DISSERTATION

The role of the tumor suppressor genes TNFAIP3 and NR4A1 in the pathogenesis of hematological malignancies

Submitted by

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Declaration

I hereby declare that this dissertation is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this dissertation. Due acknowledgement has been made in the text to all other material used. Throughout this dissertation and in all related publications I followed the guidelines of "Good Scientific Practice".

Graz, 30.11.2015 Kerstin Wenzl

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Abbreviations

7-AAD	7-amino-actinomycin D
AF1	activation function 1
AF2	activation function 2
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
B-CLL	B-cell chronic lymphatic leukemia
BCR	B-cell receptor
BL	Burkitt lymphoma
BM	bone marrow
B-PLL	B-cell prolymphocytic leukemia
BrdU	bromodeoxyuridine
cDim	methylene-substituted 3,3'-diinodylmethane
CsnB	Cytosporone-B
CSR	class switch recombination
D	diversity
DBD	DNA binding domain
DLBCL	diffuse large B-cell lymphoma
FDA	Food and Drug Administration
FL	follicular lymphoma
FLIII	follicular lymphoma grad III
GC	germinal center
GCB	germinal center B-cell like
GEP	gene expression profiling
HCL	hairy cell leukemia
HL	Hodgkin's Lymphoma
IC	immunocytome
Ig	immunoglobulin
J	joining

LBD	ligand binding domain
MALT	mucosa associated lymphoid tissue
MCL	mantle cell lymphoma
MM	multiple Myeloma
MZBCL	marginal zone B-cell lymphoma
nGCB	non germinal center B-cell like
NHL	non-Hodgkin's Lymphoma
NK	natural killer
nMZBCL	nodal marginal zone B-cell lymphoma
OTU	ovarian tumor domain
PMBL	primary mediastinal B-cell lymphoma
RIP1	recptor-interaction protein 1
RS	Richter syndrome
SHM	somatic hypermutation
SNP	single nucleotide polymorphism
SPL	spleen
TNF	tumor necrosis factor
TNFR1	TNF recptor 1
T-NHL	T-cell Non-Hodgkin's lymphoma
V	variable
wt	wild type
ZnF	zinc finger
ZnF7	zinc finger domain 7

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Abstract

Tumor suppressor genes are a large group of genes, which play a critical role in cell cycle, apoptosis and progression of disease. The loss of either one or both alleles can lead to malignant transformation of cells and cancer. Over the last few years scientists have shown particular interest in A20 –a negative regulator of NF-κB activation – known to function as tumor suppressor in lymphoid malignancies. A newly identified tumor suppressor in hematologic neoplasm is NR4A1, which acts together with NR4A3 in acute myeloid leukemia and, as recently described by our group in aggressive lymphoma. The aim of this thesis was to investigate the role of A20 and NR4A1 in lymphomagenesis. Therefore, we tested 15 different types of lymphoid malignancies for the occurrence of A20 gene polymorphism *r*s143002189 and explored the function of NR4A1 in Myc-driven lymphomagenesis *in vivo.*

We found the rs143002189 of germline origin with a significant higher incidence in diffuse large B-cell lymphoma compared to non-neoplastic controls and other lymphoma types. Further *in silico* investigations of the occurrence of the *r*s143002189 polymorphism on A20 protein structure predicted to alter the functional properties of the protein suggesting that the *r*s143002189 might contribute to lymphomagenesis.

Furthermore, we could show for the first time that the loss of NR4A1 accelerated Myc driven lymphomagenesis. Additionally, the loss of NR4A1 favors infiltration by malignant B-cells in bone marrow and spleen. We could also show that B-cells lacking NR4A1 have a better *in vitro* survival caused by a higher proliferation. Western blot analysis revealed that the oncogenes MDM2 and Bcl-xL are both overexpressed in tumors without NR4A1.

Taken together our findings we could demonstrate that A20 and NR4A1 both significantly contribute to lymphomagenesis. However, further studies in order to fully understand their functions are necessary.

Zusammenfassung

Tumorsuppressorgene sind eine große Gengruppe, die eine wichtige Rolle bei Prozessen wie dem Zellzyklus, der Apoptose und der Progression einer Krankheit spielen. Der Verlust von einem oder beider Allele kann zur malignen Veränderungen von Zellen und somit zur Entstehung von Krebs führen. In den letzten Jahren wurde für die Forschung besonders A20, ein negativer Regulator der NF-kB Aktivierung, interessant. A20 ist als Tumorsuppressor in malignen Lymphomen bekannt. Ein kürzlich identifizierter Tumorsuppressor ist NR4A1, der gemeinsam mit NR4A3 in akuter myeloischer Leukämie agiert, und kürzlich von unserer Gruppe in aggressiven Lymphomen beschrieben wurde. Das Ziel dieser Arbeit war es, die Rolle von A20 und NR4A1 in der Entstehung und Entwicklung von Lymphomen zu untersuchen. Dazu untersuchten wir 15 verschiedene Lymphomentitäten auf das Vorhandensein des A20 Genpolymorphismus rs143002189 sowie *in vivo* die Funktion von NR4A1 in Myc induzierter Lymphomentstehung.

Wir stellten, verglichen mit nicht-neoplastischen Kontrollen und anderen Lymphomarten, ein signifikant höheres Vorkommen von keimbahnentsprungenem rs143002189 bei diffus großzelligen B-Zell-Lymphomen fest. Weitere in silico Untersuchungen von rs143002189 in der A20 Proteinstruktur zeigten eine Veränderung der funktionellen Eigenschaften des Proteins. Diese Ergebnisse weisen darauf hin, dass rs143002189 signifikant zur Lymphomentstehung beiträgt.

Des Weiteren konnten wir erstmalig zeigen, dass der Verlust von NR4A1 die Myc induzierter Lymphomentstehung beschleunigt. Außerdem bewirkt der Verlust von NR4A1 einen erhöhten Anteil von malignen B-Zellen in der Gesamtzellzahl von Knochenmark und Milz. Wir konnten fernen zeigen, dass B-Zellen ohne NR4A1 durch verstärkte Proliferation *in vitro* ein besseres Überleben haben. Western Blot Analysen zeigten in Tumoren ohne NR4A1 eine Überexpression der Onkogene MDM2 und Bcl-xL.

Mit unseren Ergebnissen können wir die signifikante Bedeutung von A20 und NR4A1 in der Lymphomentstehung nachweisen. Dennoch sind weitere Untersuchungen zum vollen Verständnis ihrer Funktionsweisen unabdingbar.

Background

Lymphoma

Lymphoma is an umbrella term covering all tumors, which can emerge from B-cells, T-cells and natural killer (NK) cells. Currently, there are about 30 different subtypes of B-cell lymphomas and about 20 different subtypes of T-cell and NK-cell lymphomas classified by the WHO(1), whereby lymphomas arising from B-cells represent the most common form in adults. Furthermore, we discriminate between non-Hodgkin's Lymphoma (NHL) and Hodgkin's Lymphoma (HL). The NHL subgroup represents the majority of lymphomas and can be further divided in indolent and aggressive lymphomas (2), based on their clinical appearance as well as genetic and histopathologic criteria.

B-cell development

In the adaptive immune system, B-cells play an important role as they produce antibodies with a high affinity and diversity against a variety of different antigens.(3) The central feature in B-cell development is the production of the B-cell receptor (BCR), which distinguishes B-cells from other lymphocytes like T-cells or NK-cells. Upon binding of an antigen to the BCR, B-cells are activated and can either differentiate into long living plasma cells or memory B-cells.(4) The BCR is a membrane-bound immunoglobulin. Immunoglobulin (Ig) chains have either four or five domains depending on their isotype (IgM or IgD) (4) and consist of either heavy or light chains each having a constant and variable region.(5)

B-cell development starts in the liver of the fetus and further takes place in the bone marrow of the embryo.(6) During early stages of B-cell development more than 10 transcription factors play an important role. The transcription factors E2A, EBF and Pax5 are responsible for the differentiation at this time of development.(7) As immature B-cells they migrate from the bone marrow to secondary lymphoid tissue, where immature B-cells acquire their mature form. (3) (Figure 1)

During all stages of B-cell development, the BCR undergoes constant genetic rearrangement. Both, the light and heavy chains and their V (variable), D (diversity) and J (joining) regions are affected by this rearrangement at early stage of B-cell development in the bone marrow.(8) After bypassing several check points, immature B-cells migrate to the T-cell zones of the secondary lymphoid tissue. There they have two options after activation of T-cells. The first option is to become a short lived plasma cell, which produces IgM antibodies. The second is that immature B-cells migrate into the B-cell follicles and start undergoing different genetic rearrangement and mutations known as the germinal center (GC) reaction.

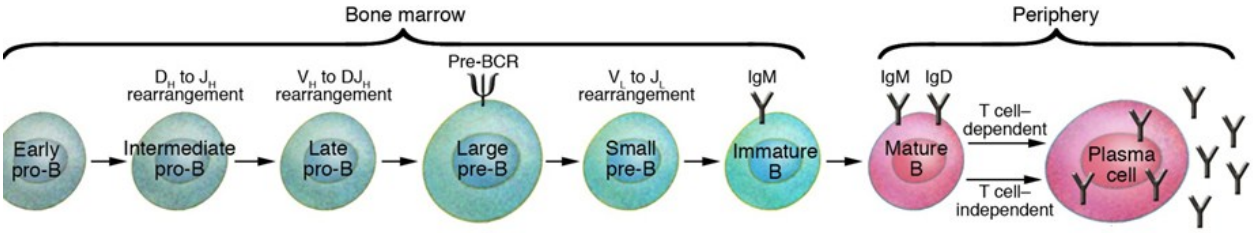

Figure 1 Schematic representation of the different stages during B-cell development – adapted from *Pillai et al.*(9) B-cell development mainly occurs in the bone marrow and the secondary lymphoid organs. During this process, the B-cell goes through different stages like pro-B-cell, pre-B-cell and immature-B-cell stages. At an immature B-cell stage, the cell leaves the bone marrow and migrates to the secondary lymphoid organs were it becomes a mature B-cell.(9)

The GC consists of two compartments – the dark zone and the light zone.(figure 2) This classification is based on a histologic criteria.(10) Furthermore, the GC can be divided genetically dependent on the modification the BCR undergoes.(6) Upon entering the GC the BCR is modified by somatic hypermutation (SHM). The SHM causes point mutations, small deletions, insertions, and duplications in variable region of the rearranged V(D)J-genes of the heavy and light Ig genes of the BCR. The SHM is needed for fine tuning of the low affinity BCR and is also called affinity maturation.(6, 10) The next process in the GC reaction is the class switch recombination (CSR), which is needed to create different classes of Ig (IgG, IgM, IgE, IgA). Both modifications are important factors during immune response to generate a wide range of different antibodies in order to recognize a variety of different antigens.(6) (Figure 2)

