µl Primer-mix

PCR-conditions:

- 95 °C 3 minutes
- 95 °C 15 seconds
- 55° C 30 seconds
- Plate read
- Repeat steps 2 – 4 39 times.

Primer:

- IAP_fo: 5'-ACTAACTCCTGCTGACTGG-3'
- IAP_re: 5'-TGTGGCTTGCTCATAGATTAG-3'

2.2.4 Protein isolation

Pelleted cells were resuspended in an appropriate amount (about two times pellet size) of HUNT lysis buffer. Resuspended cells were then frozen in liquid nitrogen and thawed at 37 °C for two to three minutes. Then the samples were frozen another time in liquid nitrogen and thawed on ice for about ten minutes, before centrifuging them at 16800 RCF for 15 minutes at 4 °C. The resulting supernatants containing the proteins were transferred into new microcentrifuge tubes and protein concentration was measured using a Bradford assay. Aliquots of the samples were stored at -80 °C.

Buffers and solutions:

HUNT-buffer (40 ml):

- 800 µl 20 mM Tris (pH = 8,0)
- 660 µl 100 mM NaCl
- 80 µl 1 mM EDTA
- 2 ml 0,5 % NP-40
- Fill up to 40 ml with ddH₂O

2.2.5 Western Blot analysis

To check for various protein levels in the cells after 4-Hydroxytamoxifen and decitabine treatment, western blots were performed.

2.2.5.1 Protocol

After protein isolation, 20 µg of protein were dissolved in 2x DTT buffer. HUNT buffer was used to fill up the samples to a final volume of 25 µl. Next, the samples were incubated at 95°C for 8 minutes. The samples were then loaded onto an 8% polyacrylamide-gel and separated by gel-electrophoresis using 120V. After electrophoresis, the separated proteins on the gel were blotted onto a nitrocellulose membrane. For this purpose a wet transfer with transfer buffer was performed and the blotting procedure lasted two hours and 30 minutes at 120V. Ponceau-red staining served as control for successful transfer and equal protein loading. The membrane was washed with 1x TBS-T for five minutes on a laboratory shaker before blocking with 5% milk-powder diluted in 1x TBS-T for at least one hour. Primary antibody (diluted in blocking solution) incubation occurred at 4°C on a shaker overnight. Next

the membrane was washed four times for five minutes with 1 x TBS-T on a laboratory shaker. After washing, incubation with the secondary antibody (diluted 1:10000 in 1 x TBS-T) for one hour at room temperature was performed. Membranes were washed another four times for five minutes with 1 x TBS-T. In order to measure protein signals, the membranes were incubated with ECL plus Western blotting reagent (Thermofisher) for three minutes and measured with a F1 LumiAnalyst imager (Roche).

Buffers and solutions:

HUNT-buffer (40 ml):

- 800 µl 20 mM Tris (pH = 8,0)
- 660 µl 100 mM NaCl
- 80 µl 1 mM EDTA
- 2 ml 0,5 % NP-40
- Fill up to 40 ml with ddH₂O

2x DTT-loading dye:

- 100 mM Tris-HCl (pH = 6,8)
- 200 mM DTT
- 4 % SDS
- 0,2 % Bromphenolblue
- 20 % Glycerol

Electrophoresis buffer (1 l):

- 14,4 g Glycine
- 3 g Tris
- 1 g SDS
- Fill up to 1 l H₂O

Transfer buffer (1 l):

- 11,2 g Glycine
- 2,46 g Tris
- 200 ml Methanol
- Fill up to 1 l H₂O

10 x TBS-T (1 l):

- 60,5 g Tris
- 90 g NaCl
- 0,1 % Tween-20
- Fill up to 1 l with H₂O

Blocking solution (500 ml):

- 25 g Instant milk powder
- 0,01% NaN₃
- Fill up to 500 ml with 1 x TBS-T

Ponceau-red staining solution:

- 0,1 % Ponceau-red
- 5% Acetic acid

8% polyacrylamide gel:

	8% separating gel (30 ml for 3 gels)	5% stacking gel (10 ml for 4 gels)
H ₂ O	13.8 ml	6.8 ml
30% acrylamide mix	7.8 ml	1.7 ml
1.5 M Tris pH = 8.8	7.8 ml	-
1.0 M Tris pH = 6.8	-	1.25 ml
10% SDS	300 µl	100 µl
10% APS	300 µl	100 µl
TEMED	30 µl	10 µl

Table 1: Reagents and volumes used for the preparation of polyacrylamide gels

2.2.5.2 Antibodies

Primary Antibodies	Company	Dilution	Protein size
DNMT1	Santa Cruz; H300 rabbit mAb	1:400 in blocking solution	184 kDa
ALK	Cell Signaling; D5F3 XP rabbit mAb	1:1000 in blocking solution	80 kDa (NPM-ALK)
Phospho-ALK	Cell Signaling; D59G10 rabbit mAb	1:1000 in blocking solution	80 kDa (NPM-ALK)
STAT3	Cell Signaling; D3Z2G rabbit mAb	1:1000 in blocking solution	79/86 kDa
Phospho-STAT3	Cell Signaling; D3A7 XP rabbit mAb	1:1000 in blocking solution	79/86 kDa
β-Actin	Cell Signaling; rabbit mAb	1:1000 in blocking solution	45 kDa
Secondary Antibodies			
Anti-rabbit Peroxidase AffiniPure IgG	Jackson; Goat anti-rabbit IgG	1:10000 in 1 x TBS-T	

Table 2: List of antibodies used for the conducted Western blot analyses.

2.2.6 RNA isolation

For RNA isolation the RNeasy Mini Kit (QIAGEN) was used and extraction was performed according to the supplied protocol. Before starting with the RNA isolation from pelleted cells, the working areas as well as pipettes were cleaned with RNase AWAY Surface Decontaminant (Thermo Scientific).

After RNA isolation the concentrations were measured on a Nanodrop 2000 spectrophotometer (Thermo Scientific).

2.2.7 Immunofluorescence

2.2.7.1 Protocol

In order to spin cells onto cover slides for immunofluorescence stainings, cell suspensions of 10^6 cells/ml in RPMI-medium were generated. 100 µl of these suspensions were then spun on each cover slide, using a Shandon Cytospin3 centrifuge (Amlab). Cover slides were checked under the microscope for correct cell density and dried for 2 minutes at room temperature, before putting the slides on ice. Using a pap-pen marker the cell-containing area was marked and fixed with 4 % paraformaldehyde for 10 minutes at 4 °C. After fixing, paraformaldehyde was removed by tapping the slides on a tissue and drying for 2 minutes at room temperature. Next the cells were permeabilized with 0,1% Triton X-100 in PBS for ten minutes at room temperature. For staining against 5-methylcytosine, incubation with 2 M HCl for 15 minutes was carried out, followed by neutralization with 100 mM Tris-HCl pH 8,5 for

ten minutes. The following steps were performed for all stainings. Slides were washed five times for five minutes with 1 x PBS and blocked with 10 mg/ml endobulin for 30 minutes at room temperature. Another washing step with 1 x PBS for ten minutes followed, before the slides were incubated with the primary antibody overnight at 4 °C in a humidified chamber. On the next day, the cover slides were washed once again three times for five minutes in 1 x PBS and incubated with the secondary antibody for one hour in a dark humidified chamber at room temperature. DAPI-staining was performed after the incubation time, by adding DAPI solution (diluted 1:50000 in 1 x PBS) directly onto the marked area. After five minutes DAPI was removed by tapping, before mounting the cover slides with geltol. The slides were finally dried in the dark at room temperature for at least 2 hours and stored at 4 °C afterwards until analysis with a LSM 5 exciter confocal microscope (Zeiss).

2.2.7.2 Antibodies

Primary Antibody	Company	Dilution
DNMT1	Santa Cruz; H300 rabbit mAb	1:100 in 2% BSA in 1 x PBS
5-methyl-cytidine	Diagenode; Mab-005-500 mouse	1:500 in 2% BSA in 1 x PBS
Phospho-H2AX	Cell Signaling; 20E3 rabbit mAb	1:400 in 2% BSA in 1 x PBS
Phospho-H3S10	Cell Signaling; D2C8 rabbit mAb	1:1600 in 2% BSA in 1 x PBS
Secondary Antibody		
Alexa fluor 546 α-rabbit	ThermoFisher; Goat anti rabbit IgG	1:500 in 2% BSA in 1 x PBS
Alexa fluor 546 α-mouse	ThermoFisher; Goat anti mouse IgG	1:500 in 2% BSA in 1 x PBS

Table 3: List of antibodies used for the conducted immunofluorescence stainings.

2.2.8 FACS-Analysis

For FACS-analysis and cell-cycle-profiling the Click-iT® EdU Alexa Fluor® 488 Flow Cytometry Assay Kit from ThermoFisher was used. The cells were treated with EdU for 20 minutes, followed by fixation with 100% ice cold methanol for 10 minutes at -20°C. Further steps were done according to the provided protocol.

2.2.9 RNA-sequencing

RNA of both treatments, either tamoxifen or 5-aza-CdR, was isolated. For tamoxifen treatment, cells were harvested four days post treatment and RNA was isolated as described in 4.2.5. Cells subjected to decitabine treatment were harvested two days post treatment.

Quality control, RNA-sequencing and statistical processing was performed by Christoph Bock's group at the biomedical sequencing facility (CeMM Research Center for Molecular Medicine, Vienna).

TopHat (<https://ccb.jhu.edu/software/tophat/index.shtml>) was used in order to identify exon-exon splice junctions. Additionally, Cufflinks (<http://cole-trapnell-lab.github.io/cufflinks/>) assembled aligned RNA-Seq reads into transcripts, estimated their abundances and tested, transcriptome-wide, for differences in gene expression and regulation. Furthermore Cuffdiff (<http://www.broadinstitute.org/cancer/software/genepattern/modules/docs/Cuffdiff/7>) found significant changes in promoter use, splicing and transcript expression. Quality plots were generated for genes and isoforms, including Dispersion plots, density plots and Scatter Matrix plots.

3. Results

3.1 Genetic deletion of *Dnmt1* in murine T-cell lymphoma cells

3.1.1 Lentiviral transduction and 4-Hydroxytamoxifen treatment

The first step in the analysis of *Dnmt1* function for murine T-cell lymphoma cell survival was to construct a genetic deletion of *Dnmt1*. In order to do so, tumors from a transgenic NPM-ALK mouse model with loxP sites at exon 4 and 5 of the *Dnmt1* gene were isolated and cultivated. Since expression of the Cre recombinase is necessary for inducing the deletion of *Dnmt1*, a lentiviral transduction with the MSCV CreERT² vector was performed. Successful transduction was ensured by puromycin selection. Lymphoma cells were treated with 4-hydroxytamoxifen, resulting in a complete deletion of *Dnmt1* 3 days post treatment as confirmed by genotyping and Western blot analysis (figures 9 and 11). Genotyping of DNA samples of tamoxifen treated B57 cells after lentiviral transduction with the MSCV CreERT² vector resulted in two possible bands, *Dnmt1* 2lox and *Dnmt1* 1lox (figure 10). While the 2lox band included exons 4 and 5 and therefore the functional *Dnmt1* alleles, the 1lox alleles underwent Cre mediated recombination and lack exons 4 and 5 resulting in the *Dnmt1* knockout.