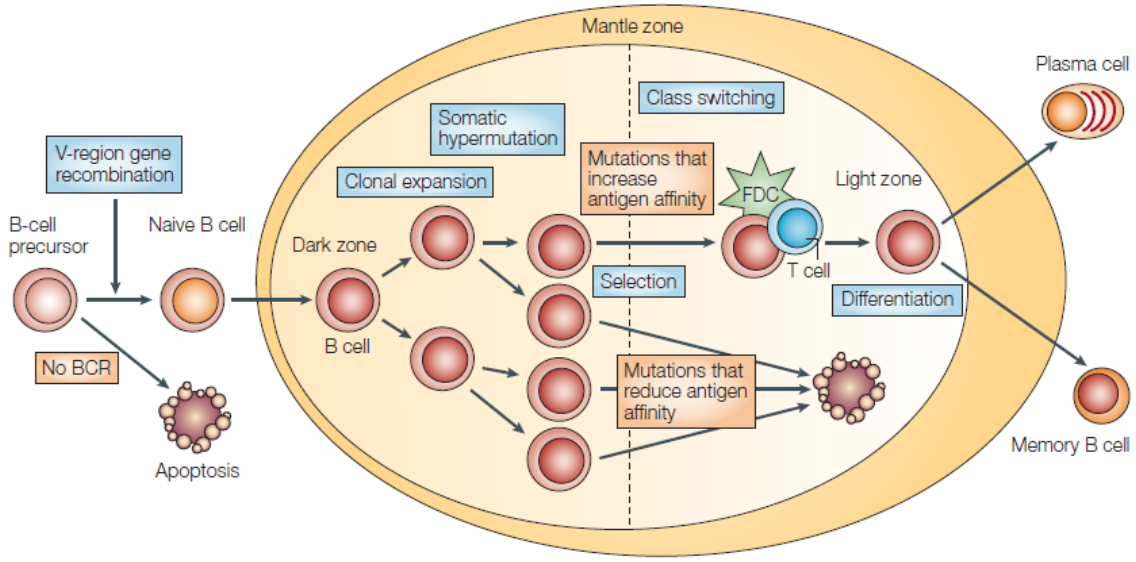

Figure 2 Schematic representation of the germinal center reaction – adapted from *Küppers et al.*(2) The immature B-cell enters the germinal center after activation. During the maturing process the cell undergoes different genetic modifications. The first modification is the somatic hypermutation followed by the class switch reaction.(2)

Today there is ample evidence that most lymphomas arise from the GC as during GC reaction B-cells experience a lot of genetic modifications like SHM and CSR that could lead to malignant transformations.(11)

Aggressive B- cell lymphomas

Aggressive B-cell lymphoma is a term, which has been used for years, for lymphomas with a high proliferation rate. These lymphomas often need clinical intervention, immediately.(12) Aggressive B-cell lymphoma can be cured in 80% of the patients. However, there is a great variation in results reported from individual clinical trials. These differences can be attributed to unrecognized heterogeneity in this kind of malignancy. Recent clinical and molecular studies introduced a new classification of aggressive B-cell lymphoma e.g. by gene expression profiling (GEP) and by the modified Ann Arbor staging classification.(13, 14)

The three most common forms of aggressive B-cell lymphoma are - diffuse large B-cell lymphoma (DLBCL), follicular lymphoma grade III (FLIII) and Burkitt's lymphoma (BL) - all arising from GC B-cells.

Diffuse large B-cell lymphoma

DLBCL is the most common type of lymphoma accounting for 30%-40% of all lymphomas in adults. Clinical outcome is diverse, with a 5-year survival rate between 30% and 80%.(15)
GEP showed that all DLBCL cluster in three different subtypes based on similarity in expression patterns to their cellular origin: germinal centre B-cell-like (GCB-DLBCL), activated B-cell-like DLBCL (ABC-DBLCL) and primary mediastinal B-cell lymphoma (PMBL).(15) These subtypes of DLBCLs are associated with distinctly different overall survival rates after anthracycline-based chemotherapy. Overall survival is favorable in patients with GCB subtype and PMBL and inferior in those with the ABC subtype. Addition of rituximab to standard chemotherapy has improved the survival rates of DLBCL patients. Nevertheless, the ABC subtype still remains less curable than GCB.(16)
The modified Ann Arbor staging classification is used to describe the extent of disease and is based on number of involved sites and presence of extralymphatic involvement - stage I: Involvement of a single lymph node region; stage II: Involvement of two or more lymph node region on the same side of the diaphragm; stage III: Involvement of lymph node regions on both sites of the diaphragm accompanied with local involvement of an extralymphatic organ; and stage IV: multifocal involvement of one or more extralymphatic organ.(14) With standard chemotherapy, aggressive B cell lymphoma, even when in advanced stage, is a curable disease. Nonetheless, despite the improvements in therapy, approximately one third of patients with advanced-stage DLBCL still is refractory to therapy or will relapse.(17, 18)

Follicular lymphoma

Follicular lymphoma (FL) represents one of the most common subtypes of indolent lymphomas. The hallmark of this type of lymphoma is the translocation of the BCL2 gene

(t(14;18)(q32;q21)) to the enhancer of the IgH-chain.(19) This translocation occurs almost in 90% of all FL but is not sufficient for malignant transformation since it has been found in B-cells from healthy patients as well.(20) Additionally to the BLC2 translocation, other genetic alterations have been found. The most common ones are loss of 1p36 and 6q chromosomes and gains on chromosomes 7, 18, and X (21) Although FL is an indolent disease previous reports show that FL tends to undergo transformation to more aggressive types of lymphoma (e.g. FLIII) or even a histological change to DLBCL. (22) There are two major events that play a critical role in the transformation process of FL. The first would be abnormality of neoplastic cells. In these cells tumor suppressors like p53 or CDKN2A are lost or over expression of the known oncogene c-Myc occurs.(20, 23) The second key factor in the development of a more aggressive form is the microenvironment. Studies have shown that loss of the follicular dendritic cell meshworks, increased microvessel density as well as an increased number of intrafollicular $CD4^+$ T cells are associated with the transformation process of FL. (19, 21)

Burkitt's lymphoma

Named after Denis Burkitt, who first described these lymphoma in African children, the Burkitt's lymphoma (BL) is a highly aggressive lymphoma with a high proliferation rate of the malignant cells.(24) Throughout the years, epidemiological studies recognized three different subtypes of BL - endemic, sporadic, and immunodeficiency-associated.(25) Endemic BL refers to those lymphomas diagnosed in African children. These lymphomas are often associated with an infection of Epstein-Bar-Virus. All BL outside of Africa but showing the same characteristics as endemic BL are called sporadic BL. The third form, immunodeficiency-associated BL, is mainly found in patients, who are infected with the human immunodeficiency virus (HIV).(25) A hallmark of BL is the translocation of c-Myc on one of the Ig-chains.(26) This translocation is not exclusively for BL since it has also been found in cases of DLBCL. This fact led to the suggestion that the translocation of c-Myc occurs during the GC reaction.(27) Additionally to the c-Myc translocation, other genetic aberrations have been described. One of most frequent mutated genes is p53which have been found in 35% of BL samples. (28) Recent studies showed that in BL the TCF3/ID3 pathway is

also altered.(29, 30) This pathway is a key regulator of cell survival and proliferation in the dark zone of the GC. This alteration may play an important role in cooperation with c-Myc and the survival of BL cells since no alterations have been found in DLBCL samples.(28)

Tumor suppressor genes

Tumor development is a multi-step process involving genetic transformation of oncogenes and tumor suppressor genes.(31) Tumor suppressor genes are involved in cell division, cell cycle and DNA repair. Once a tumor suppressor function is lost, it often results in uncontrollable cell division which could lead to tumor development.(32) One of the early models for tumor suppressor was the "two hit" model postulated by Knudson.(33) He hypothesized, supposed that both copies of the gene have to be lost to affect the tumor suppression function of the gene.(33) This may be true for some tumor suppressors but not all of them. For example for p53, the most mutated gene in cancer, it was shown that the loss of one allele is sufficient to lead to cancer in mice at the age of nine month.(33)

In the past few years, several studies have tried to therapeutically target tumor suppressor genes and/or their pathways.(31) p53 is one of the main targets in these approaches. One of the concepts is to restore p53 wilde type function via gene therapy.(34) Other approaches are to use inhibitors for epigenetic modulators. There are already first clinical trials for small molecules that inhibit 2-hydroxyglutarate production by isocitrate dehydrogenases. (35)

Apart from the already well known tumor suppressor genes, there a new ones, which should be considerated as potential drug targets, like A20 or the NR4A1 nuclear orphan receptor.

A20

A20 also known as tumor necrosis factor (TNF) α induced protein 3 or TNFAIP3 is an ubiquitin-modifying enzyme acting as negative regulator of NF-κB. It was first identified in 1990 as a cytokine response gene in the human umbilical vein endothelial cells.(36) It is located on chromosome 6 and has eight coding exons.(37) On the A20 C-terminal region seven zinc finger (ZnF) domains are located, wherebyone of those ZnFs, the ZnF domain 4, functions as an ubiquitin ligase or E3 ligase.On the N-terminal region the protein contains an ovarian tumor domain (OTU).(36) (Figure 3) A20 can be induced by various NF-κB stimuli through the NF-κB binding sites in the A20 promotor. Furthermore, multiple studies have showed that A20 is a negative regulator of the NF-κB pathway.(38)

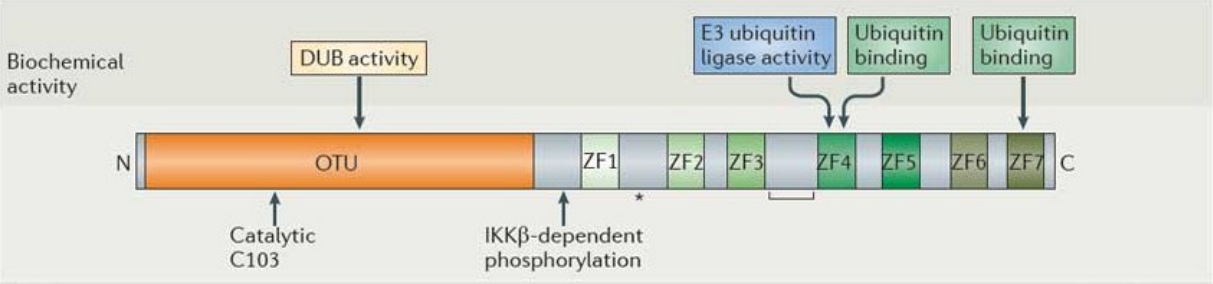

Figure 3 Schematic representation of the A20 protein structure - adapted from *Ma et al.*(39) On the N-terminal site the A20 protein has an ovarian tumor domain (OTU) harbouring a deubiquitinating enzyme activity. On the C-terminal the protein contains seven zinc finger regions with an E3 ubiquitin ligase activity on zinc finger domain 4. (39)

Regulation and function of A20

The main regulator of A20 is the CARMA1-BCL10-MALT1 complex. This complex connects the T-cell receptor signalling with the canonical IKK/NF-κB pathway.(36) The way A20 restricts NF-κB pathway is very complex and unique.(40) Like other E3 ligases and deubiquitinating enzymes, A20 is involved in ubiquitylations of several proteins.(40) The polyubiquitiylation process represents a post transcriptional modification which causes proteasomal degradation. The OTU of A20 functions as a deubiquitiylating enzyme by cleaving lysine-63 linked polyubiquitin chains from the receptor-interaction protein 1 (RIP1), which is an essential factor for the TNF receptor 1 (TNFR1) signaling pathway.(38) This

reaction is followed by adding lysing-43 linked polyubiquitin chains and degradation of RIP1 through the E3 ligase of A20 .(38) This reaction suggests that A20 has a dual function in the TNFR1 pathway.(41) Furthermore, A20 does not only regulate the ubiquitiylation of RIP1 in the TNFR1 pathway but it also suppresses the activation of NF-κB through TNF by deubiquitinating TRAF6.(42, 43)

A20 and hematological neoplasms

The role of A20 as a tumor suppressor is highly discussed. However, recent studies in lymphoid neoplasms have shown alterations in A20. They could detect frequent inactivation of A20 through mutations and/or deletions in several different subtypes of hematological neoplasms like DLBCL, FL or HL.(44-46) In one of our most recent studies frequent downregulation of A20 in multiple myeloma (MM) was shown. We could demonstrate that A20 is mutated in MM patients and that deletion of the A20 loci is a frequent event in MM patients.(47) Promoter methylation is an additional mechanism to inactivate A20. This type of inactivation has been shown in mucosa associated lymphoid tissue (MALT) lymphoma and the ABC subtype of DLBCL Additionally, re-expression of A20 in A20 deficient DLBCL lymphoma cell lines induced apoptosis(46, 48) demonstrating its function as a tumor suppressor gene.

The constitutive activation of the NF-κB pathway is a characteristic for lymphoid neoplasms and the fact that A20 is frequently inactivated in lymphoma suggest that there is a connection between those two events.

A20 as a drug target

Until now, numerous drugs have been developed to inhibit the NF-κB pathway, E3 ligase or deubiquitinating enzymes. However, no substance that mimics the effect of A20 has been identified.(49) However, there has been a lot of research in finding drug targets to inhibit deubiquitinating enzymes which play a role in the activation of the NF-κB pathway. The first inhibitor which was approved by the Food and Drug Administration (FDA) was bortezomib. Bortezomib is a protease inhibitor which inhibits deubiquitinating enzymes. It was first approved for patients with MM and is now also approved for patients with mantle cell

lymphoma.(50, 51) Carfilzomib is one of the newest approved FDA drugs. It is only allowed for MM patients who had already failed two therapies. Carfilzomib, a protease inhibitor like bortezomib, targets deubiquitinating enzymes. Beside those two, many deubiquitinating enzyme inhibitors and E3 ligase inhibitors are in phase I or II studies.(51)

NR4A nuclear orphan receptors

Nuclear receptors are a set of transcription factors regulating cellular differentiation, reproduction, homeostasis, and metabolism. The nuclear receptor superfamily shares the same structural receptor characteristics (Figure 4) and within the subgroups of the NR superfamily the receptors share a high homology in their amino acid sequence. All NR consists of an amino-terminal region encoding activation function 1 (AF1), DNA binding domain (DBD), the ligand binding domain (LBD) and a ligand-dependent activation function (AF2) transactivation domain located on the C-terminal region. Their transactivation is mainly mediated by AF1, while AF2 is dispensable.(52, 53)

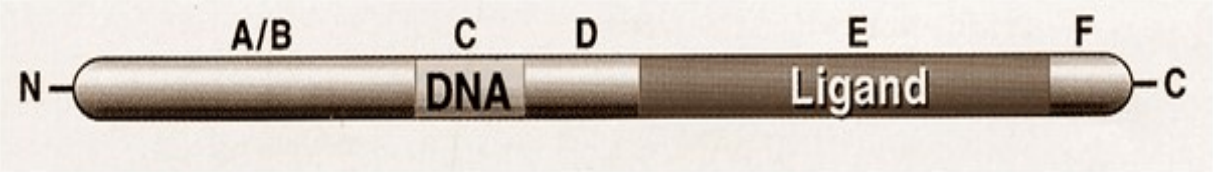

Figure 4 Schematic representation of the nuclear receptors structure. - adapted from *Mangelsdorf et al.* (53) All nuclear receptors have the same structure. The all have a N-terminal activation function 1 domain, DNA binding domain, ligand binding domain and a C-terminal ligand-dependent activation function 2.(53)

NR4A1 (Nur77), NR4A2 (Nurr1) and NR4A3 (NOR-1) belong to the nuclear orphan receptors superfamily. NR4As are classified as orphan receptors because no physiological or endogenous ligand has been identified. (54)

NR4A1, NR4A2 and NR4A3 (NR4A) share a high homology within their amino acid sequence. (Figure 5)

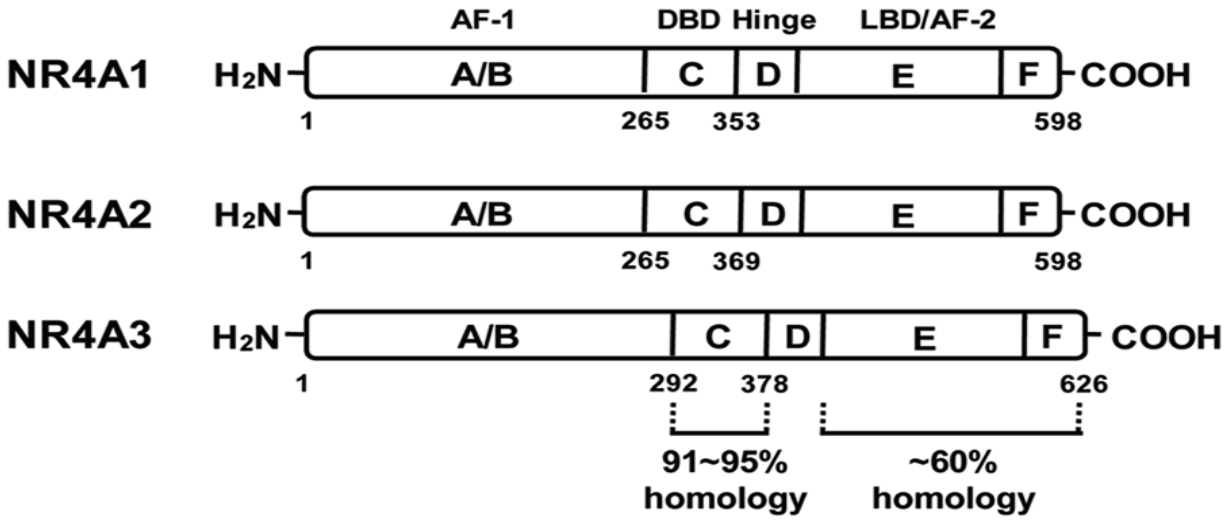

Figure 5 Illustration of the sequence homology within the NR4As. - adapted from Safe *et al*. (55)

Overall, the NR4A gene expression effect is general a short one. The cellular outcome is a stimulus- and cell context-dependent differential activation of NR4A target genes that regulate cell cycle, apoptosis, inflammation, atherogenesis, metabolism or DNA repair and, as described more recently, are involved in tumorgenesis. Additionally, NR4A1 and NR4A3 play a key role in negative selection of T-lymphocytes, as well as IgM mediated- and viral induced B-cell apoptosis.(54) NR4As are expressed in different types of tissues, such as skeletal muscle, adipose tissue, heart, kidney, T-cells, liver and brain. They are classified as early immediate- response genes, which are induced by a wide range of different stimuli. These stimuli include prostaglandins, growth factors, fatty acids, neurotransmitters and other cellular stressors.(56, 57)

Regulation of NR4As

NR4A1 gene expression can be regulated on both the transcriptional and post-transcriptional level. On the transcriptional level, negative regulation of NR4A1 gene expression can be influenced by histone deacetylases, which function as co-repressors of NR4A1. cAMP/CREB, calcium flux and ERK5 can reverse this repression. c-Fos and c-Jun, which form a complex bound to the AP-1 binding site of the NR4A1 promoter, enhance NR4A1 gene expression on the transcriptional level. The post-transcriptional regulation is an important mechanism in

regulation of NR4A1 activity. NR4A1 can be phosphorylated by different post-transcriptional factors including c-Jun-N-terminal kinase (JNK), ERK2, ERK5, the Protein kinase C, the p90-kDa ribosomal S6 kinase (RSK), the Akt protein kinase and the glycogen synthase kinase 3β, respectively. Phosphorylation of its N-terminal amino acid residues by JNK causes NR4A1 translocation to the cytoplasm. Other phosphorylation sites like Ser105 within the MAP kinase pathway, at Thr142 of NR4A1 by ERK2 and at Ser350 (Ser351 in human NR4A1) by Akt induce inhibition of its DNA binding activity, nuclear export of NR4A1, and induction of NR4A1 mediated apoptosis. Cytoplasmic located NR4A1 targets and binds to the hydrophobic groove of BCL2 at the mitochondria – called mitochondrial targeting. This results in a BCL2 conformation change by the conversion from a cytoprotective to a cytodestructive molecule, which triggers a cytochrome C release followed by induction of apoptosis.(54, 58)

There is very little known about the regulation of NR4A3. Since it has been shown that NR4A3 is functionally redundant with NR4A1 at least in T-cell apoptosis and has similar tissue expression pattern than those of NR4A1, it is speculated that transcriptional regulation is similar to that of NR4A1. Additionally it was demonstrated that the NK6 homebox 1 protein induces NR4A1 and NR4A3 by binding in both promoter.(59, 60)

NR4As and hematological neoplasms

The first study that showed that NR4As may play an important part in the development of hematological neoplasms was from *Mullican et al.*. This study showed that NR4A1 and NR4A3 double knockout mice developed acute myeloid leukaemia (AML) and died within two to four weeks.(61) Additionally, they could demonstrate that transplantation of bone marrow cells of double knockout mice into irradiated wild-type mice results in development of AML as well. Furthermore, comparison of NR4A1 and NR4A3 gene expression of leukemic blasts from human AML patients compared to $CD34^+$ progenitor cells of healthy bone marrow donors demonstrated that down regulation of these two genes is a common feature in AML patients.(61) Hypoallelic (NR4A1+/-NR4A3-/- or NR4A1-/-NR4A3+/-) mice develop mixed myelodysplastic/myeloproliferative neoplasms with a more aggressive form in NR4A1+/-NR4A3-/- than in NR4A1-/-NR4A3+/- mice demonstrating indicating that gene dosage of

NR4A1 and NR4A1 is a key factor for the development of hematological neoplasms. Microarray analysis of NR4A1 and NR4A3 over-expressing AML cell lines showed that 97% of genes deregulated by NR4A1 are as well deregulated by NR4A3. These findings demonstrate a nearly complete redundancy of both NRs.(62) Overexpression of NR4A1 and NR4A3 induced tumor growth factor-β (TGF-β)-, tumor necrosis factor-, and JUN-expression and supressed MYC-expression by occupying its promoter. Additionally, NR4A1 and NR4A3 over-expression caused up-regulation of the TGF-β -activated cell cycle arrest gene p57 and up-regulation of FOXO1 and GATA2. These results identified a large number of molecular targets of NR4A1 and NR4A3 in AML and highlighted a central role for suppression of MYC in NR4A mediated tumor suppression in AML.(63)

In our previous comprehensive study, we showed that NR4A1 and NR4A3 expression was significantly down regulated in chronic lymphocytic B-cell leukaemia (CLL, 71%), in FL (70%), and DLBCL (74%) compared to normal controls. Furthermore, down regulation of NR4A1 and NR4A3 was significantly associated with low apoptotic signals (Trail, Bim and Puma). Survival analysis revealed that low NR4A1 expression is associated with poor cancer specific survival in our cohort of aggressive lymphomas and additionally in the public available gene expression data set of *Lenz et al*..(64, 65) Over-expression of NR4A1 in lymphoma cell lines caused induction of apoptosis and abrogated tumor growth in xenografts. By restoring NR4A1 expression, Trail, Bim and Puma expression was induced.(64) Furthermore, over-expression of NR4A3 in aggressive lymphoma cell lines had the same effect as NR4A1 over-expression suggesting a pro-apoptotic and tumor suppressive function of NR4A1 and NR4A3 in aggressive lymphoma.(66)

NR4As as drug target

A great number of agents/chemotherapeutics affecting NR4A expression has been identified (table 1). Their effect is either mediated by inducing apoptotic stimuli through activation of apoptotic target genes or by translocation to the cytoplasm and mitochondrial targeting. In the NR4A family, NR4A1 is the best studied receptor. (54)