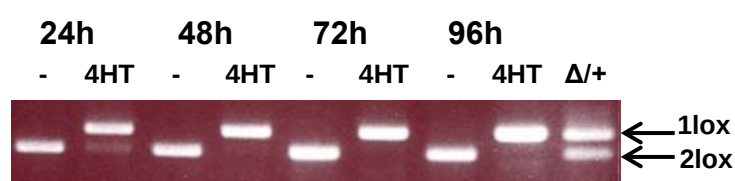


Figure 9: Genotyped DNA samples of tamoxifen treated B57 cells. DNA was isolated 24, 48, 72 and 96 hours post treatment. “4HT” indicates treatment, whereas “-” represents DNA from control cells. After 24 hours, cells have a nearly complete *Dnmt1* deletion, as the *Dnmt1* 1lox signal is stronger than the *Dnmt1* 2lox signal. Complete knockout is reached after 48 hours post tamoxifen treatment.

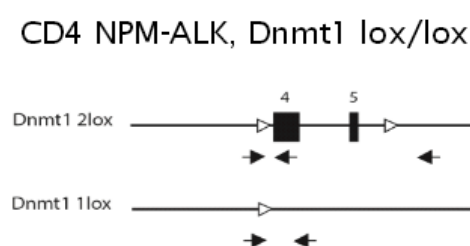


Figure 10: LoxP sites depicted as open arrow heads flank exons 4 and 5 of *Dnmt1*. Upon tamoxifen treatment Cre recombinase will exert its function and remove these exons resulting in a *Dnmt1* 1lox allele. Black arrows show primer sites for PCR.

Western blot analysis of tamoxifen treated Cre expressing B57 cells with an antibody against DNMT1 showed the impact of the genetic *Dnmt1* knockout on the protein level. DNMT1 was already reduced 24 hours after the treatment and was no longer detectable after 48 hours (figure 11).

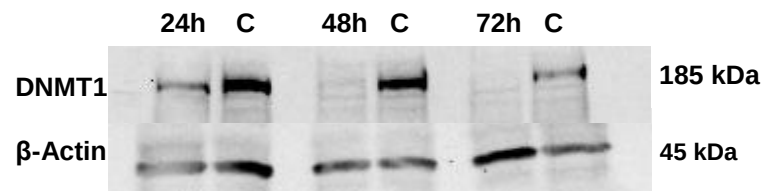


Figure 11: Western Blot analysis of tamoxifen treated, Cre expressing B57 cells. DNMT1 is slightly reduced 24 hours post treatment, but no longer detectable after 48 hours. β-Actin served as loading control.

To analyze the effect of *Dnmt1* deletion we measured the expression levels of the retrotransposon intracisternal A-particle (IAP), which is usually silenced by DNA methylation (Figure 12). [46] Expression levels of this retrotransposon were about 45 times higher six days and about 10 times higher after four days post tamoxifen treatment, compared to control cells. However, there seemed to be no difference in the expression levels between control and treated cells after one day of *Dnmt1* deletion.

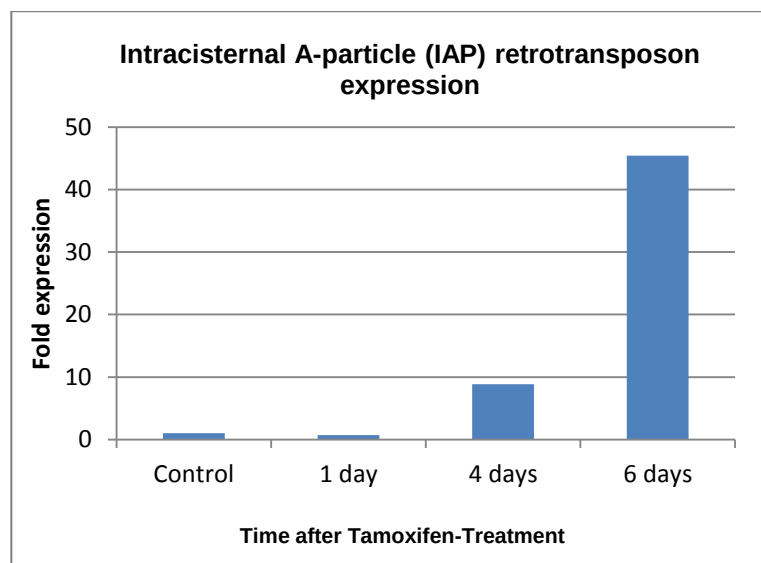


Figure 12: IAP retrotransposon expression after *Dnmt1* deletion. Deletion of *Dnmt1* in the murine NPM-ALK T-cell lymphoma cell line B57 results in re-expression of the usually silenced retrotransposon IAP. Expression is elevated in cells four and six days after *Dnmt1* deletion.

We also performed immunofluorescence staining to monitor the levels of DNMT1 depletion after tamoxifen treatment (figure 13). We observed a strong loss of the specific nuclear DNMT1 signal in treated cells confirming the successful deletion of *Dnmt1*.

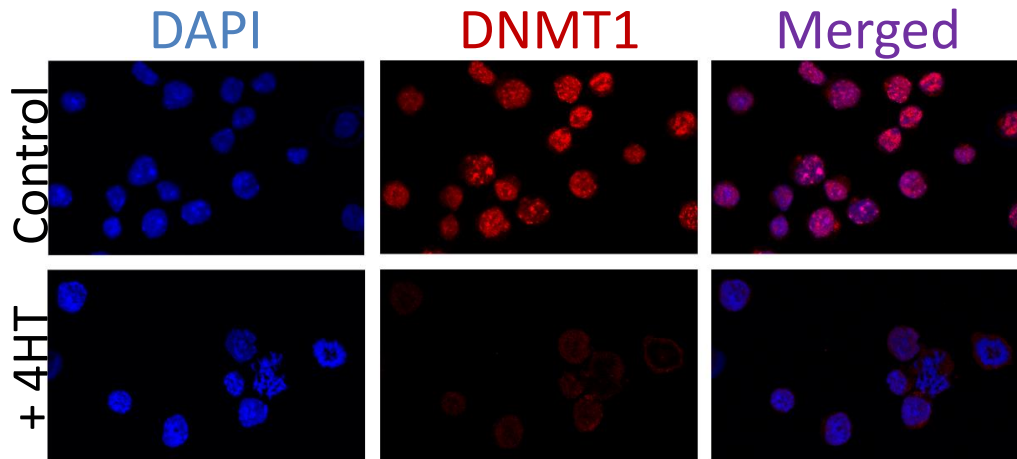


Figure 13: Immunofluorescence staining against DNMT1 after tamoxifen treatment. DNMT1 levels are clearly reduced in the NPM-ALK+ T-cell lymphoma cell line B57 after tamoxifen treatment (bottom) when compared to untreated control cells (top). DAPI indicates nuclear staining. Cells were analyzed 96 hours after tamoxifen treatment.

Since absence of DNMT1 leads to passive demethylation over continuous replication cycles, a global loss of 5-methylcytosine was observable by immunofluorescence staining with an antibody recognizing 5-methylcytosine (figure 14). The typical dotted 5-methylcytosine staining caused by strong methylation of heterochromatin was visible in the control cells, whereas treated cells only showed a ring-shaped signal in a subset of cells. This circular structure might be an artifact.

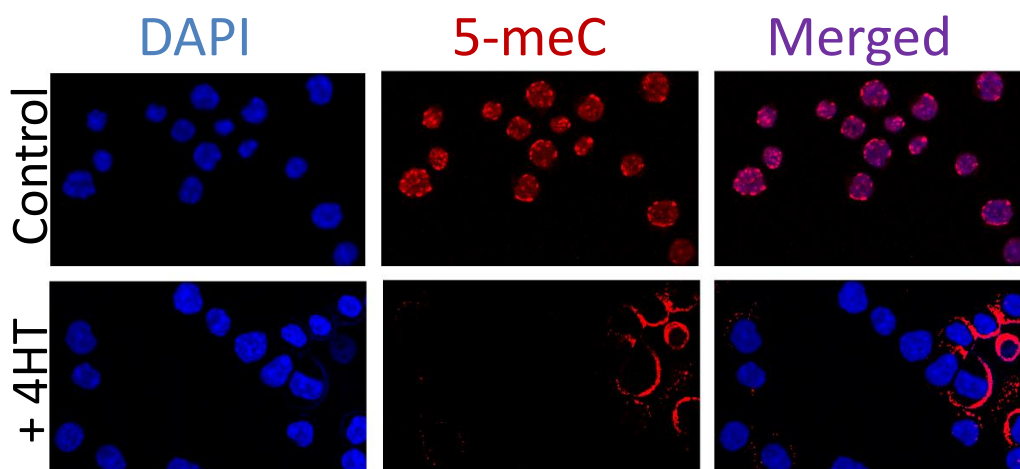


Figure 14: Immunofluorescence staining against 5-meC after tamoxifen treatment. 5-methylcytosine levels show a distinct reduction in NPM-ALK+ T-cell lymphoma cells after tamoxifen treatment (bottom) when compared to untreated control cells (top). DAPI indicates nuclear staining. Cells were analyzed 96 hours after tamoxifen treatment.

These data suggest that deletion of *Dnmt1* with an inducible Cre recombinase was successful resulting in a complete knockout 48 hours after tamoxifen treatment and subsequent highly reduced DNA methylation.

3.1.2 Characterization of the *Dnmt1*^{Δ/Δ} phenotype

Since *Dnmt1* was successfully deleted by 4-hydroxytamoxifen treatment and the knockout was confirmed by Western blot analysis, genotyping and immunofluorescence, the next task was to analyze the cells' phenotype. First, we analyzed cell survival rates. For this purpose, the cells were initially stained with 0,4% trypanblue staining solution (Gibco) every day after 4-Hydroxytamoxifen treatment. Percentage of viable cells was calculated for both treated and control cells by dividing the number of stained cells through the total number of cells counted.

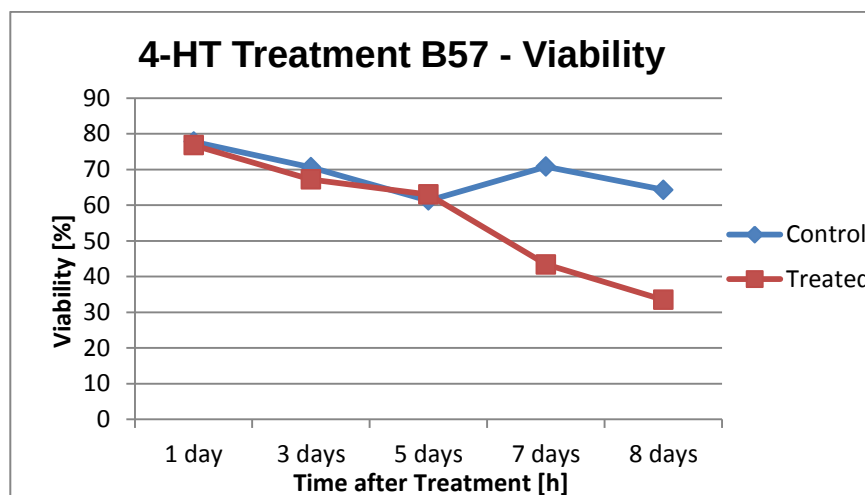


Figure 15: Viability of B57 cells after *Dnmt1* deletion. Viability starts to decrease after five days post tamoxifen treatment (red), when compared to control cells (blue).

While the viability of the control cells fluctuated between 80% and 60% over the course of eight days, treated cells showed a distinct decrease of their viability starting five days post-treatment to nearly 30% viability at day eight.

Next we investigated the levels of DNA damage following DNMT1 disruption by immunofluorescence staining against the DNA damage marker γ H2AX (figure 16). We observed a strong increase of γ H2AX positive signals in tamoxifen treated cells, suggesting higher levels of DNA damage upon deletion of *Dnmt1*.