For treatment of DLBCL, a chimeric anti-CD20 monoclonal antibody, Rituximab (Rituxan or MabThera) was recently approved by the FDA.(67) The *in vivo* mechanisms of Rituximab is not fully established, but potential mechanism are discussed: Rituximab dependent cyctotoxity could be induced in B-cells by antibody-dependent cell mediated cytotoxicity, the complement dependent cytotoxicity, Erk1/2 and /or p38 mediated induction of apoptosis or cell proliferation inhibition of malignant cells.(67-72) Gene expression profiles of Rituximab treated lymphoma cell lines showed a similar expression pattern as those of IgM-mediated BCR ligation with Erk1/2 dependent NR4A1 mRNA induction. Together with the evidence of NR4A1 playing a central role in surface IgM-mediated BCR ligation accompanied by apoptosis induction and virally induced apoptosis of B-cells, these findings suggest that NR4A1 is essential for the apoptotic effects of Rituximab.(73-76)

The identification of NR4A1 mimetics (NUBCP-9) and agents inducing NR4A1 expression like Cytosporone-B (CsnB) and methylene-substituted 3,3'-diinodylmethane (cDim) raised the question if NR4As can serve as therapeutic targets.(54, 58) First attempts were performed by our group and by the group of *Liu et al*. by treating aggressive lymphoma cell lines with CsnB and inducing NR4A1-dependent apoptosis. This led to the suggestion that NR4As could serve as potential target for cancer therapy.(54, 58) Furthermore silencing of NR4A1 by shRNA entirely reversed the apoptotic effects and confirmed the central role of NR4A1 in lymphomagenesis.(64)

Table 1 NR4A1 or NR4A3 inducing agents and their effect on NR4A1 - adapted from *Wenzl et al.* (77)

Agent:	NR4A:	Type of agent	Cell system	Effects on NR4A1
Fenretinide	NR4A1	Retinoid	Liver cancer cell lines	Induction of NR4A1 and mitochondrial targeting
Vitamin D3	NR4A1		PBMNC	Induction of NR4A1
Vitamin K2	NR4A1		Ovarian cancer cells	Mitochondrial targeting
Lithium treatment	NR4A1	Chemotherapeutics	Follicular thyroid cancer cell line	Induction of NR4A1
HDACi –HDAC7 HDACi & ErB blockade HDACi, hydroxamate 5Aza	NR4A1	Chemotherapeutics	T cell lymphoma cells Prostate and breast cancer cells AML cell line – HL60	Induction of NR4A1 & mitochondrial targeting Induction of NR4A1 Induction of NR4A1
Staurosporine	NR4A1	Chemotherapeutics	T cell lymphoma cells	Induction of NR4A1
Rituximab	NR4A1	Chemotherapeutics	B cell lymphoma	Induction of NR4A1
Indomethacin	NR4A1	NSAID	Colon cancer cells	Induction of NR4A1
Ionizing radiation	NR4A1	Radio therapy	Hepatoma cells	-
Cadmium	NR4A1	Environment toxin	Lung cancer cell line (WI-38, A549)	Induction of NR4A
di-n-butyltin dichloride (DBTC)	NR4A1	Environment toxin	Thymocyte	Induction of NR4A1
Cartenolides	NR4A1	Plant extract	Cancer cells	Induction of NR4A1
Acetylshikonin and analogs	NR4A1	Plant extract derivate	lung cancer and HeLa cervical cancer cells	Mitochondrial targeting
n-Butylenephtalide	NR4A1 NR4A3	Plant extract derivate	hepatocellular carcinoma, glioma and glioblastoma cell line	Induction of NR4A& mitochondrial targeting of NR4A1
Palmitate	NR4A1 NR4A3	Fatty acid	pancreatic β-cells	Induction of NR4A1 expression

Aim

The aim of the study was to investigate the role of two known tumor suppressor genes in hematological neoplasms. In the first part we studied the contribution of A20 in lymphomagenesis. We could recently describe the rs143002189 polymorphism in multiple myeloma patients in combination with a reduced A20 activity.(47) To further investigate this polymorphism and its contribution to lymphomagenesis we screened different lymphoma entities for this polymorphism.

In the second part of the study we investigated the tumor suppressor gene NR4A1. Although there is good evidence that *NR4A1* and *NR4A3* act as cooperating tumor suppressors in AML, investigations of *NR4A1* loss *in vivo* in combination with other genetic alteration causing lymphoid neoplasms are lacking. Therefore, we established a Myc induced lymphoma mouse model in which NR4A1 is lost.

Part I

The role of the tumor suppressor A20 in hematological malignancies

Materials and Methods

Patient samples and DNA extraction

For genotype analysis of the rs143002189 polymorphisms, DNA of the most common lymphomas - including acute lymphoblastic leukemia (ALL), CLL, BL, DLBCL, FL, HL, hairy cell leukemia (HCL), Immunocytoma (IC), PMBL, mantel cell lymphoma (MCL), Marginal-zone B-cell lymphoma (MZBCL), MALT lymphoma, B-cell prolymphocytic leukemia (B-PLL), Richter syndrome (RS), and T-cell non Hodgkin lymphoma (T-NHL) - and non-neoplastic controls were used (table 2). DNA purification was performed on fresh frozen material using the DNA Mini Kit (Qiagen GmbH, Hilden, Germany). Additionally, control subjects were recruited from local health screening studies, the presence of known current or previous malignant disease was excluded anamnestically. All lymphoma samples were collected and stored at the Institute of Pathology at the Medical University Graz. DLBCL were classified as GCB and non-GCB DLBCL according to the Hans algorithm.(78) The study was approved by the ethics committee of the Medical Univeristiy of Graz (20/388/08/09-1).

Genotyping analysis of rs143002189 polymorphism

Genotypes were determined by 5'-exonuclease assay (TaqMan). Primer and probe sets for rs143002189 were designed and manufactured using Applied Biosystems 'Assay-by-Design' custom service (Life Technologies, Vienna, Austria). General TaqMan reaction conditions were according to the manufacturer of the assays. Endpoint fluorescence was measured in a POLARstar plate reader (BMG Labtech). The data were exported into Excel format and depicted and analyzed as scatter plot. In the plot, genotype groups were identified as separate and distinguishable clusters. As a control for consistency of genotyping methods, determination of genotypes was repeated in at least 10% of the samples and no discrepancies were observed.

Structural analysis of the zinc finger 7 domain

Structural information about A20 was available in the PDB structure database, an international repository for 3-D structure files.(79) Protein structures were determined with crystallographic methods or by nuclear magnetic resonance spectroscopy. The structural information was stored in the PDB as data files, which contain mainly the Cartesian coordinates of all atoms involved in the protein. From the PDB database these coordinate files can be downloaded via internet, and the protein visualized at the local place. In this study we used the 3VUW18 and the 3DKB19 data file. The visualization of the A20 protein was done with the Swiss-Pdb viewer20. The electron behavior in molecules was analysed by methods based on quantum theoretical calculations. Quantum theoretical calculations aid the exploration of processes that provide the link between structural analysis and physicochemical and biological functions.(80, 81)

Statistical Analyses

All statistical analyses were performed using the Statistical Package for Social Sciences version 17.0 (SPSS Inc., USA) as previously described.(82)

Results

Higher occurrence of Rs143002189 in DLBCL

In a recent study we could show that the A20 gene is significantly reduced in MM patients. Furthermore, we could detect a missense single base pair substitution- c.2364G>A in one of the patients (Case MM26).(47)

In order to investigate whether the germline mutation c.2364G>A which is affecting the amino acid sequence at position 788 (methionine (Met) to isoleucine (Ile)), is involved in lymphoma predisposition, we screened 479 samples of the most common lymphoid neoplasm and 528 non neoplastic controls, by using the genotyping technique. Case MM26 –known to harbor the A20 c.2364G>A mutation- was used to validate the genotyping assay and served as positive control throughout the whole analysis. From the NCBI SNP database it is known that the rs143002189 polymorphism was present in 1 out of 4370 chromosomes, resulting in minor allele frequency lower than 0.001. However, we could find the A20 heterozygous c.2364G>A mutation in three cases (table 2) among 15 different lymphoid malignancies. All three cases belonged to the group of DLBCL: the single nucleotide polymorphism (SNP) was found in one GCB subtype patient sample and in two non-GCB subtype patient samples. Moreover, two out of 529 non-neoplastic controls also exhibited the heterozygous A20 c.2364G>A mutation. Comparing the incidence of rs143002189 between DLBCL and non-neoplastic controls, it was significantly higher in DLBCL ($p=0.031$), suggesting that A20 c.2364G>A mutations might predispose to play a critical role in the tumorigenesis of DLBCL.

Table 2 Rs143002189 in lymphoid neoplasms and non-neoplastic controls

	Mutated	**Tested**	**Chi-Square**
ALL	0	8	0,97
B-CLL	0	7	1
BL	0	4	1
DLBCL	3	101	**0.031**
GCB-DLCBL	1	35	0.175
nGCB-DLCBL	2	54	**0.045**
unclassified DLBCL	0	12	1
FL	0	45	1
FL II	0	8	1
FL III	0	37	1
HL	0	9	1
HCL	0	7	1
IC	0	19	1
PMBL	0	10	1
MCL	0	8	1
MZBCL	0	14	1
MALT	0	7	1
nMZBCL	0	7	1
MM	1	53	0.284
B-PLL	0	5	1
RS	0	9	1
T-NHL	0	10	1
non neoplastic	2	528	

Structural analysis of the zinc finger 7 domain

A20 contains ZnF domains on the C-terminal region and an OTU on the N-terminal region. To study putative structural effects of the rs143002189 mutation in the ZnF domain 7 (ZnF7), the affected residues at position 788 (Met $\rightarrow$ Ile) inside the domain were interchanged (figure 6). Methionine and isoleucine are neutral, hydrophobic and aliphatic amino acids. The force field energy was = -772KJ/mol for the non-mutated domain and -751KJ/mol for the mutated domain. Additionally, the electron density (figure 7I and II) and the electrostatic potential (figure 7 III and IV) were altered by the mutation in ZnF7.

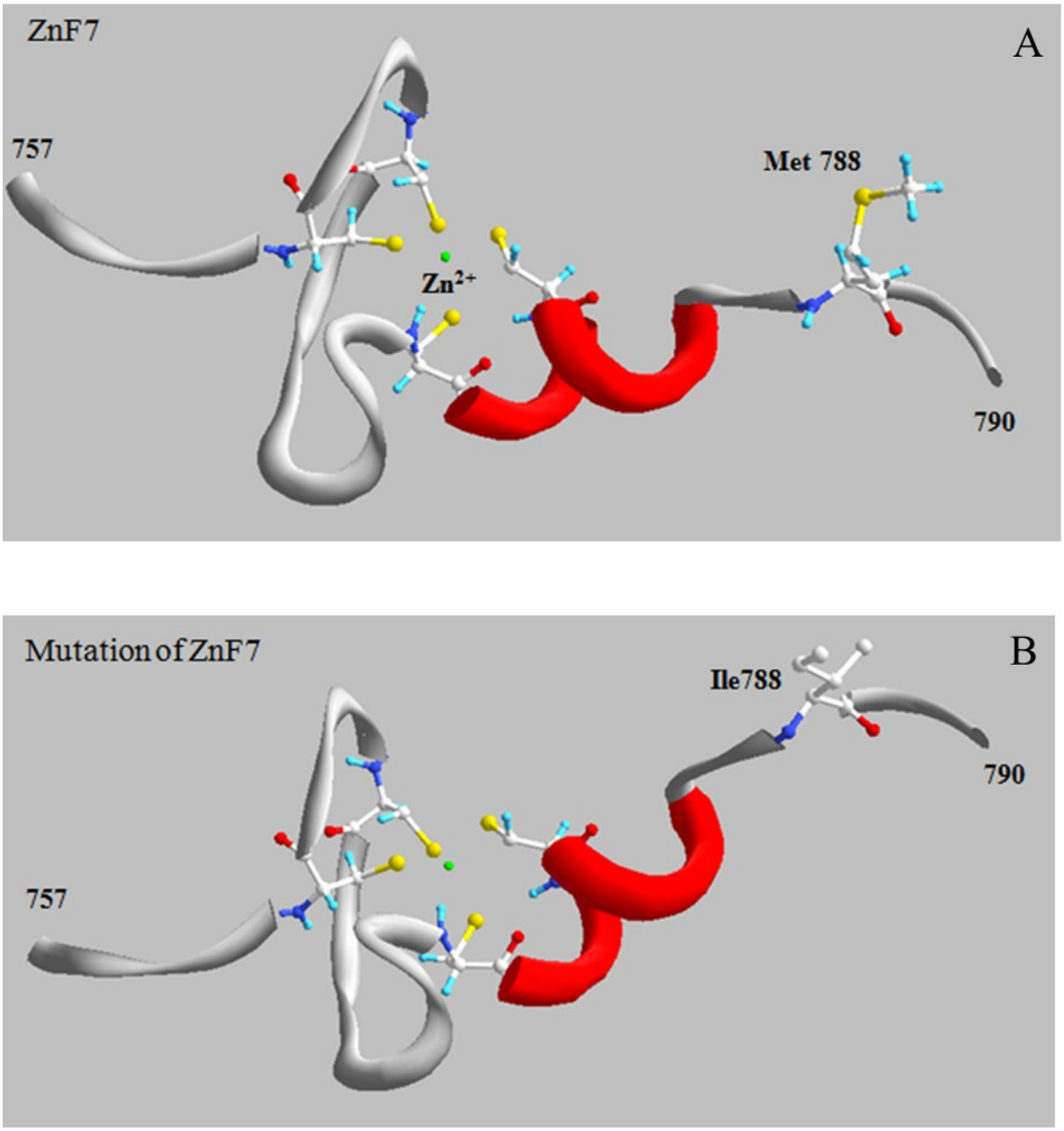

Figure 6 Structural analysis of A20 A.With the Methionine on position 788, B. The protein with the Isoleucine on positon 788.

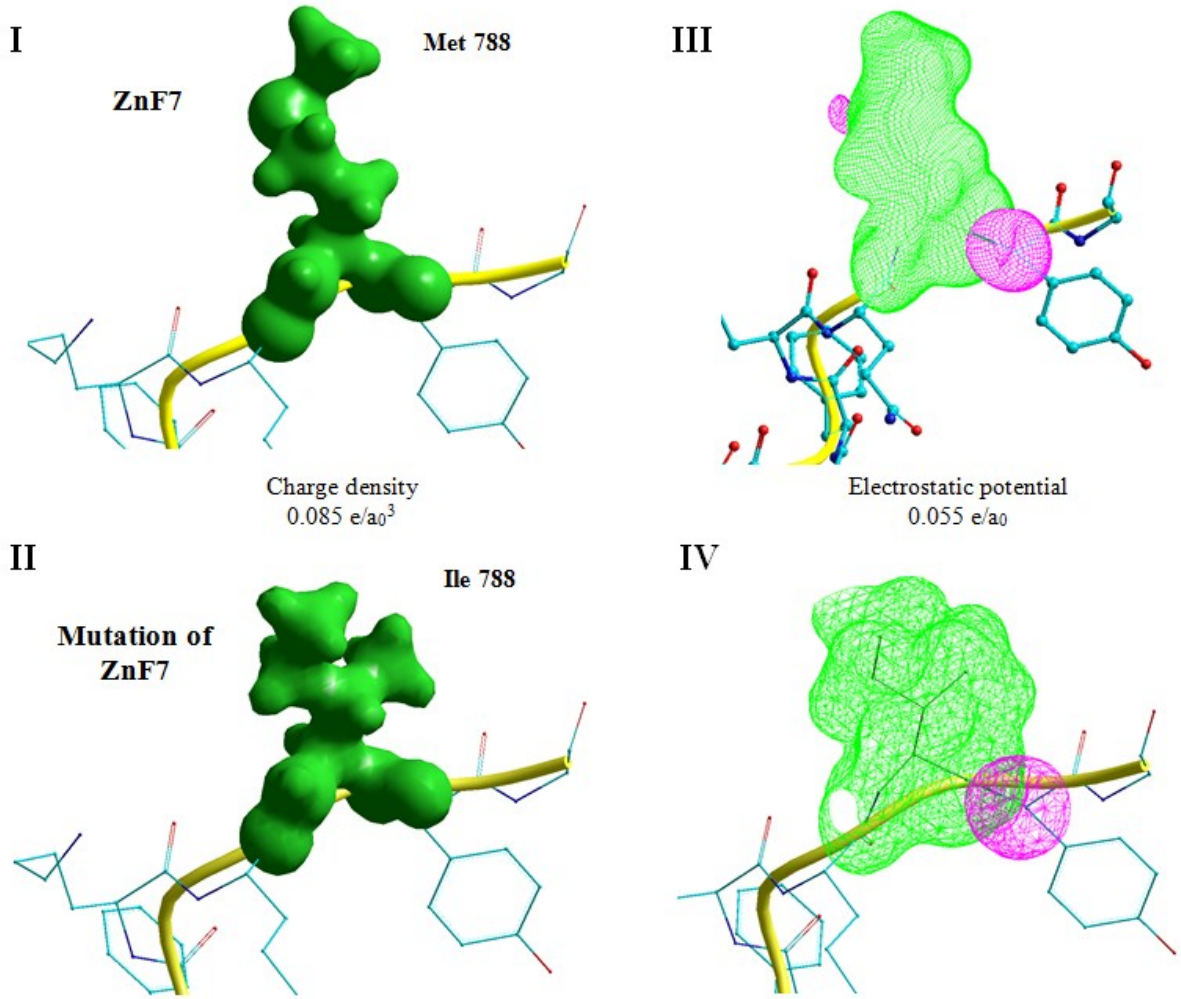

Figure 7 Density plots of A20 with and without the rs143002189 polymorphism. Plots show the different charge density distributions of the unmutated- (I) and the mutated ZnF7 (II) as well as the changed electrostatic potential for the normal (III) and the mutated form (IV) form of the A20 protein.

Discussion

This study was designed to investigate the SNP rs143002189 of A20, which was recently found in MM patients and in other lymphoid neoplasia.(47) Therefore, we screened 15 different lymphoma entities. We could detect a higher incidence of the rs143002189 polymorphism in DLBCL compared to non-neoplastic controls.

It is known that A20 is a negative regulator for the NF-κB-pathway. Additionally, A20 functions as a tumor suppressor in several lymphomas. Different studies have shown that A20 is inactivated as a result of somatic mutations, deletions and/or promoter hypermethylation in various B-cell malignancies, causing constitutive NF-κB activation.(45, 46, 83-88). Often, both of the A20 alleles are inactivated by deletions or mutations, which is a hallmark for tumor suppressor genes (89). Inactivation of A20, caused by any mechanism, promotes uncontrolled NF-κB activity and enhanced cell survival, thereby contributing to lymphomagenesis.

Our genotype analysis for rs143002189 polymorphisms in various lymphoid neoplasms revealed a significantly higher incidence of the c.2364G>A mutation in DLBCL (2.97%) compared to non-neoplastic controls (0.37%), suggesting a predisposing role of this mutation in the pathogenesis of DLBCL. The same mutation was found in one HL sample by *Schmitz et al.*.(90) The rs143002189 polymorphism was not detected in any other type of cancer (http://cancer.sanger.ac.uk/cosmic) and there are no studies where the rs143002189 polymorphism was detected in DLBCL. This led to the suggestion that the rs143002189 polymorphism is exclusively associated with the development of lymphoid malignancies.

The c.2364G>A mutation was found to be located within exon 9 of the A20 gene. This location is the coding region for the ZnF7 of the A20 protein and the place where the A20 ligase activity is presumably located. Due to the fact that the aliphatic, nonpolar amino acid methionine is substituted by isoleucine with a more compact appearance, it might be speculated that a steric interaction results in a possibly reduced function of A20 induced by this missense polymorphism.

In conclusion, we could show for the first time that the rs143002189 polymorphism in the A20 gene occurs in a higher frequency in DLBCL than in any other lymphoid neoplasms. It can be presumed that this mutation has an impact on lymphomagenesis in a fraction of patients with DLBCL. Furthermore, structural analysis of the ZNF7 protein with and without amino acid substitution revealed that replacement of methionine by isoleucine changes the appearance of the protein. Hence, this SNP is predicted to alter the functional properties of the protein.

Part II

The role of the tumor suppressor NR4A1 in hematological malignancies

Materials and Methods

Mouse experiments

All mice have been generated on a C57/BL6 background. The EμMyc transgenic mouse and NR4A1 knockout mouse were obtained from The Jackson Laboratories. Health status and signs of lymphoma development were assessed three times a week. Moribund mice were humanly euthanized and tumors and organs were either fixed in formalin for histopathology or snap frozen for protein and DNA/RNA extraction. For genotype analysis the KAPA2G Fast Hot Start Kit (Peqlab, Erlangen, Germany) was used according to manufacturer's instructions. All mouse experiments were approved by the Austrian Federal Ministry of Science, Research and Economy.

Quantitative Real time PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen GmbH; Hilden; Germany) according to the manufacturer's protocol. cDNA was synthesized using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Waltham, MA, USA). Quantitative reverse transcriptase-polymerase chain reaction was performed using Kapa Probe Fast reaction mix (Peqlab, Erlangen, Germany) and TaqMan® probes to Myc, NR4A1 and NR4A3 (Applied Biosystems, Invitrogen, Carlsbad, CA). qPCR reactions were performed using an ABI Prism 7000 Detection system (Applied Biosystems, Invitrogen, Carlsbad, CA). GAPDH (Applied Biosystems, Invitrogen, Carlsbad, CA), PPIA (Applied Biosystems, Invitrogen, Carlsbad, CA), and HPRT1 (Applied Biosystems, Invitrogen, Carlsbad, CA), served as housekeeping genes. The results are expressed as relative units based on calculation $2^{-\Delta\Delta CT}$, which gives the relative amount of target gene normalized to the endogenous control.

Table 3 List of TaqMan® gene expression assays for qPCR

Gene Symbole	Gene Name	Assay ID
GAPDH	glyceraldehyde 3-phosphate dehydrogenase	Mm99999915_g1
PPIA	peptidylprolyl isomerase A	Mm02342430_g1
HPRT	hypoxanthine guanine phosphoribosyl transferase	Mm01545399_m1
NR4A1	nuclear receptor subfamily 4, group A, member 1	Mm01300401_m1
NR4A3	nuclear receptor subfamily 4, group A, member 3	Mm00450074_m1
Myc	myelocytomatosis oncogene	Mm00487804_m1

Cell culture, treatment with NR4A agonists and LPS and cell cycle analysis

B220 positive cells, from the EμMyc transgenic mice with and without NR4A1 were isolated from the tumor, by passing through a 70μm cell strainer (Becton Dickinson, Heidelberg, Germany) and sorted by FACS analysis. The isolated cells were cultured in RPMI 1640 (Sigma Life Science, St. Louis, MO) medium with 20% fetal calf serum, antibiotics and 50μM beta-mercaptoethanol (all Gibco Life Technologies, San Francisco, CA). For the Lipopolysaccharide (LPS) (Sigma Life Science, St. Louis, MO) treatment, B220 positive cells were treated with 10μg/ml LPS. Cells were counted after 24h, 48h and 72h on the Biorad TC20 cell counter and were stained for $B220^{+}$ and 7-amino-actinomycin D (7-AAD) (Becton Dickinson, Heidelberg, Germany) and detected on a LSRII (Becton Dickinson, Heidelberg, Germany). To determine cell proliferation, $1x10^{6}$ cells were incubated for 1 hour at 37°C with 10 μM bromodeoxyuridine (BrdU) solution. BrdU and 7-AAD staining was performed according to the BrdU Flow kit manual (Becton Dickinson, Heidelberg, Germany). A total of 1×10^{5} events were collected on a LSR II and the cellular DNA content was analyzed by FlowJo software (TreeStar, Ashland, OR, USA).