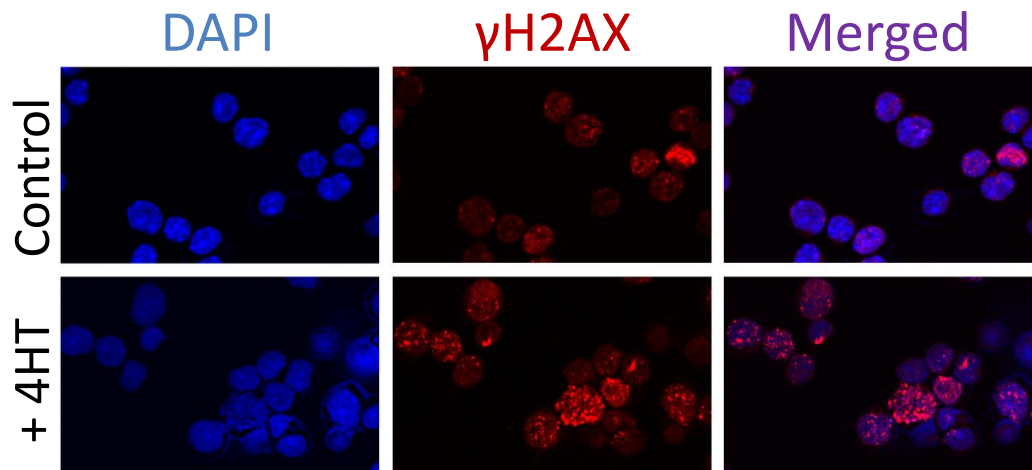


Figure 16: Immunofluorescence staining against γ H2AX after tamoxifen treatment. γ H2AX levels are increased in B57 cells after tamoxifen treatment (bottom) when compared to untreated control cells (top). DAPI indicates nuclear staining. Cells were analyzed 96 hours after tamoxifen treatment.

In addition to elevated γ H2AX levels and DNA damage, cellular stress could be detected in cells with deleted *Dnmt1* by phospho-H3S10 staining (figure 17). When compared to control cells, cells with deleted *Dnmt1* showed more intense fluorescence signals. Besides mitosis, phosphorylated H3S10 is an indicator for cellular stress. Therefore the stronger signal in treated cells for phosphorylated H3S10 could suggest increased cellular stress levels.

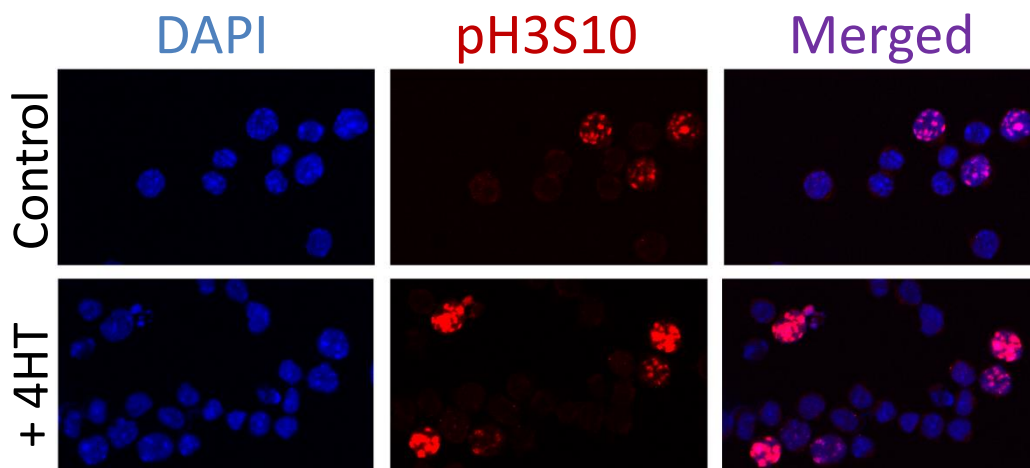


Figure 17: Immunofluorescence staining against phospho-H3S10 after tamoxifen treatment. Phospho-H3S10 staining results in a stronger signal in B57 cells after tamoxifen treatment (bottom) when compared to untreated control cells (top). DAPI indicates nuclear staining. Cells were analyzed 96 hours after tamoxifen treatment.

To monitor changes in cell cycle distribution and apoptosis, we performed cell cycle profiling using the Click-iT® EdU Alexa fluor® 488 Imaging Kit. This kit is based on a

modified thymidine analogue called EdU which is incorporated into newly synthesized DNA and labeled with an Alexa Fluor® dye in a fast and highly specific reaction. During this procedure the cell cycle profiles of control cells, cells two days, four days and seven days after tamoxifen treatment, were measured and analyzed (figure 18).

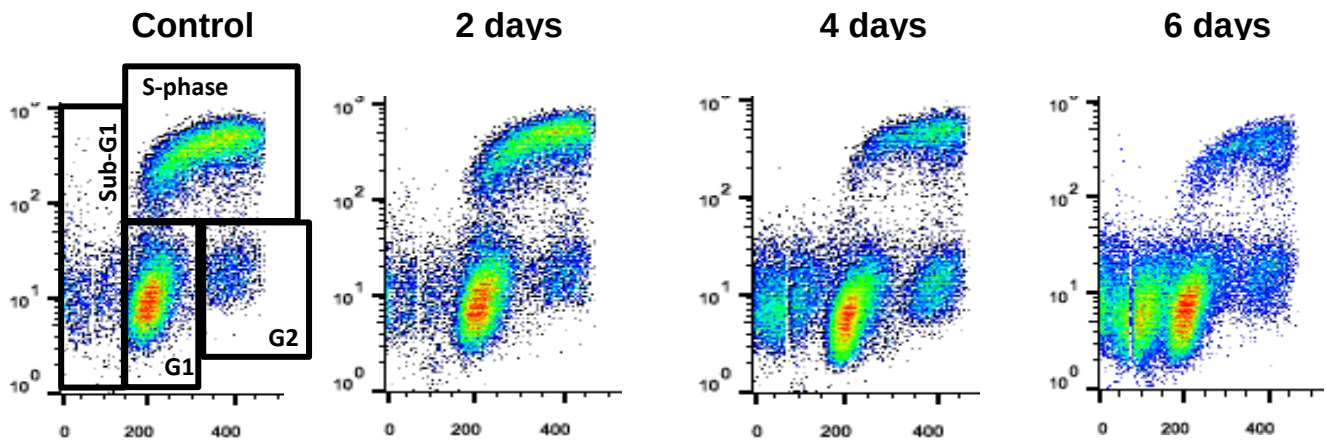


Figure 18: Cell-cycle profiling of tamoxifen treated B57 cells. The amount of S-phase cells starts to decrease 4 days after the treatment, while apoptotic Sub-G1 cells increase.

The cell cycle profile can be divided into four sections as displayed by the control cells in figure 18: S-phase cells, sub-G1 cells, G1 cells and G2 cells, with sub-G1 cells reflecting apoptotic cells. The profile of cells two days post treatment was similar to the profile of the control cells. After four and seven days, however, a decrease in S-phase cells and an increase in sub-G1 cells was observed as visualized in a bar chart (figure 19). Furthermore a slight increase in G2 cells, four days after the treatment occurred.

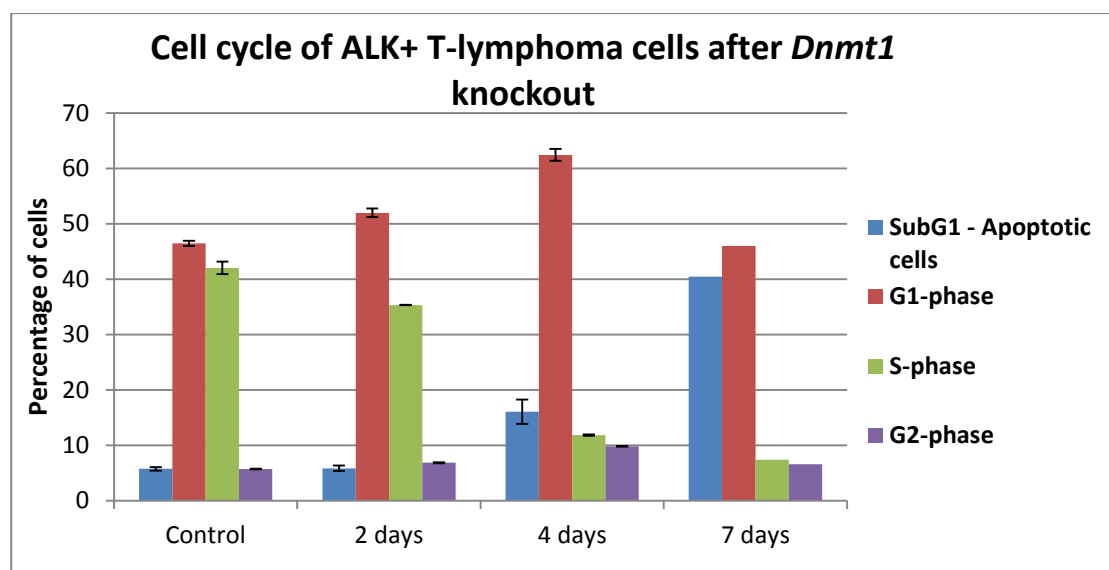


Figure 19: Cell cycle analysis after *Dnmt1* deletion. A cell-cycle profile of B57 cells after *Dnmt1* deletion shows a clear reduction of S-phase cells (green bars) over 7 days post-treatment, while apoptotic cells (blue bars) increase.

While S-phase cells decreased from 40% in the control to below 10% after seven days post treatment, the amount of apoptotic cells started to increase from approximately 5% to 40%. The biggest decrease of S-phase cells occurred from two days to four days post-treatment and the biggest gain of subG1 cells from four to seven days. The amount of G2-phase cells shows a slight increase four days after treatment. Also G1-phase cells were about 10% higher in cells after four days of tamoxifen treatment when compared to the remaining timepoints.

Taken together, our results show that NPM-ALK lymphoma cells are highly sensitive to genetic deletion of *Dnmt1* as indicated by high proportion of apoptotic cells and a marked decrease of S-phase cells after successful depletion of DNMT1. The slight increase in G2-phase cells and the induced staining against phospho-H3S10 might indicate a mitotic defect, reminiscent of a phenomenon described in colon cancer cells after *Dnmt1* deletion.

3.2 Inhibition of DNMT1 using 5-aza-2-deoxycytidine

Besides genetic deletion of *Dnmt1* a second approach was used, in which DNMT1 was inhibited chemically on the protein level. For this purpose the DNMT1 inhibitor 5-aza-2'-deoxycytidine (5-aza-CdR) was used. As this drug induces cytotoxic effects when used in higher concentrations, the viability of the NPM-ALK⁺ cells was initially tested at different concentrations (figure 20). For this, the cells were counted after staining with 0,4% trypanblue staining solution. As displayed in figure 20, control cells showed a viability of about 90% after two days, whereas a concentration of 0,01 μ M of 5-aza-CdR treatment led to 60% viability and only around 20% for higher concentrations indicating a high sensitivity of these cells against DNMT inhibitors.

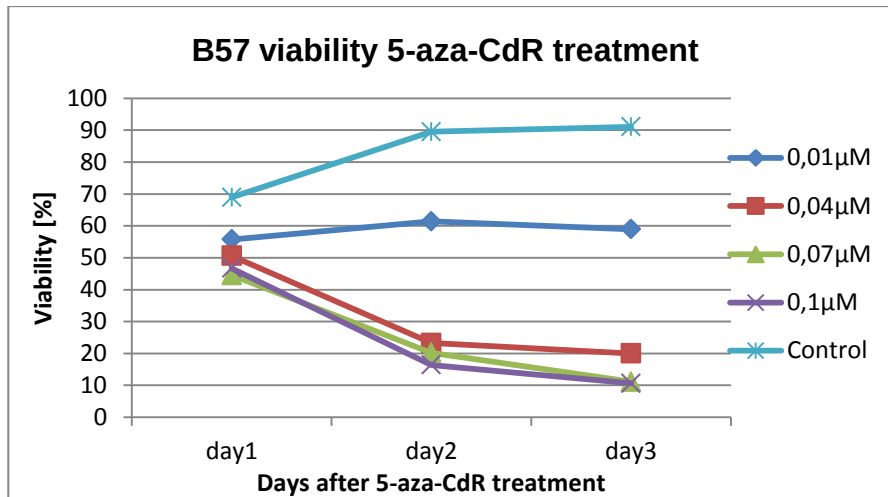


Figure 20: Viability of B57 cells after 5-aza-CdR treatment. Different concentrations of 5-aza-CdR were used to treat B57 cells in order to exclude improper concentrations with possible cytotoxic effects. Control cells reach 90 % viability three days after treatment, while 0,01 μ M 5-aza-CdR results in 60% viability. Higher concentrations reach 20 % (0,04 μ M) viability and below.

In order to make sure that DNMT1 is inhibited, the IAP retrotransposon expression was measured by quantitative PCR, as it was done with tamoxifen treated cells. The outcome showed no elevated IAP expression levels for control cells, whereas a concentration of 0,04 μ M and 0,07 μ M 5-aza-CdR led to a 4,5 times higher expression. 0,01 μ M 5-aza-CdR resulted in slightly higher expression levels of about 1,3 and 0,1 μ M to 20 times higher IAP expression. Based on these results, we used 0,04 μ M 5-aza-CdR for further experiments.

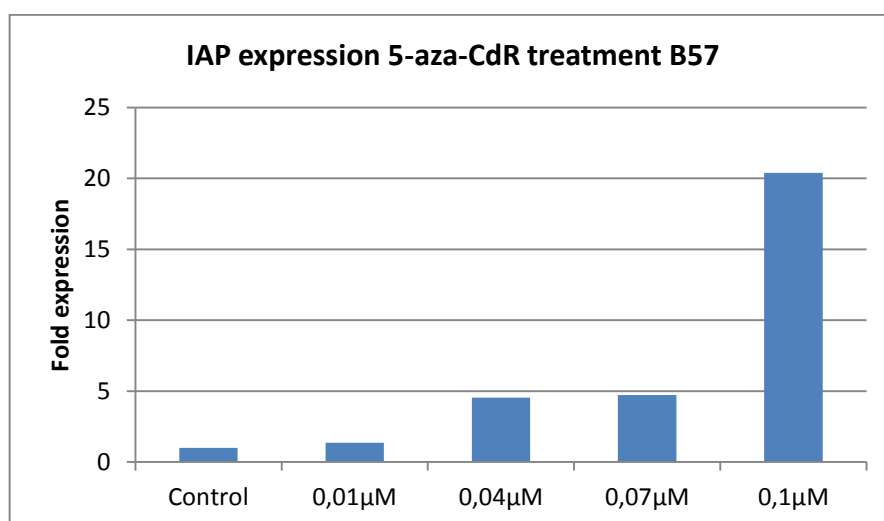


Figure 21: IAP retrotransposon expression of B57 cells after 5-aza-CdR treatment. Inhibition of DNMT1 in the murine NPM-ALK T-cell lymphoma cell line B57 results in re-expression of the usually silenced retrotransposon IAP, two days after 5-aza-CdR treatment. Higher 5-aza-CdR concentrations result in higher IAP expression levels.

As it was done for the experiments with genetic *Dnmt1* deletion, immunofluorescence stainings against DNMT1, 5-methylcytosine and γ H2AX were performed with 5-aza-CdR treated cells in order to analyze the effect of this drug.

Figure 22 is a staining against DNMT1 with control cells (top row) and 5-aza-CdR treated cells (bottom row). Comparing the merged and DNMT1 stainings from control and treatment cells, no significant differences were detected.

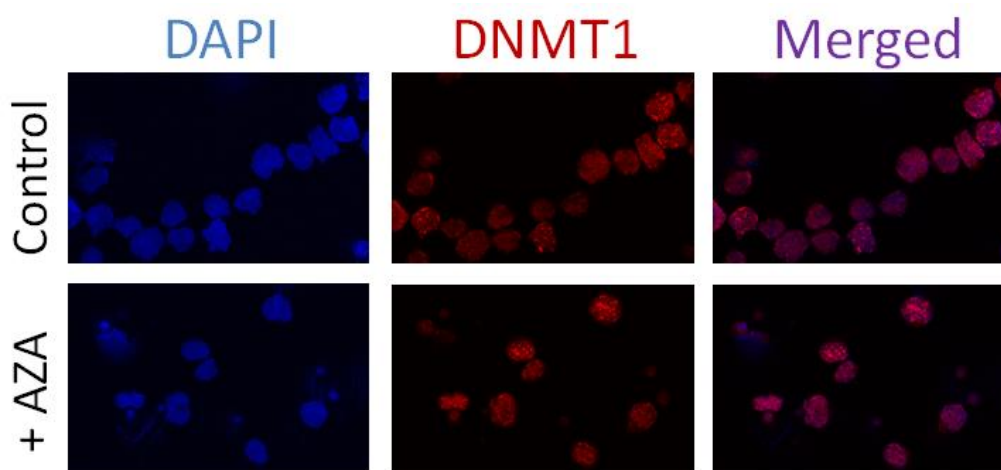


Figure 22: Immunofluorescence staining against DNMT1 after 5-aza-CdR treatment. DNMT1 levels show no difference in B57 cells after 5-aza-CdR treatment (bottom) when compared to untreated control cells (top). DAPI indicates nuclear staining. Cells were analyzed 52 hours after 5-aza-CdR treatment.

Since 5-aza-CdR is a DNMT inhibitor, its effects on global 5-methylcytosine were analyzed by immunofluorescence. We detected highly reduced 5-methylcytosine levels in cells treated with 5-aza-CdR compared to the control (figure 23).

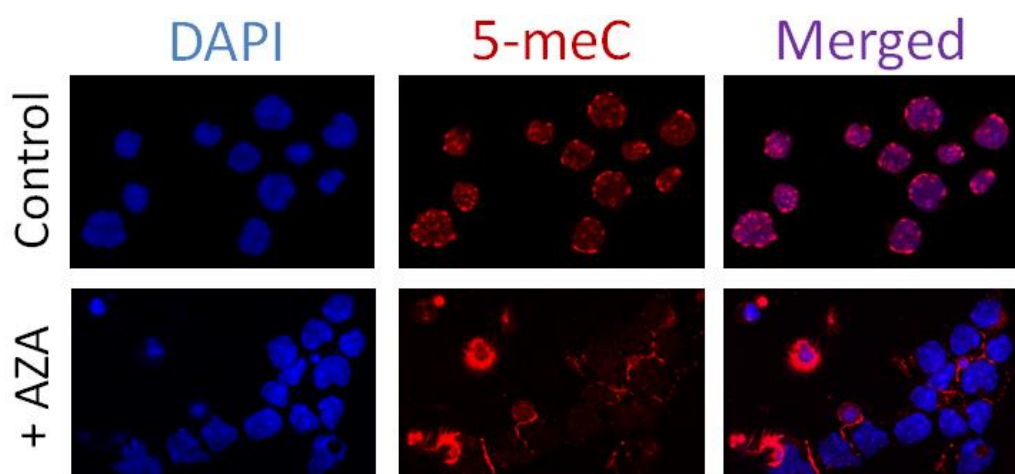


Figure 23: Immunofluorescence staining against 5-meC after 5-aza-CdR treatment. 5-methylcytosine levels are decreased in B57 cells after 5-aza-CdR treatment (bottom) when compared to untreated control cells (top). DAPI indicates nuclear staining. Cells were analyzed 52 hours after 5-aza-CdR treatment.

Besides DNMT1 and 5-methylcytosine, γ H2AX levels were analyzed via immunofluorescence staining after 5-aza-CdR treatment to evaluate the effects on DNA damage. DNA damage levels of murine NPM-ALK⁺ T-cell lymphoma cells seem to be slightly elevated upon treatment with 5-aza-CdR when measured via γ H2AX staining.

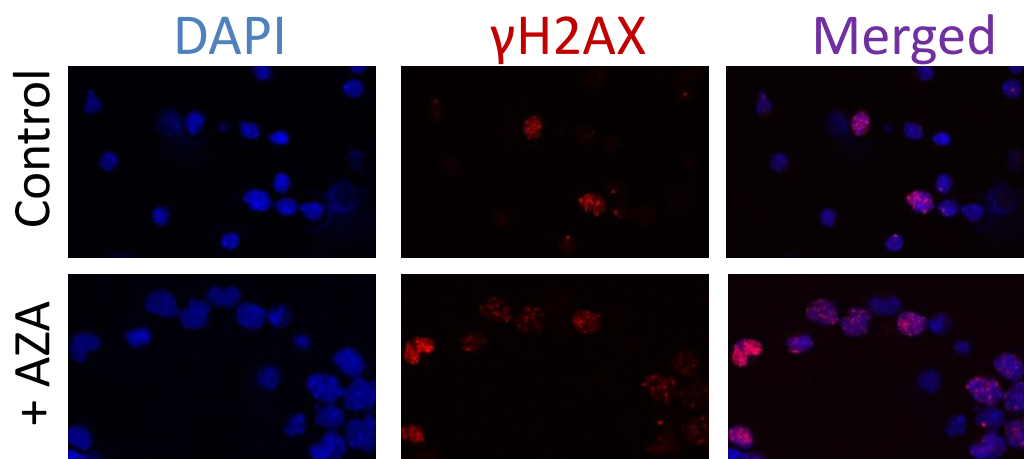


Figure 24: Immunofluorescence staining against γ H2AX after 5-aza-CdR treatment. γ H2AX levels are increased in B57 cells after 5-aza-CdR treatment (bottom) when compared to untreated control cells (top). DAPI indicates nuclear staining. Cells were analyzed 52 hours after 5-aza-CdR treatment.

Together, these data suggest that the cell line used for these experiments is very sensitive to the DNMT inhibitor 5-aza-CdR. The cells' survival rates showed a strong reduction starting two days after the treatment, after usage of low 5-aza-CdR concentration. This reduction in cell survival was accompanied by global loss of 5-methylcytosine levels. DNMT1 is not degraded by 5-aza-CdR but covalently bound to the DNA, explaining the observed signals of DNMT1 by immunofluorescence staining. IAP retrotransposon expression levels were increased with higher 5-aza-CdR concentrations leading to higher expression levels. Elevated DNA damage measured by γ H2AX levels was observable when compared to untreated control cells. While genetic *Dnmt1* deletion resulted in cell death starting five days after tamoxifen treatment (figure 15), chemical DNMT1 inhibition led to induction of cell death after two days. The reason for the delayed cell death upon tamoxifen treatment probably lies in the fact that these cells still had DNMT1 as proteins available.