For the NR4A1 agonist treatment, EμMyc mouse lymphoma cells were seeded at $1x10^6$/ml and treated with either CsnB, an analogue of CsnB (n-Amyl 2-[3,5-dihydroxy-2-(1-nonanoyl)phenyl]acetate) or cDim-pOCH_3 (all Sigma Life Science, St. Louis, MO) Cell viability and apoptosis assays were performed after 24, 48 and 72h. For apoptosis assays, cells were stained with Annexin V-APC/7-AAD. Briefly, cells were washed and centrifuged in binding buffer (0.1 M HEPES/NaOH (pH 7.4), 1.4 M NaCl, 25 mM $CaCl_2$), and the pellet was resuspended in 5 µL Annexin V-APC , 5 µL 7-AAD and 200 µL binding buffer, followed by incubation for 15 min at room temperature in the dark. Measurement was performed by flow-cytometer using the LSR II Percentage of double negative cells was taken to determine viability.

Flow cytometry immunophenotyping

Tumor cells from Eμ-Myc transgenic mice with and without NR4A1 were isolated by passing through a 70µm mesh and immunophenotype was determined by FACS. Fresh isolated tumor cells were incubated with Mouse Fc-block (Becton Dickinson, Heidelberg, Germany) for 10 min at 4°C. Afterwards, cells were incubated with an antibody mix at 4°C for 20 min. Then cells were centrifuged, washed and resuspended in FACS buffer including 7-AAD. During antibody incubation cells were kept on ice and in the dark. Used antibodies for immunophenotyping analyses are listed in table 3. Flow cytometry was performed on a LSRII and data was analyzed with FlowJo software

Table 4 List of used antibodies for tumor immunophenotyping

Marker	Dye	Company
B220	APC	BD Biosciences
B220	APC-Cy7	BD Biosciences
CD117	BV605	BD Biosciences
CD127	Pe-Cy7	BD Biosciences
CD138	APC	BD Biosciences
CD19	PE	BD Biosciences
CD21	APC	BD Biosciences
CD23	Pe-Cy7	BD Biosciences
CD24	BV421	BD Biosciences
CD24	PerCp-Cy5	BD Biosciences
CD38	APC	BD Biosciences
CD4	Pe-Cy7	BD Biosciences
CD43	APC	BD Biosciences
CD5	PerCp-Cy5	BD Biosciences
CD8	APC	BD Biosciences
CD93	PerCp-Cy5	BD Biosciences
FAS	FITC	BD Biosciences
GR-1	PE	BD Biosciences

IgD	BV605	BD Biosciences
IgM	EF450	BD Biosciences
IgM	FITC	BD Biosciences
Sca-1	FITC	BD Biosciences
TCR	FITC	BD Biosciences
Ter119	BUV395	BD Biosciences

Immunoblotting and antibodies

For western blot, fresh frozen tumor sections from the EμMyc transgenic mice with and without NR4A1 were lysed in RIPA buffer (Thermo Scientific, Waltham, MA) with protease and phosphatase inhibitor cocktail (Thermo Scientific, Waltham, MA). Protein extracts were clarified by centrifugation, resolved by SDS-PAGE using Mini-PROTEAN® TGX™ gels (Bio-Rad Laboratories, Hercules, USA) and transferred to PVDF membranes. Used antibodies can be found in table 4. Rabbit, mouse and rat secondary antibody (Santa Cruz, USA) conjugated to Horseradish peroxidase were used. Peroxidase activity was detected by using WesternBright chemiluminescence detection (Advansta, USA). Membranes were stripped with Restore PLUS Western Blot Stripping Buffer (Thermo Scientific, Waltham, MA) for 30 min by 37°C. Protein concentration was measured using Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, USA). Protein band intensity was quantified using digital image densitometry analysis using the ImageJ software (National Institutes of Health, Bethesda, MD).

Table 5 List of used antibodies for immunoblotting

	Dilution	Company
Beta-Actin	1:1.000	Cell Signaling
p53	1:1.000	Cell Signaling
$p19^{ARF}$	1:500	Santa Cruz
BIM	1:1.000	Cell Signaling
Bcl-xL	1:1.000	Cell Signaling
BCL-2	1:1.000	Cell Signaling
MDM2	1:500	Santa Cruz
MCL-1	1:10.000	Cell Signaling

Statistical methods

All statistical analyses were performed using the Statistical Package for Social Sciences version 17.0 (SPSS Inc., USA) as previously described.(82)

List of primers and cycling conditions used for the genotyping of the mice and additionally plots for the experiments can be found in the supplementary part.

Results

Loss of NR4A1 accelerates Myc driven lymphomagenesis in vivo

To explore whether NR4A1 suppresses tumor formation in oncogene-driven B-cell lymphoma development, mice lacking the NR4A1 protein were crossed with the EμMyc transgenic mice, which develop malignant monoclonal lymphomas (mean latency of 12-16 weeks).(91-93) For comparison, we generated a cohort of EμMyc NR4A1 +/+ mice (n=75) and EμMyc NR4A1 -/- mice (n=46). Cohorts of EμMyc mice with and without NR4A1 loss were monitored until onset of overt disease. EμMyc mice with NR4A1 loss (EμMyc NR4A1 -/-) developed significantly faster visible tumors compared to EμMyc mice without NR4A1 loss (EμMyc°NR4A1 +/+) (median = 44 days for EμMyc NR4A1 -/- vs. 107 days for EμMyc°NR4A1 +/+; $p<0.001$; figure 8A). Additionally, EμMyc NR4A1 -/- mice showed a significantly shorter life span (median survival = 77 days) compared to EμMyc NR4A1 +/+ mice (median survival = 156 days; $p<0.001$, figure 8B). Taken together, these findings demonstrate that NR4A1 possesses tumor suppressing properties. Comparing tumor and spleen weight between the two different mice subgroups, no differences were detected suggesting that the NR4A1 loss has no impact on tumor load.

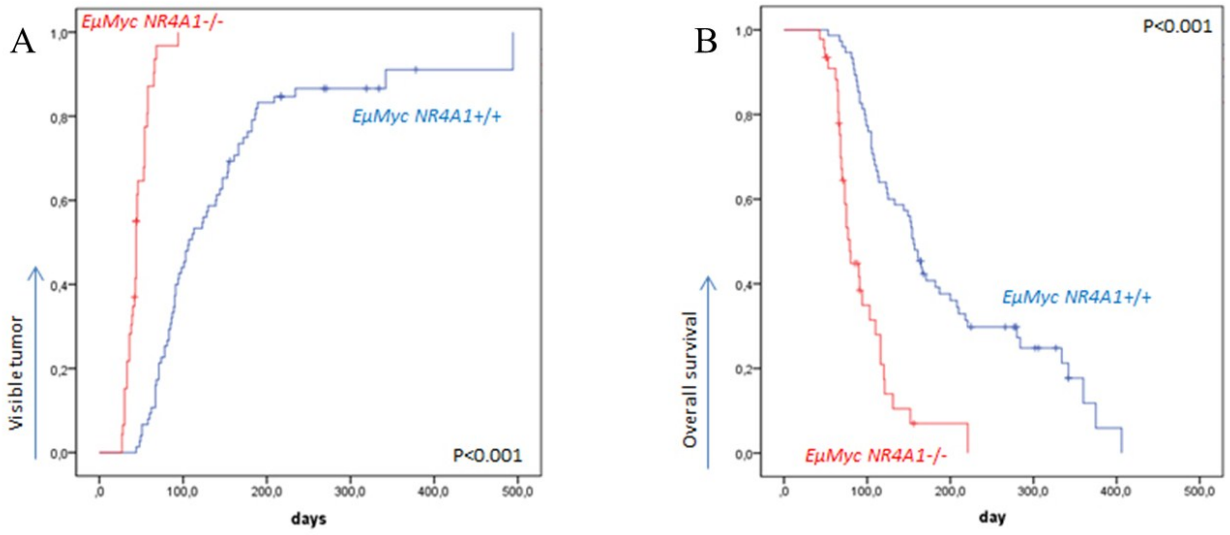

Figure 8 NR4A1 accelerates the development of B-cell lymphoma in EμMyc transgenic mice. A. Lymphoma-free survival of Eμ-myc with or without NR4A1. The red line represents the mice without NR4A1 (EμMyc NR4A1 -/-) and the blue line represents the mice with NR4A1 (EμMyc NR4A1 +/+). B. Overall survival of of EμMyc with or without NR4A1. The red line represents the mice without NR4A1 (EμMyc NR4A1 -/-) and the blue line represents the mice with NR4A1 (EμMyc NR4A1 +/+).

No difference between tumor phenotype of Myc induced lymphomas with or without NR4A1 loss

Tumors developing in EμMyc mice normally have an immature B-cell lymphoma immunophenotype (Pro B-cell-, Pre B-cell- or an immature B-cell lymphoma phenotype).(91-93) Therefore, we isolated tumor cells developed in the EμMyc NR4A1 +/+ mice (n=14) and EμMyc NR4A1 +/+ mice (n=10) and phenoytpically analysed those by flow cytometry (using antibodies against B-cell-, T-cell and myeloid cell markers). All lymphoma cells stained positive for B220, CD19 and CD24. IgM, IgD, CD43, CD93 and Sca-1 were heterogeneously expressed within the two groups (EμMyc NR4A1 +/+ and EμMyc NR4A1 -/-). None of the lymphomas expressed Gr-1, CD127, CD23, CD21, CD117, CD5, TCR, CD4, CD8 and CD138. Furthermore, classifying all analysed lymphomas as pro B-cell ($B220^{+}/IgM^{-}/CD43^{+}$), pre B-cell- ($B220^{+}/IgM^{-}/CD43^{-}$) or immature B-cell- immunophenotype ($B220^{+}/IgM^{+}$), we detected 40% pro B-cell tumors (4 of 10), 30% pre B-cell tumors (3 of 10) and 30% immature

B-cell in EμMyc NR4A1 -/- mice (table 6). In the EμMyc NR4A1 +/+ mice approximately 35.7% (5 of 14) of tumors exhibited the pro B-cell- immunophenotype, 21.5% (3 of 14) the pre B-cell- immunophenotype and 42.3% (6 of 14) the immature B-cell- immunophenotype (table 5). By comparing the two groups, no significant difference in the frequency of different lymphoma immunophenotypes was observed. Interestingly, EμMyc NR4A1 -/- mice showed an increased frequency of strong CD93 expression (5 of 10, respectively, vs. 2 of 14 EμMyc NR4A1 +/+ mice, p=0.066), which is usually expressed in the early stages of B cell development in the bone marrow.(94) Since most of the EμMyc NR4A1 -/- lymphomas that strongly express CD93 were IgM negative (7 of 10), these results indicate that NR4A1 loss leads to a more immature phenotype in Myc driven lymphomagenesis.