3.3 Differences between genetic *Dnmt1* deletion and chemical inhibition

After characterization of the genetic deletion of *Dnmt1* and chemical inhibition by 5-aza-CdR, the next question was if there are any differences between them. To address this issue, Western blot analyses were performed in order to gain insight into effects of both treatments onto ALK signaling. For this, antibodies against ALK and its active phosphorylated form phospho-ALK were used. Furthermore, one of the most important downstream targets of ALK signaling, STAT3 and its active form phospho-STAT3, were measured. In addition an antibody against β -actin was used as loading control. As depicted in figure 25, DNMT1 is clearly reduced in the cells treated with tamoxifen (+) compared to control cells (-). For the 5-aza-CdR treatment (right side) the signal for DNMT1 is weak in general and seems to be only slightly reduced after treatment. However, having a look at the β -actin signals a strong reduction of DNMT1 is present after inhibition with 5-aza-CdR. The ALK protein level is similar for tamoxifen treatment and a slight reduction after 5-aza-CdR treatment could be observed. Phospho-ALK (pALK) levels are similar in the tamoxifen treatment, but a clear reduction is detected in cells treated with 5-aza-CdR. The same finding can be observed for STAT3 and phospho-STAT3 (pSTAT3): whereas STAT3 levels are similar for both treatments, pSTAT3 is reduced in cells treated with 5-aza-CdR, but no difference is observed in tamoxifen treated cells when compared to controls. However, it has to be taken into account that β -actin levels are different for the western blot with 5-aza-CdR treatment, as control cells show a weaker signal.

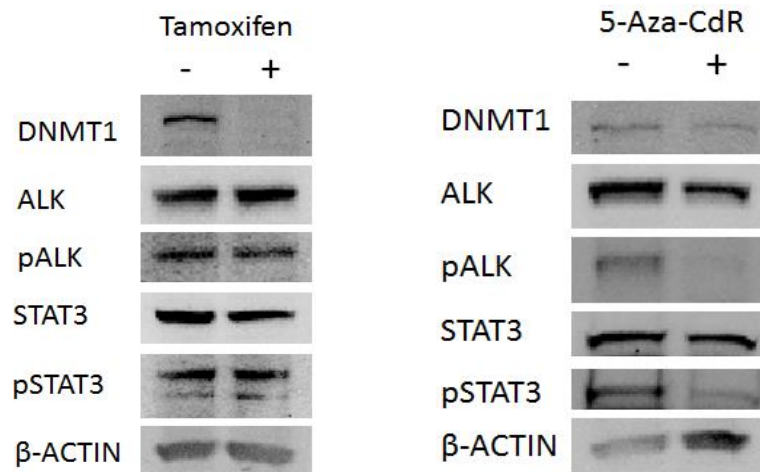


Figure 25: Western blot analysis after genetic *Dnmt1* deletion (left side) and chemical inhibition (right side). DNMT1 is strongly reduced after tamoxifen treatment, but ALK and STAT3 show no differences for both phosphorylated and normal forms. Chemical inhibition of DNMT1 shows slight reduction of DNMT1, ALK and STAT3 and a stronger reduction for phosphorylated forms of ALK and STAT3. β-Actin served as loading control for both blots.

These data suggest that 5-aza-CdR and tamoxifen treatments have different effects on ALK signaling. Chemical inhibition using the DNMT inhibitor seems to negatively regulate the activation of ALK phosphorylation and therefore STAT3 activation, while the genetic *Dnmt1* deletion doesn't show effects on ALK signaling.

For more detailed information of how inhibition of DNMT1 influences the cellular expression patterns, genome-wide RNA sequencing (RNA-Seq) was performed using massive parallel sequencing on the Illumina platform. We identified 81 deregulated genes between control and *Dnmt1*^{-/-} cells and 61 between control and 5-aza-CdR treated cells (tables 4 and 5). Both phenotypes had eight deregulated genes in common (table 6). Table 5 displays the eight genes, which are deregulated in both treatment groups. Interestingly all of them are upregulated after tamoxifen treatment and downregulated after 5-aza-CdR treatment. This list includes among others the cytotoxic granule granzyme A. Most of the deregulated genes after tamoxifen treatment are upregulated (table 4) while 5-aza-CdR treatment leads especially to downregulation of genes (table 5).

4HT treatment		4HT treatment	
Gene name	log2 fold change	Gene name	log2 fold change
Grb10	6,914	Trat1	1,910
Gm11382	6,175	Tuba8	1,855
Gm9864	5,969	Etv5	1,827
Gm10040	5,814	Folr4	1,774
Inadl	5,686	Adh1	1,726
Wdr31	5,653	Prkcq	1,682
Ttll9	5,535	Tmsb4x	1,663
Lgals1	4,970	Cdh19	1,650
Pdcd1	4,769	Trp53inp1	1,622
Slc38a6	4,571	Cpt1a	1,581
Ccrn4l	4,434	Gem	1,477
Gm11669	4,203	H2-K1	1,477
Strc	4,113	S100a16	1,440
Ggt1	4,017	Cryga,D630023F18Rik	1,405
Rbpms	4,003	Sectm1a	1,227
Sema4a,mmu-mir-7011	3,756	4930550L24Rik	1,069
Camk2b	3,476	Tnfsf4	0,877
Xlr	3,387	Gtsf1	0,714
Ccr9,Gm17200	3,323	Bcas1	0,675
Armxc2	2,956	3830403N18Rik	0,653
Olfr1357	2,944	Dppa2	0,603
Gal	2,890	Cald1	0,502
Cd53	2,874	Xlr3a	0,423
Trim9	2,844	Gm364	0,423
Mid1	2,837	Serpinh1	0,331
Sox13	2,673	1700024B05Rik	0,309
Igfbp4	2,650	5730559C18Rik	0,262
Sgip1	2,553	Fkbp6	0,136
Gzma	2,523	M1ap	-0,044
Gm16352,Sorbs2	2,500	Pira2	-0,113
Plac8	2,317	Rnf183	-0,126
Ahnak	2,263	Pnliprp2	-0,349
Npffr1	2,135	Pet2	-0,617
Tox2	2,095	Hc	-0,635
Il6ra	2,068	Enthd1	-1,163
Itgb3	2,015	Slc9b1	-1,195
Serpinb1c	2,009	Ces2e	-1,261
Gstp2	1,977	Rpl27a-ps1	-2,311
Pycr2	1,959	Scd1	-2,665
Itk	1,919	Dnmt1	-2,793

Table 4: Genome-wide RNA sequencing resulted in a list of 81 deregulated genes after tamoxifen treatment. 69 genes were upregulated (red) and twelve downregulated (green).

5-aza-CdR treatment	
Gene name	log2 fold change
Fabp5	2,469
Srm	1,659
Gsr	1,655
Agfg1,Gm22396	1,607
Mthfd1	1,425
Ffar2	0,725
Crybb3	0,336
Lcn2	0,029
Il6	-0,534
2210408F21Rik	-0,884
Arg1	-1,354
Tmsb4x	-1,376
Hid1	-1,456
Nabp1	-1,602
Gm12505	-1,652
Gm12840	-1,700
Ero1l	-1,718
Dusp6	-1,777
Lipg	-1,798
Mxd4	-1,813
Insl3,Jak3	-1,825
Dzip1	-1,869
B230206L02Rik	-1,892
Sesn1	-1,901
Gm20400	-1,908
Egr1	-1,943
Gem	-2,068
Atp1b1	-2,141
Ckb	-2,159
Itgb3	-2,198

5-aza-CdR treatment	
Gene name	log2 fold change
Gbp4,Gbp8,Gbp9	-2,300
Gbp2	-2,401
Col6a1	-2,429
5430421N21Rik	-2,441
Folr4	-2,472
Trib3	-2,473
Sp100,n-R5s215	-2,537
Akap12	-2,545
Gimap1,Gimap5,Gimap7,RP24-315D19.2	-2,549
Itgb7	-2,587
Tmem176b	-2,603
Osgin1	-2,726
Fam129a	-2,748
Gzma	-2,773
Txnip	-2,797
Cox6a2	-2,836
Rrm2b	-2,844
Gimap6	-2,872
Cd53	-2,933
Ccr9,Gm17200	-2,950
Tmem176a	-3,015
Rora	-3,114
Myl10	-3,230
Ccnd2	-3,556
Mmp12	-3,760
Epas1	-3,808
Gimap8	-3,843
Igfbp4	-3,892
Fam102a	-4,036
Cd69	-4,308
Tenm4	-6,218

Table 5: Genome-wide RNA sequencing resulted in a list of 61 deregulated genes after 5-aza-CdR treatment. Eight genes were upregulated (red) and 53 downregulated (green).

	4HT treatment	5-aza-CdR treatment
Common deregulated genes	log2 fold change	
Igfbp4	2,650	-3,892
Gem	1,477	-2,068
Tmsb4x	1,663	-1,376
Folr4	1,774	-2,472
Itgb3	2,015	-2,198
Gzma	2,523	-2,773
Cd53	2,874	-2,933
Ccr9,Gm17200	3,323	-2,950

Table 6: Genome-wide RNA-sequencing resulted in a list of eight genes, which are deregulated in both treatment groups. While all of them are upregulated (red) after tamoxifen treatment, 5-aza-CdR led to downregulation of those genes (green).

Deregulated genes after both treatments consisted of genes playing roles in different processes: pseudogenes, genes involved in different metabolic processes, transmembrane genes and genes involved in signaling processes. The eight genes, which are deregulated upon both treatments (table 6) are playing roles in IGF regulation (Igfbp4), regulation of signal transduction (Gem, CD53), cytoskeleton organization (Tmsb4x), folate metabolism (Folr4), cell surface mediated signaling (Itgb3), cytotoxic T-lymphocyte mediated cell lysis (Gzma) and chemokine signaling (Ccr9). These differentially regulated genes might cause the observed differences in phospho-STAT3 and phospho-ALK levels between genetic deletion of *Dnmt1* and chemical inhibition with 5-aza-CdR.

A core analysis of the data was conducted by using ingenuity pathway analysis (IPA) (<http://www.ingenuity.com>; QIAGEN). The purpose of IPA analysis is to identify common pathways and functions or upstream transcriptional regulators that might be responsible for, or could explain the observed gene expression changes. Among the top biological functions for the genes differentially expressed after genetic *Dnmt1* deletion were antigen presentation, hematopoiesis, lymphoid tissue structure and cancer (figure 26). For the genes differentially expressed after 5-aza-CdR treatment the top bio functions were cellular growth and proliferation, cancer, inflammatory response and lymphoid tissue structure (figure 27).

Analysis: 4HT_vsC - 2015-11-02 11:37 AM

■ 4HT_vsC - 2015-11-02 11:37 AM

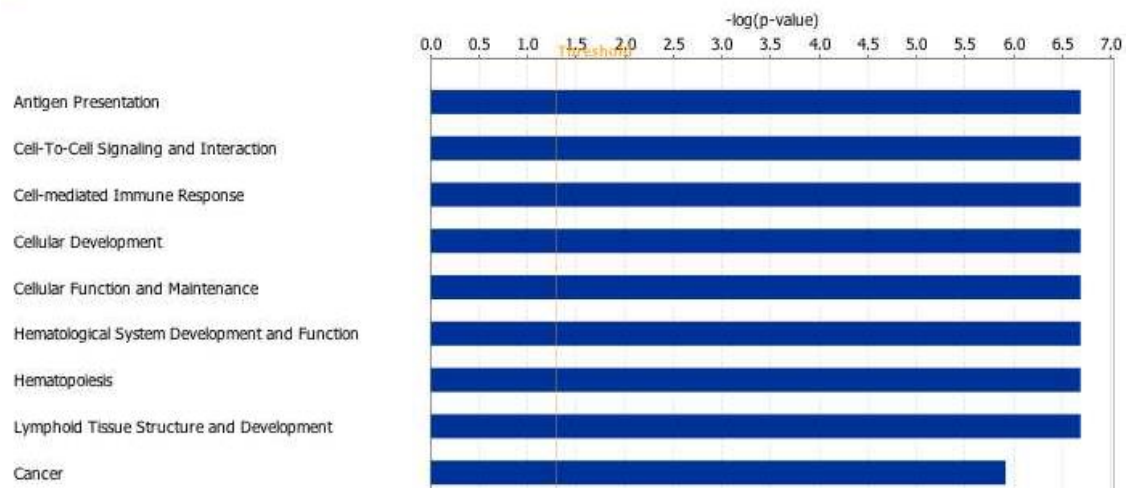


Figure 26: Top biological functions of differentially expressed genes after genetic *Dnmt1* deletion in B57 cells, including antigen presentation, hematopoiesis and cancer.

Analysis: AZA_vsC - 2015-11-02 11:42 AM

■ AZA_vsC - 2015-11-02 11:42 AM

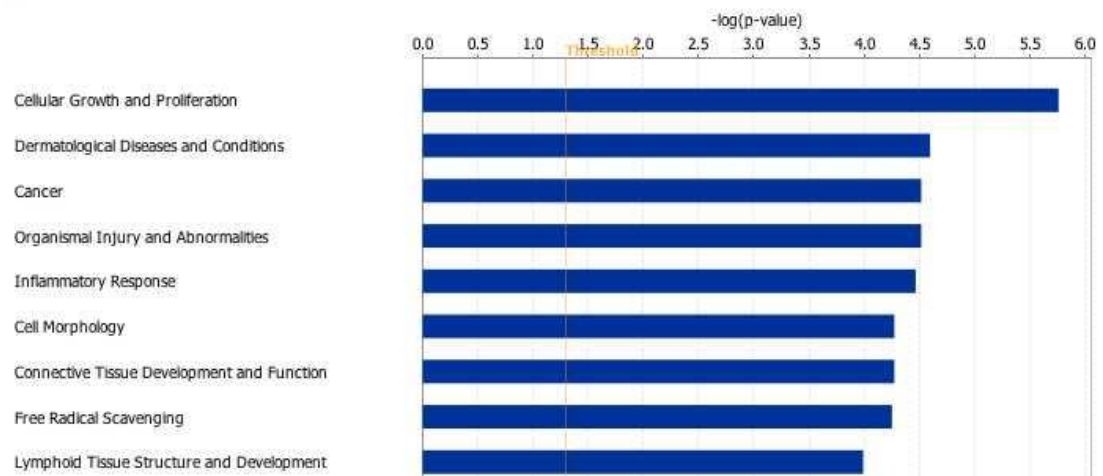


Figure 27: Top biological functions of differentially expressed genes after chemical inhibition of DNMT1 in B57 cells, including cellular growth and proliferation, cancer and inflammatory response.

Furthermore, the IPA core analysis predicted biological functions represented as networks of the differentially expressed genes and their relationships to these functions. Figure 28 shows the prediction legend, used for figures 31, 32 and 33. While differences in gene expression after loss of *Dnmt1* predicted activation of T-lymphocyte apoptosis based on upregulated genes, 5-aza-CdR treatment results mostly in down-regulation of genes leading to apoptosis (Figure 29).

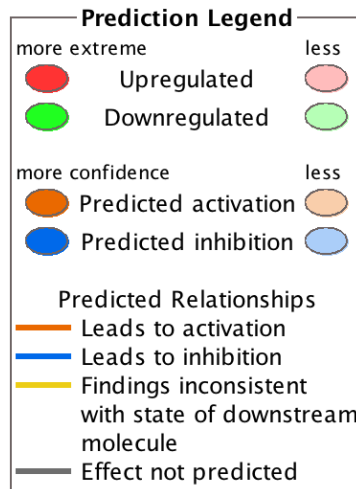


Figure 28: Prediction Legend for IPA core analysis networks of biological function predictions.

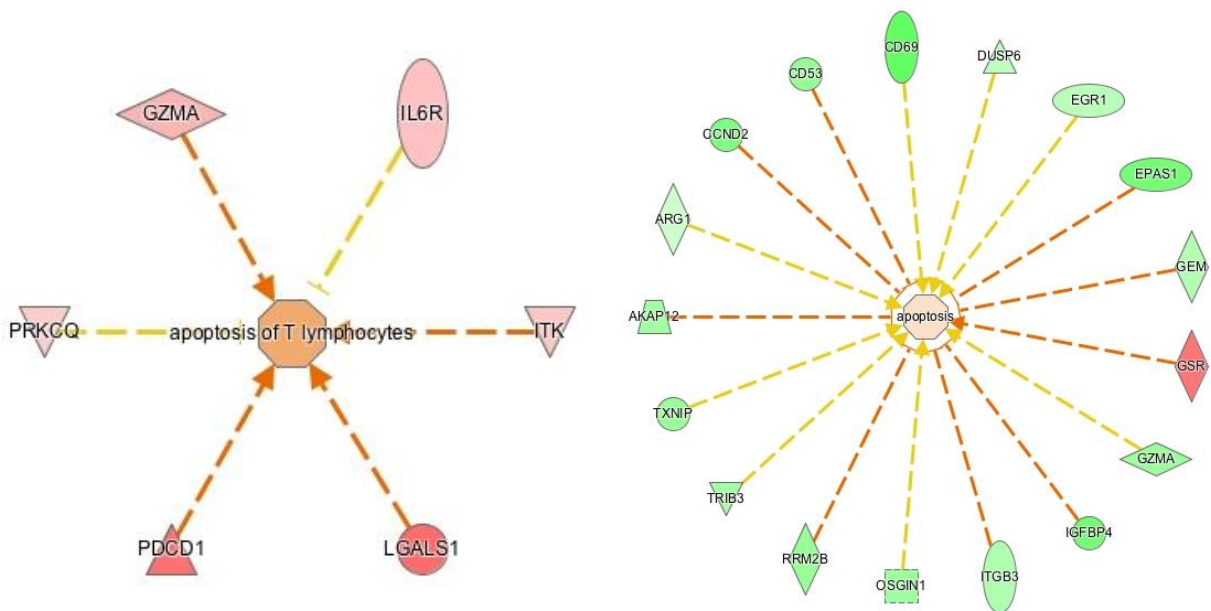


Figure 29: IPA core analysis of RNA-sequencing data after tamoxifen (left) and 5-aza-CdR (right) treatment of B57 cells. Differentially expressed genes in both treatments lead to predicted activation of apoptosis.

According to the prediction legend (figure 28), the genes displayed in figure 29 are either upregulated (red) or downregulated (green) after treatment. Upregulation of some genes including *GZMA* (Granzyme A), *PRKCQ* (Protein kinase C theta), and *PDCD1* (Programmed cell death 1) after tamoxifen treatment, predict activated apoptosis of T-lymphocytes indicated by the red color. In cells treated with 5-aza-CdR, however, the predicted activation of apoptosis is caused especially due to downregulated genes, including *ITGB3* (Integrin Beta 3), *CD53* (CD53 antigen) and *IGFBP4* (Insulin-Like Growth Factor Binding Protein 4).

In addition to these findings, the core analysis predicted activation of T-lymphocytes after genetic *Dnmt1* loss and inflammation of organ after chemical DNMT1 inhibition (figure 30). Based on the same color code, the networks predicted activation of T-lymphocytes after tamoxifen treatment, by upregulation of *GZMA*, *PRKCQ* and *ITK* (IL-2 inducible T-cell kinase), among other genes. The activation of inflammation after 5-aza-CdR was predicted to be caused by mostly downregulated genes including *MMP12* (Matrix Metallopeptidase 12), *ITGB3*, *EGR1* (Early Growth Response 1), and others.

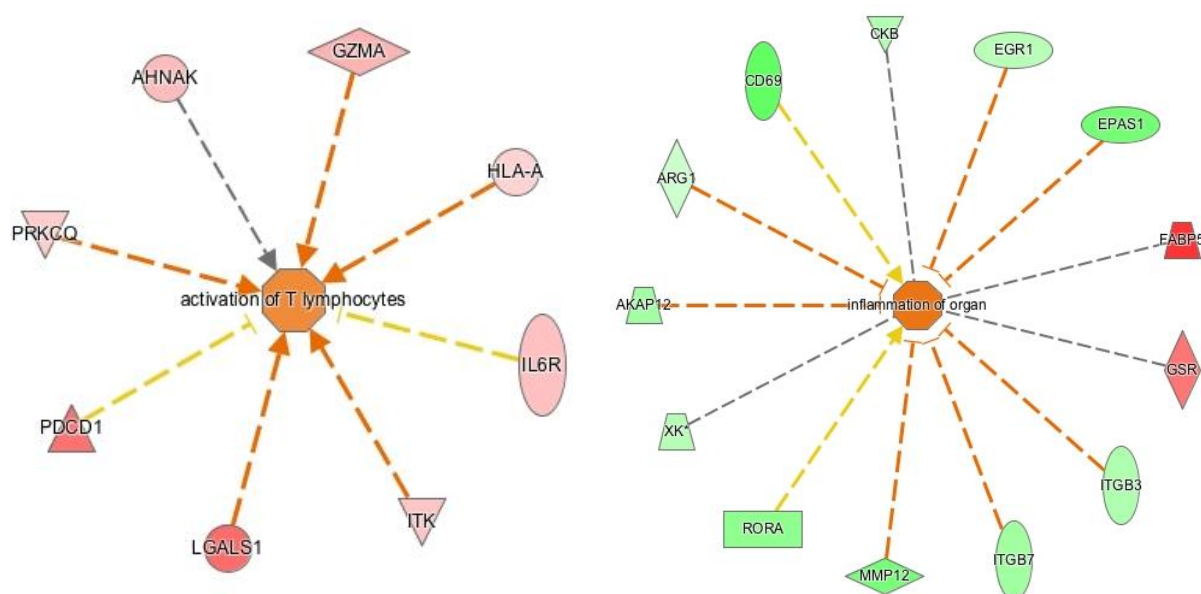


Figure 30: IPA core analysis of RNA-sequencing data after tamoxifen (left) and 5-aza-CdR (right) treatment of B57 cells resulted in predicted activation of T-lymphocytes and activation of inflammation.