Table 6 Phenotypical analysis from tumors with or without NR4A1

		EμMyc NR4A1-/-	EμMyc NR4A1+/+	p-value
Phenotype	**Pro B**	4	5	1.0
	Pre B	3	3	0.665
	B-cell	3	6	0.678

NR4A1 loss impacts on dissemination of EμMyc lymphoma cells

Flow cytometry analysis of bone marrow (BM) and spleens (SPL) of EμMyc NR4A1 -/- (n=10), EμMyc NR4A1 +/+ (n=13) and wild type (wt; n=4) mice was performed. In BM the ratio of Gr-1 positive cells to B220 positive cells was decreased in the group of EμMyc NR4A1 -/- mice compared to EμMyc NR4A1 +/+ (p=0.047) and wt mice (p=0.045) (figure 9A). More than 95% of the B220 positive cells isolated from BM of EμMyc NR4A1 -/- and EμMyc NR4A1 +/+ exhibited the same immunophenotype like the lymphoma. Additionally, in SPL the ratio of Gr-1 positive cells to B220 positive cells was decreased in EμMyc NR4A1 -/- (p=0.027) and EμMyc NR4A1 +/+ (p=0.046) mice (figure 9B) and the ratio of

B220 positive cells to TCR positive cells was increased in EμMyc NR4A1 -/- (p=0.005) and EμMyc NR4A1 +/+ (p=0.013) mice compared to wt mice (Figure 9C). Moreover 95% of the B220 positive cells isolated from SPL of both lymphoma mice types exhibited the same immunophenotype like the primary lymphoma. Combining all findings, our data suggest that in the EμMyc NR4A1 -/- and EμMyc NR4A1 +/+ mice the lymphoma infiltrate BM and SPL. The fact that more lymphoma cells are detectable in BM of EμMyc NR4A1 -/- mice compared to EμMyc NR4A1 +/+ mice suggests that loss of NR4A1 increases the dissemination potential of EμMyc lymphoma cells.

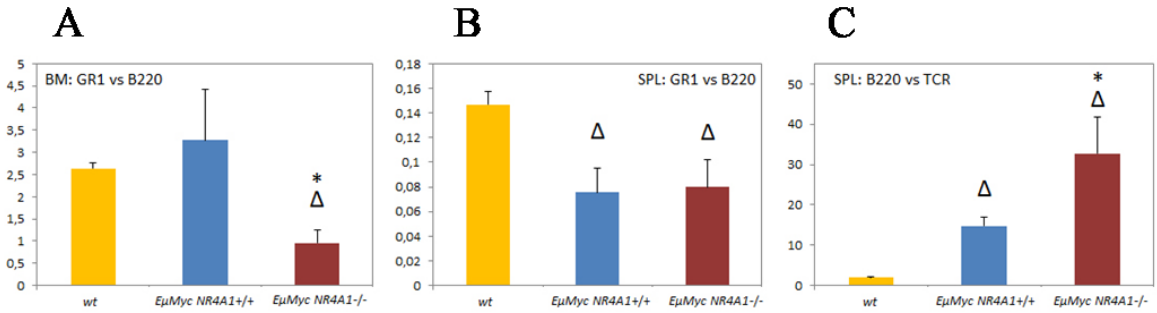

Figure 9 Loss of NR4A1 has an impact on cell ration in bone marrow and spleen. A. Ratio of $GR1^+$ cells to $B220^+$ cells in the bone marrow. B. Ratio of $GR1^+$ cells to $B220^+$ cells in the spleen. C. Ratio of B220+ cells to TCR cell in the spleen. Data represent mean ± standard deviation. *p < 0,05 to to EμMyc NR4A1 +/+, Δ p< 0,05 to wt mice.

NR4A1-/- lymphoma cells exhibit a higher proliferation rate

To further investigate the impact of NR4A1 loss on the oncogenic potential of EμMyc lymphoma cells we isolated viable tumor cells ($B220^+$ and $7AAD^-$) from EμMyc NR4A1 -/- (n=5) and EμMyc NR4A1+/+ (n=8) mouse tumors, cultured them for 72h with or without LPS stimulation and determined the number of viable cells and the viability (B220 and 7AAD-staining by flow cytometry analysis) each day. As expected, around 65% of B220 positive cells stained positive for 7AAD after 24h, 79% after 48h and 82% after 72h and the number of viable cells decreased through culturing B220 positive tumor cells isolated from EμMyc

NR4A1+/+ mouse tumors (Figure 10). The percentage of 7-AAD positive of the B220 positive tumor cells isolated from EμMyc NR4A1-/- mice was around 56% after 24h of culturing, 58% after 48h and 52% after 72h (Figure 10). Interestingly, the number of viable cells increased and was significantly higher after 72h compared to cultured B220 positive tumor cells isolated from EμMyc NR4A1+/+ mouse tumors ($2.05*10^6$/ml vs $1.5*10^5$/ml, p=0.052; figure 10B&C). We performed a BrdU assay to gain insight into the proliferation rate of the B220 positive tumor cells either isolated from EμMyc NR4A1-/- or EμMyc NR4A1+/+ mouse tumors after 72h of culturing time. B220 positive tumor cells isolated from EμMyc NR4A1-/- mouse tumors exhibited a significant higher percentage of BrdU positive cells compared to EμMyc NR4A1+/+ (19.28% vs 4.28%, p=0.038; figure 10C). LPS stimulation caused an increase of BrdU incorporation in $B220^+$ tumor cells isolated from both type of mouse lymphomas, however, the percentage of BrdU positive cells was higher in B220 positive tumor cells isolated from EμMyc NR4A1 -/- mouse tumors compared to EμMyc NR4A1 +/+ (32.8% vs 15.2%, p=0.064, Figure 10C). Furthermore, the number of viable cells was significantly higher after culturing $B220^+$ isolated from EμMyc NR4A1 -/- mouse tumors compared to EμMyc NR4A1 +/+ mice ($4.18*10^6$/ml vs $9.4*10^5$/ml, p=0.056; figure 10C). These data indicate that loss of NR4A1 accelerates the proliferation of tumor cells *in vitro*.

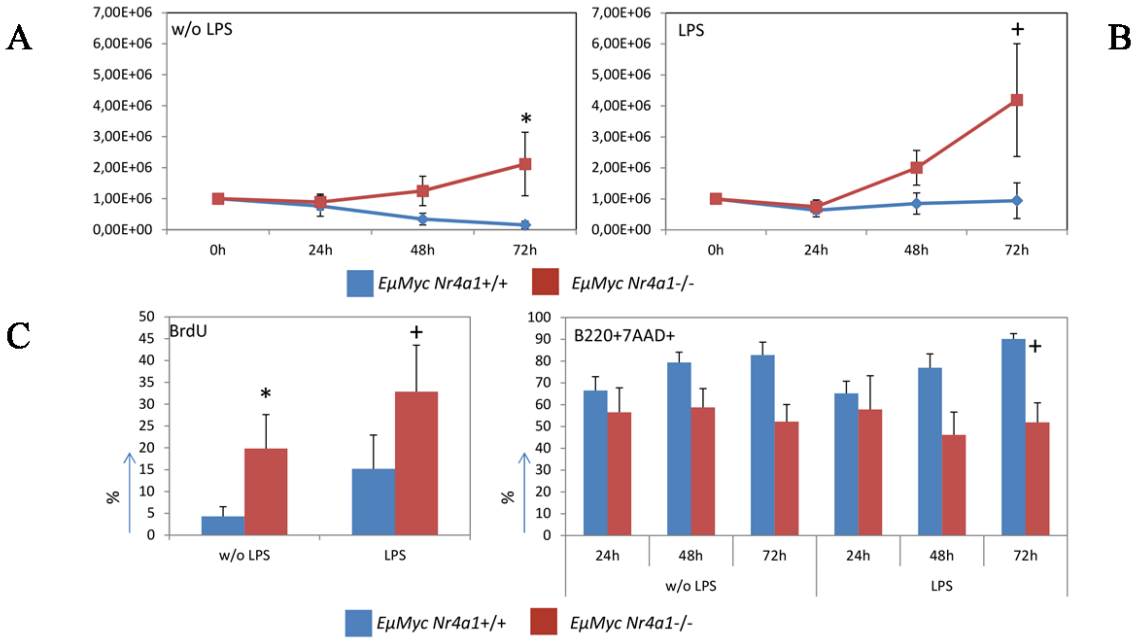

Figure 10 Loss of NR4A1 results in a higher viability and proliferation rate of B200$^+$ cells. A B220$^+$ cells treated without LPS. B. B220$^+$ cells treated with 10µg/ml LPS. C. Apotose assay and BRdU incoperation messerud with FACS after treatment with and without 10 mg/ml LPS. *p < 0,05 without LPS treatment, + p< 0,05 with LPS treatment.

Tumors with NR4A1 loss show a higher expression of NR4A3

To explore if there is a difference in the mRNA expression level of NR4A1, NR4A3 and Myc in the different mouse strains, we performed qPCR analysis on lymphomas of EμMycNR4A1+/+ (n=14) and EμMyc NR4A1 -/- (n=12) mice. As expected, there was a significant downregulation of NR4A1 in the mice with NR4A1 loss (figure 11, p<0,05). Furthermore, we could detect a significant upregulation of NR4A3 in mouse tumors from mice with NR4A1 loss (figure 11, p<0,05). We could not detect any differences regarding the Myc expression between the two mice strains (figure 11). These data indicate that NR4A3 overexpression is a result of the NR4A1 loss in EμMyc NR4A1 -/- mice since it is speculated that NR4A1 and NR4A3 have a genetic redundancy.

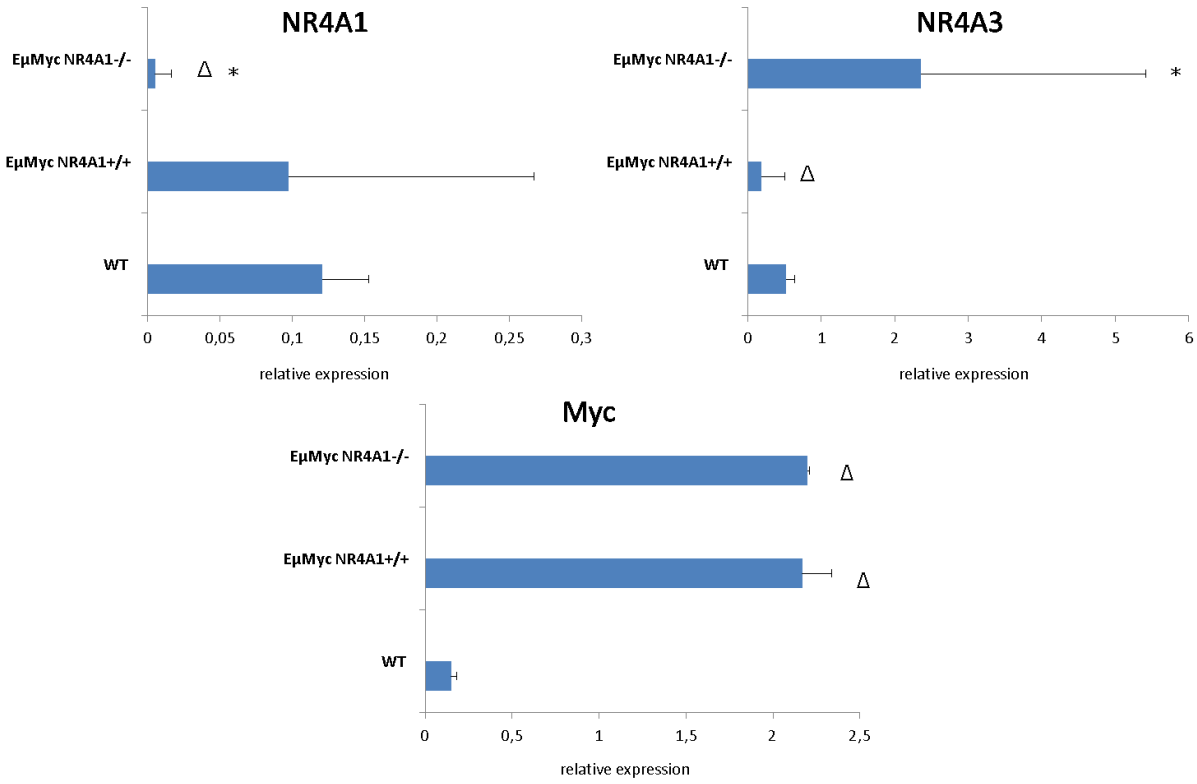

Figure 11 Relative expression of NR4A1 and NR4A3 in Myc induced lymphomas and wild type B220+ postive cells. Bars represent mean and standard deviation. *p < 0,05 to EμMyc NR4A1 +/+, Δ p< 0,05 to wt mice.