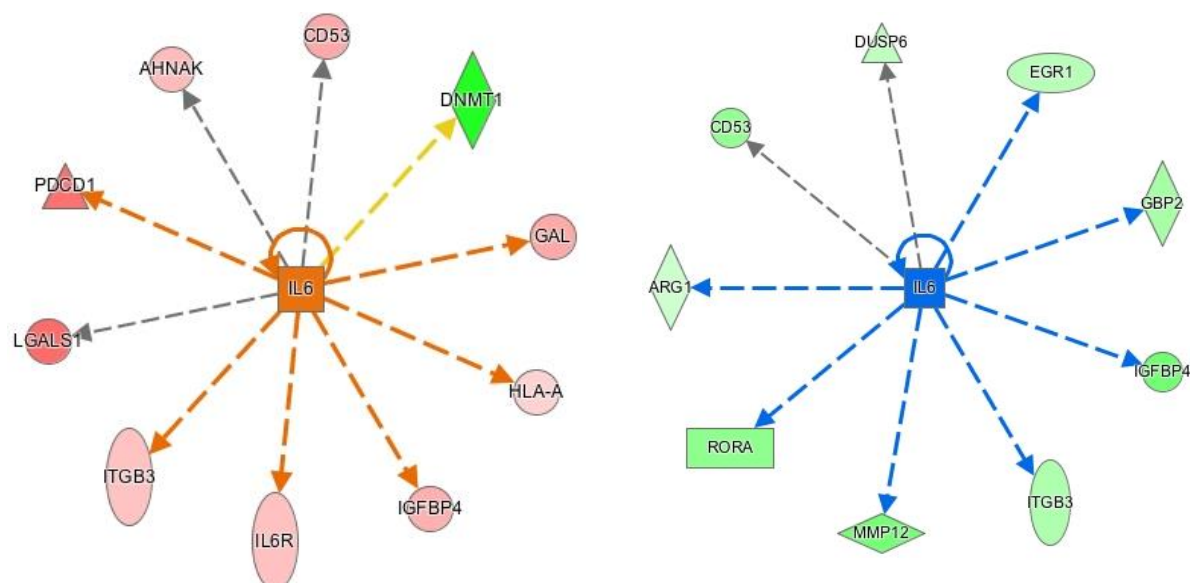


Figure 31: Prediction of Interleukin 6 being an activated upstream regulator for cells with genetic *Dnmt1* deletion (left), but inhibited upon chemical DNMT1 inhibition (right).

Further core analysis results predicted the cytokine Interleukin 6 (IL6) to be activated in cells with genetic loss of *Dnmt1* but inhibited in cells when DNMT1 is inhibited with 5-aza-CdR (figure 31). This might serve as a possible explanation for the differences observed in STAT3 activation, since IL-6 is known to activate STAT3 via downstream signaling pathways. [49] Similar results were found for Lipopolysaccharide (LPS) as depicted in figure 32, showing predicted activation of this upstream regulator. This means that according to the upregulation of the genes shown in figure 32, signaling pathways activated by LPS are predicted to be activated. LPS signaling includes the Toll like receptor 4 (TLR4) signal transduction pathway that is responsible for the release of proinflammatory cytokines and also known to induce STAT3 activation via IL-10 activation in macrophages. [67, 68] This could be an additional explanation for the observed differences in STAT3 activation.

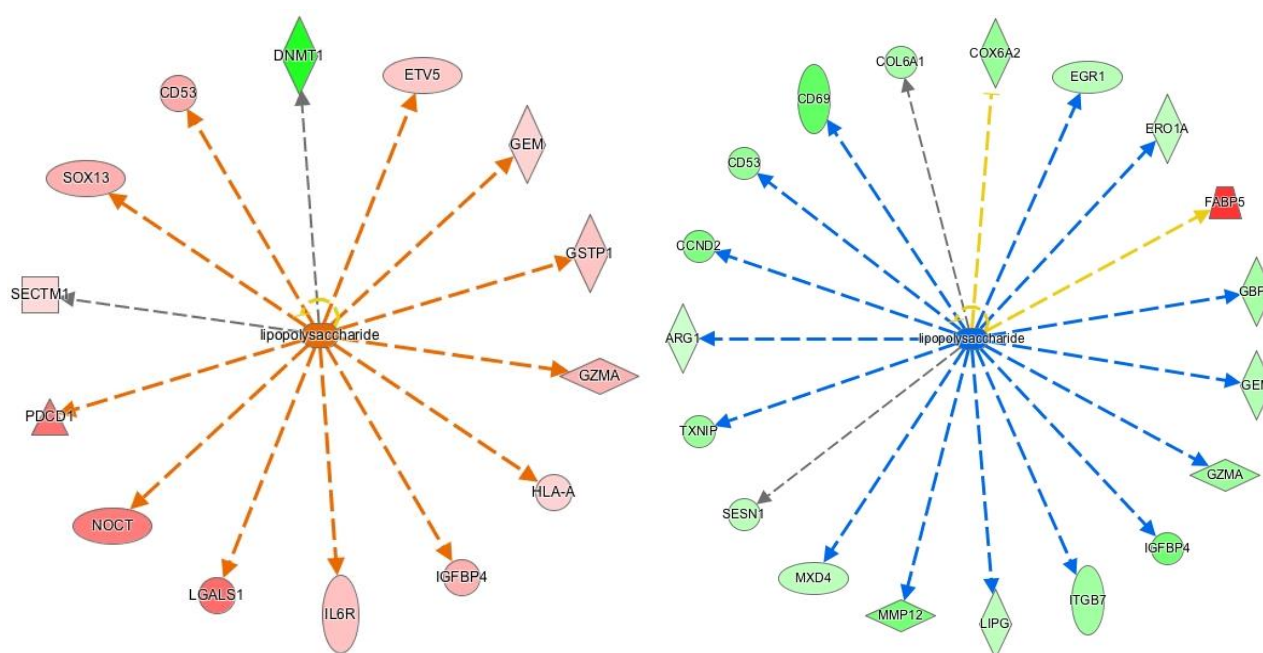


Figure 32: Prediction of Lipopolysaccharide being an activated upstream regulator for cells with genetic *Dnmt1* deletion (left), but inhibited upon chemical DNMT1 inhibition (right).

4. Discussion

4.1 Genetic deletion of *Dnmt1* in murine NPM-ALK+ T-cell lymphoma cells

Preliminary results of our lab have shown that thymus specific deletion of *Dnmt1* in a transgenic NPM-ALK+ mouse model results in prolonged survival and inhibition of lymphomagenesis.

The group of En Li found that colon cancer cells undergo a so called mitotic catastrophe upon loss of *Dnmt1*. [48] They showed that these cells have elevated DNA damage levels and arrest at the G2-stage, ultimately resulting in the programmed cell death of these cancer cells. [48] The results obtained in this study correlate with the findings of En Li's group, since increased DNA damage was observed by γ H2AX and the amount of apoptotic cells was increased, while S-phase cells decreased upon loss of *Dnmt1*. Usually elevated DNA damage levels induce a cell-cycle arrest at the G2 checkpoint. [72] This was also seen as a slight increase in G2-cells four days after tamoxifen treatment, further promoting the view that genetic *Dnmt1* deletion results in a mitotic catastrophe like cell death.

The observed stronger signal in phospho-H3S10 levels after genetic knockout of *Dnmt1* might have a causal connection to cellular stress or mitotic catastrophe. Li's group reported that cells with disrupted *Dnmt1* undergo an abnormal mitotic entry after G2-arrest. [48] Since H3S10 phosphorylation is a marker of chromatin during mitosis. [73] The increased phospho-H3S10 signal of *Dnmt1* deleted cells (figure 17) could indicate cells undergoing an abnormal mitotic entry, further promoting the suggestion of NPM-ALK positive cells with disruption of *Dnmt1* undergoing mitotic catastrophe.

4.2 Chemical inhibition of DNMT1 in murine NPM-ALK+ T-cell lymphoma cells

5-Aza-2'-deoxycytidine or decitabine is a cytidine analog and thus a DNA methyltransferase inhibitor. [62] Besides genetically deleting *Dnmt1*, 5-aza-CdR was used to inhibit DNMT1 in the cell line B57 in this study. For most cancer cell lines, usually a concentration of about 1 μ M 5-aza-CdR is used in order to block DNA methylation. However, 1 μ M of decitabine resulted in cell death only 24 hours after the treatment. Since a concentration of 0,04 μ M 5-aza-CdR already resulted in inhibition of

DNMT1, the cell line of this study appeared to be very sensitive to DNMT inhibitors. 5-Aza-CdR is incorporated into the DNA as cytidine analog and DNMT1 is covalently bound to the nucleotide, therefore inhibiting the enzyme. [62] Our data confirm that DNMT1 is only covalently bound but not degraded by 5-aza-CdR, which makes it detectable after drug treatment in the immunofluorescence staining but not in Western blot analysis, where DNMT1 is depleted from the protein extract with DNA. As it was done for cells with genetically deleted *Dnmt1*, the expression of IAP retrotransposon was measured. Caused by the inhibition of DNMT1 and therefore passive DNA demethylation, IAP expression levels were elevated.

Recent data suggest that treating the human ALCL cancer cell lines KARPAS-299, SR-786 and MAC-2A with 5-aza-CdR results in a distinct reduction of cell population doublings and therefore apoptosis. [61] These findings correlate with the observed reduction of viability of murine NPM-ALK+ T-cell lymphoma cells upon decitabine treatment.

4.3 Comparison of genetic *Dnmt1* deletion and pharmacological inhibition

In order to compare the two methods for DNMT1 depletion, western blot analysis and RNA sequencing were performed.

As shown by Western blot analysis (figure 25) DNMT1 is strongly reduced after tamoxifen treatment in B57 cells. Taking into account the uneven β -actin loading of the 5-aza-CdR Western, one could see a reduction of DNMT1 in this blot as well. The phosphorylated and therefore activated forms of ALK and its downstream target STAT3 are clearly reduced in 5-aza-CdR treated cells. This is, at least for STAT3, consistent with findings of Zhang et al. who observed STAT3 dephosphorylation in T-cell malignancies upon DNMT1 depletion. [33] The reason why this could not be observed in the genetic *Dnmt1* deletion is unclear.

Ingenuity pathway analysis (IPA) is a tool for identification of upstream transcriptional regulators that might be responsible for or could explain the found gene expression changes. An IPA core analysis with the data obtained by genome-wide RNA-sequencing of both treatments resulted in 61 differentially expressed genes after 5-aza-CdR treatment and 81 genes differentially expressed upon *Dnmt1* knockout. Eight of those expression changes were observed in both groups. In addition to these

different changes in gene expression, signaling activated by the upstream regulators IL-6 (Interleukin 6; figure 31) and LPS (Lipopolysaccharide; figure 32) was predicted to be activated in tamoxifen treated cells, but inhibited after 5-aza-CdR treatment. Since STAT3 becomes activated via the IL-6 signaling pathway [49], the results of the IPA analysis correlate with the findings of the Western blot analysis (figure 25), where STAT3 phosphorylation is lost after inhibition of DNMT1 with 5-aza-CdR. However, this does not explain the lost phosphorylation of STAT3s upstream regulator ALK. Furthermore LPS is known to activate the TLR4 signaling pathway. [67] This pathway leads to release of proinflammatory cytokines and was shown to activate STAT3 together with TLR9 via downstream signaling. [69] The findings of the IPA analysis that predict LPS mediated signaling to be inhibited in 5-aza-CdR treated cells, could be an additional explanation for the loss of STAT3 activation observed by Western blot analysis. The release of proinflammatory cytokines by the TLR4 pathway might also explain the predicted activation of T-lymphocytes after genetic *Dnmt1* deletion. Since most of the genes after 5-aza-CdR are downregulated predicting an inhibited LPS induced signaling, the activated inflammation after chemical DNMT1 inhibition might be caused by another signaling pathway.

Besides IL-6, the anchor protein AKAP12 (A-kinase Anchoring Protein 12) was shown to be responsible for STAT3 phosphorylation and activation and, additionally, to have a regulatory feedback loop with STAT3. [77] Genome wide RNA-sequencing showed that AKAP12 was among the downregulated genes after 5-aza-CdR treatment. This might be another explanation of the reduced phospho-STAT3 levels. Furthermore downregulation of IL-6 and AKAP12 was also shown after 5-aza-CdR treatment of the human colon cancer cell line HCT116 by Feinberg et al. [74] The protein kinases PRKCQ (Protein Kinase C Theta) and ITK (IL-2 induced T-cell Kinase) are suggested to contribute to phosphorylation and activation of STAT3, which would explain why there is no reduction of phospho-STAT3 in cells with genetic *Dnmt1* deletion. [75, 76] Another possibility for differences in STAT3 activation might be caused by IGFBP4 (Insulin like growth factor binding protein 4), which regulates IGF (Insulin like growth factor) both negatively and positively. [78] IGF was suggested to activate STAT3, giving yet another possible explanation for different phospho-STAT3 levels in the two treatment groups. [79] The deregulated genes after 5-aza-CdR detected by genome wide RNA-sequencing, were mostly downregulated while tamoxifen treatment led to mostly upregulated genes. This outcome is unexpected and not consistent with

findings of Feinberg et al. who observed the same amount of up – and downregulated genes after 5-aza-CdR treatment of the human colon cancer cell line HCT116. Moreover, a *DNMT1* knockout of HCT116 cells showed about the same amount of up and downregulations, when compared to normal HCT116 cell. [74] Furthermore, only eight of the deregulated genes were common for both treatments with all of them upregulated in tamoxifen treated cells, but downregulated after 5-aza-CdR treatment. Since the genome-wide RNA-sequencing was not performed with technical replicates, the obtained results may not reflect the actual situation of deregulated genes after chemical and genetic DNMT1 depletion.

A further explanation for the differences between the two approaches might be that although DNMT1 loses its function after 5-aza-CdR inhibition, it is still present in the cells. This means that interactions with other proteins or enzymes remain possible, while genetic deletion of *Dnmt1* abolishes this possibility. For instance DNMT1 is known to form a complex with Rb (Retinoblastoma), E2F1 (E2F transcription factor 1) and the histone deacetylase 1 (HDAC1) that represses transcription of E2F-responsive promoters. [53] A study from 2005, however, showed that DNMT1 is degraded rapidly upon 5-aza-CdR treatment [54]. This finding might nullify the possibility of DNMT1 to form and act via complexes, although immunofluorescence stainings (figure 22) did not show a reduction of DNMT1 levels at all. Moreover, recent studies have shown that low doses of DNA demethylating agents like 5-aza-CdR target cancer initiating cells in colon cancer by viral mimicry. This is associated with the induction of dsRNAs derived from endogenous retroviral elements and the activation of MDA5/MAVS signaling (Melanoma Differentiation Associated Protein-5; Mitochondrial Antiviral Signaling Protein). [70] Another study showed that DNMT inhibitors upregulate immune signaling in cancer cells by activation of the viral defense pathway. [71] Both findings might have occurred in the NPM-ALK positive cells treated with 5-aza-CdR and effected ALK and STAT3 signaling, serving as an additional explanation for the different outcomes of genetic and chemical DNMT1 depletion. Among the top biological functions predicted by the IPA core analysis for 5-aza-CdR treatment is inflammatory response. This event may be caused by upregulated immune signaling through viral defense pathways.

The results for genetic disruption of *Dnmt1* show similarities to cell death by mitotic catastrophe, as reported by En Li. [48] Since other mechanisms are known for cellular

death in cancer cells [72], the cause for the reduction of survival rates for 5-aza-CdR treated tumor cells might be a different than mitotic catastrophe.

Besides differences, there are also similarities between genetic and chemical DNMT1 depletion. IPA analysis predicted the activation of apoptosis in both groups (figure 29), which is consistent with previous findings. Also, activation of T-lymphocytes (for tamoxifen treated cells) and activation of inflammation (for 5-aza-CdR treatment) were predicted to be activated (figure 30). Among the top biological functions, as displayed in figures 26 and 27, were “cancer”, “antigen presentation”, “cellular growth and proliferation” and other cancer- and T-cell related functions. However, no epigenetically associated biological function or gene was identified by IPA analysis, leaving targets of ALK mediated aberrant DNA methylation a subject of further research.

4.4 Conclusion and outlook

The aim of this project was to analyze the role of DNMT1 for the survival of murine NPM-ALK⁺ T-cell lymphoma cells. For this purpose a genetic and a chemical approach were used in order to deplete DNMT1.

Both approaches showed that DNMT1 has a high importance for the survival of NPM-ALK⁺ T-cell lymphoma cells. The genetic deletion of *Dnmt1* led to cellular death, which resembled mitotic catastrophe, as reported by Li et al. [48] RNA-sequencing and Western blot analysis showed that there are differences between the two approaches, with different phospho-STAT3 levels after the treatments, being the most important one. Genome-wide RNA-sequencing and performed IPA analyses didn't reveal differences of epigenetic processes. Taken together, the underlying cause of the different observations concerning phospho-ALK and phospho-STAT3 levels and especially the differences in deregulated genes, after tamoxifen and 5-aza-CdR treatment still remains unclear and warrants further investigation.

The results of this study showed the importance of DNMT1 for the survival of ALCL cells. In order to identify targets of aberrant DNA methylation mediated by ALK, which might be caused by upregulation of *DNMT1* via STAT3 [33], further experiments ought to be performed.

This could include experiments *in vitro* as well as *in vivo* with the transgenic NPM-ALK⁺ mouse model with T cell specific *Dnmt1* deletion and tumors isolated and cultivated from these mice. Reduced representation bisulfite sequencing of tumors

taken into culture can provide more insight into aberrant DNA methylation patterns caused by ALK and STAT3 mediated upregulation of *Dnmt1*. Furthermore, ChIP-sequencing can identify targets of STAT3 transcription factor binding to specific DNA sites. All these experiments should be performed for both approaches, genetically deleted *Dnmt1* and pharmacological inhibition with 5-aza-CdR *in vivo* and *in vitro*.

The aim of this project was to analyze the effect of DNMT1 depletion on the survival of NPM-ALK positive lymphoma cells. Aberrant ALK expression caused by translocations, amplifications and mutations is not only observed in ALCL but also in non-hematological cancers like neuroblastoma and epithelial cancers of the breast, colon and lung. [63] This means that aberrant ALK signaling might be an important mechanism for malignant transformation of cells not only in ALCL but as a general oncogenic mechanism. Further studies with different types of tumors should be performed in order to gain more insight into the connection between ALK signaling and control of epigenetic modifications by DNA methylation.

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6. Appendix

6.1 Acknowledgements

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I express my gratitude to my girlfriend Hanna who was always there, in good and bad times not only during my master thesis but my whole master's program. A special thank also to my family who always supported and encouraged me during my studies.

6.2 Abstract

With about 14 million new cases and approximately 8.2 million cancer related deaths in 2012, cancer is among the leading causes of death. One of the most common hematologic cancer types are Non-Hodgkin-Lymphomas which present 90% of lymphoma-type cancers. NPM-ALK-positive Anaplastic Large Cell Lymphoma is a type of Non-Hodgkin's-Lymphoma that is characterized by the presence of the NPM-ALK fusion protein. In these lymphomas, the receptor tyrosine kinase anaplastic lymphoma kinase (ALK) is fused to the nuclear transport protein Nucleophosmin1 (NPM1), which constitutively activates the kinase. The kinase then leads to malignant transformation by activation of multiple downstream signaling pathways. One of its most important downstream targets is the transcription factor STAT3. STAT3 has been described to upregulate the transcription of the DNA methyltransferase 1 gene (*Dnmt1*) in ALK+ ALCL, and to recruit DNMTs to promoters of tumor suppressor genes, therefore silencing them. We have shown that thymic-specific deletion of *Dnmt1* in a transgenic NPM-ALK mouse model leads to inhibition of lymphomagenesis, and thereby sustains survival of the mice. Furthermore, the inhibition of DNMT1 *in vitro* in human ALCL cell lines was shown to result in the loss of STAT3 phosphorylation and activity.

The aim of this study was to investigate how depletion of DNMT1 affects NPM-ALK tumor cells. To address this, we analyzed tumor cells from NPM-ALK mice by inhibiting DNMT1 chemically with 5-aza-2-deoxycytidine. In addition, an inducible Cre recombinase was used to delete *Dnmt1* on the genetic level. Depletion of DNMT1 resulted in the reduction of cellular viability and increased cell death. Furthermore, enriched DNA-damage as well as loss of overall methylation levels was observed. Western Blot analysis showed reduced levels of the phosphorylated, activated forms of ALK and its downstream target STAT3 for chemical DNMT1 inhibition, whereas this reduction was not observed when *Dnmt1* was genetically deleted. RNA-sequencing resulted in divergent findings for chemical inhibition of DNMT1 and genetic *Dnmt1* deletion but confirmed the importance of the DNA methyltransferase for cellular survival for both approaches.

6.3 Zusammenfassung

Mit etwa 14 Millionen neuen Fällen und ca. 8.2 Millionen Krebs-assoziierten Todesfällen im Jahr 2012, gehört Krebs zu den häufigsten Todesursachen weltweit. Eine der häufigsten hämatologischen Krebsarten sind Non-Hodgkin-Lymphome, die etwa 90% aller Lymphome darstellen. Das NPM-ALK-positive anaplastische großzellige Lymphom ist ein Non-Hodgkins-Lymphom, charakterisiert durch das NPM-ALK Fusionsprotein. Die Rezeptor Tyrosinkinase Anaplastic Lymphoma Kinase (ALK) bildet in diesen Lymphomen ein Fusionsprotein mit dem nuklearen Transportprotein Nucleophosmin1 (NPM1), was die Kinase konstitutiv aktiviert. Daraufhin führt die Kinase zur malignen Transformation durch die Aktivierung mehrerer Signalwege. Der Transkriptionsfaktor STAT3 ist eines der wichtigsten Zielgene von ALK. STAT3 induziert die Transkription der DNA Methyltransferase 1 (DNMT1) in ALK+ ALCL, rekrutiert DNMTs zu Promotoren von Tumorsuppressor-genen und inaktiviert diese dadurch. Wir haben gezeigt, dass spezifische Deletion von *Dnmt1* im Thymus von transgenen NPM-ALK Mausmodellen, zur Hemmung der Lymphomagenese und somit zum Überleben der Mäuse führt. Inhibiert man DNMT1 außerdem *in vitro* in humanen ALCL Zelllinien, so verliert STAT3 an Aktivität und Phosphorylierung.

Das Ziel dieser Studie war es zu analysieren, wie sich der Verlust von DNMT1 auf NPM-ALK Tumorzellen auswirkt. Dafür wurden Tumorzellen von NPM-ALK Mäusen analysiert, in denen DNMT1 chemisch auf Proteinebene mit 5'-aza-2-deoxycytidine inhibiert wurde. Außerdem wurde mit Hilfe einer induzierbaren Cre Rekombinase *Dnmt1* auf genetischer Ebene deletiert. Der Verlust von DNMT1 resultierte in einer Abnahme der zellulären Viabilität und somit in erhöhter Zelltod-Rate. Zudem konnte sowohl ein Anstieg an DNA-Schäden als auch ein globaler Verlust der DNA-Methylierung beobachtet werden. Western Blot Analysen zeigten reduzierten Gehalt der phosphorylierten, aktiven Formen von ALK und dessen downstream target STAT3 nach chemischer DNMT1 Hemmung. Dies konnte für die genetische Deletion von *Dnmt1* nicht beobachtet werden. Genomweite RNA Sequenzierung zeigte zudem unterschiedliche Resultate für beide Ansätze und bestätigte die wichtige Rolle von DNMT1 für das Überleben der behandelten Zellen.

6.4 Curriculum vitae

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Education

Master's program "Molecular Biology"

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Project name: The role of SERPINB1 for prostate cancer

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Practical course

Max F. Perutz Laboratories Vienna: Group of Prof. Dr. Karl Kuchler

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Language Skills

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