Overexpression of MDM2 and Bcl-xL in EμMyc NR4A-/- tumors

It is known that at least 80% of the emerged lymphomas in EμMyc mice have an inactivated Arf-Mdm2-p53 pathway.(95) Furthermore, it has been shown that the antiapototic BCL2 family proteins are overexpressed in EμMyc mice.(96, 97) To evaluate the effect of NR4A1 loss in Myc induced lymphomas regarding the status of $p19^{ARF}$, MDM2, p53 expression as well as the expression of pro (Bim) and anti-apoptotic (BCL2, Bcl-xL and MCL-1) members of BCL2 family we performed western blot analyses. Comparing lymphomas of the EμMyc NR4A1+/+ (n=7) to EμMyc NR4A1-/- (n=12) mice, we could detect a significant upregulation of MDM2 (p=0,030) and Bcl-xL (p=0,001) proteins in tumors developed in EμMyc mice with NR4A1 (figure 12) whereas no difference in the protein levels of p53, p19ARF, MCL-1, Bim and BCL2 was found.

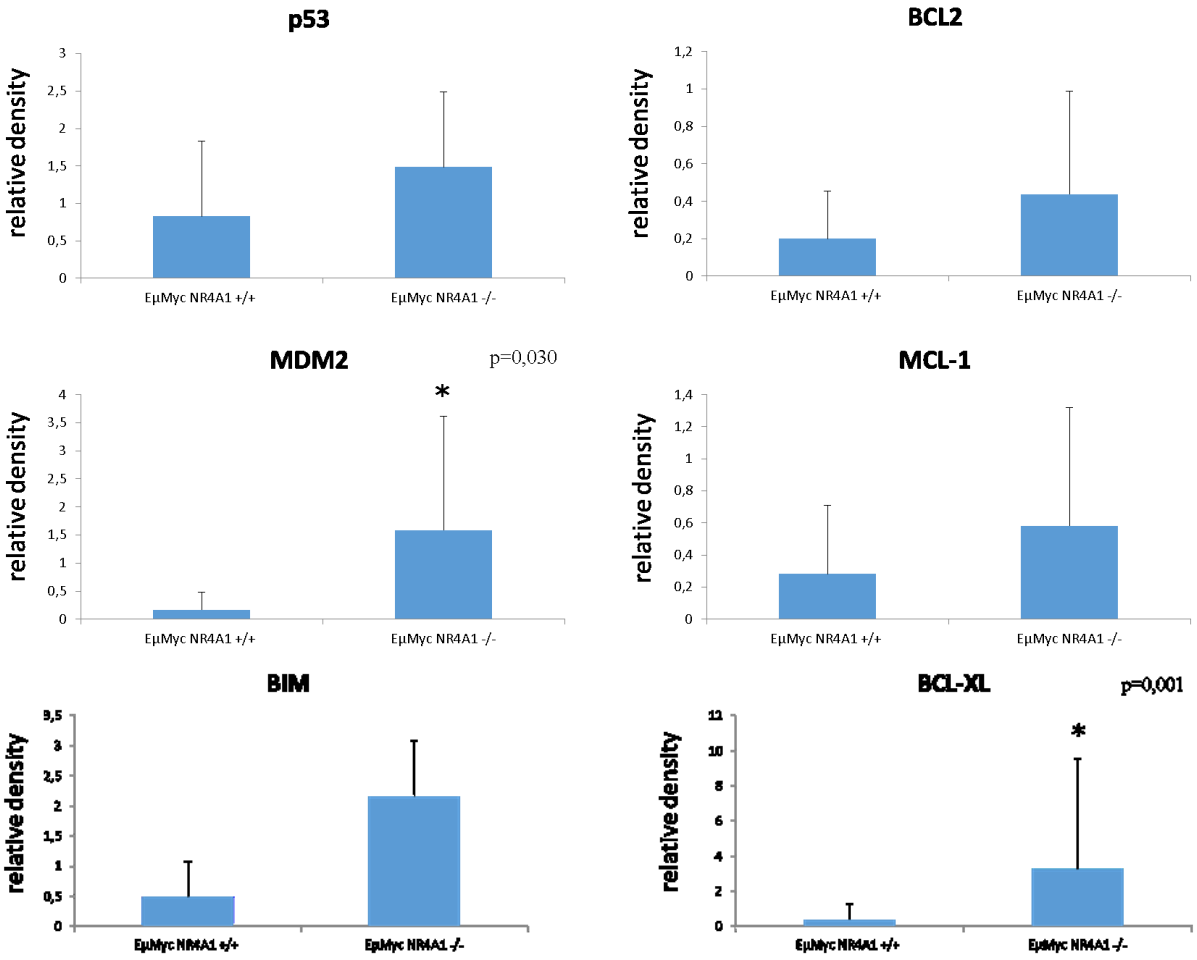

Figure 12 Relative density plots from tumors with or without NR4A1. Status of protein levels from tumors with or without NR4A1 loss. Density was calculated using the ImageJ software and row data was normalized to loading control. Bars represent mean and standard deviation. *p < 0,05 to EμMyc NR4A1 +/+.

NR4A1 agonists and their derivatives induce cell death in vitro

To further investigate NR4A1 and its role in lymphomagenesis we generated primary cell lines from EμMyc NR4A+/+ mice and treated them with NR4A1 agonists. As non-physiologic agonists CsnB and cDims (all binding the ligand-binding domain of NR4A1) increase the pro-apoptotic and/or tumor suppressive properties of NR4A1, which suggests that NR4A1 could serve as potential target for lymphoma therapy,(54, 77) In our first attempts we treated aggressive lymphoma cell lines with CsnB and were able to induce a NR4A1-dependent apoptosis.(64) EμMyc NR4A1+/+ lymphoma cell lines generated from the primary lymphoma were treated with either 75μM CsnB, 50μM CsnB-analog or 50μMl cDim-pOCH3. Treatment with NR4A1 agonists resulted in a reduction of primary lymphoma cell viability. The highest reduction of cell viability was observed in lymphoma cells treated with CsnB. the cytotoxic effects of cDim-pOCH3 and CsnB-analog were lower compared to CsnB (Figure 13).

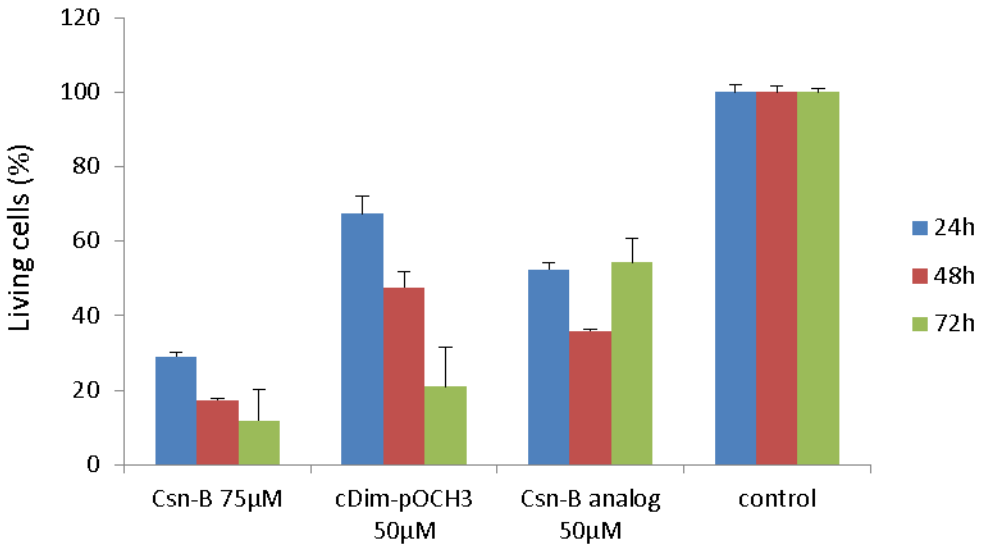

Figure 13 Percentage of Annexin V and 7AAD negative cells (living cells) estimated by FACS analysis. Primary EμMyc lymphoma cell lines were treated with different NR4A1 inducing agents. Bars represent mean and standard deviation from at least two independent experiments.

Discussion

In a previous study we showed that NR4A1 and NR4A3 are significantly down regulated in aggressive B-cell lymphomas and that overexpression of NR4A1 in lymphoma cell lines resulted in apoptosis.(64) Although, there is growing evidence that NR4A1 plays an important role in the development of lymphoid malignancies (77), studies investigating this topic and especially *in vivo* investigations of *NR4A1* loss in combination with other genetic alteration causing lymphomas are rare. Therefore, we crossed NR4A1 -/- mouse with transgenic mice expressing the c-myc oncogene under control of the Eμ heavy chain enhancer –so called EμMyc NR4A1-/- mouse- and compared it with the effects observed in the EμMyc NR4A1+/+ mouse. The EμMyc transgenic mouse model is a well established model in order to study genetic alterations which could contribute to tumor development (91, 93, 95, 98, 99). EμMyc NR4A1-/- mice showed a significantly accelerated tumor development and a shorter life span compared to EμMyc NR4A1+/+ mice indicating that NR4A1 functions as tumor suppressor in Myc driven lymphomagenesis.

The EμMyc transgenic mouse develops aggressive immature B-cell lymphoma (Pro B-cell-, Pre B-cell- or an immature B-cell lymphoma).(91-93) Additionally, studies have shown that EμMyc transgenic mice lacking different BH3 only proteins favor the immature pre B-cell or IgM^+ B-cell lymphoma phenotype.(100, 101) However, we detected no significant differences in the frequency of different lymphoma immunophenotypes comparing EμMyc NR4A1 +/+ to EμMyc NR4A1 -/- tumors indicating that NR4A1 loss has an impact on the phenotype of the lymphoma. As we detected more lymphoma cells in bone marrow and spleen specimens of EμMyc NR4A1 -/- mice compared to EμMyc NR4A1 +/+ mice, and the fact that NR4A1 down-regulation is associated with metastasis in various solid cancers, our data suggest that NR4A1 has an impact on the dissemination process of various malignancies.(102).

Our *in vitro* experiments demonstrated that $B220^+$ EμMyc NR4A1 -/- lymphoma cells have and a better *in vitro* survival caused by a significant higher proliferation rate compared to EμMyc NR4A1 +/+ lymphoma cells. Hence, these data as well as the role of NR4A1 in T cell apoptosis and negative selection indicate that loss of NR4A1 positively influences *in vitro* survival. (103, 104)

NR4A1 and NR4A3 possess functional redundancy at least in T-cell apoptosis.(105) We could detect an upregulation of NR4A3 in tumors of the EμMyc NR4A -/- mice. This finding led to the suggestion that over-expression of NR4A3 is trying to compensate the NR4A1 loss but it is not able to suppress accelerated Myc-driven lymphomagenesis.

In EμMyc transgenic animals, lymphoma formation requires additional genetic alterations, which frequently comprise loss of p53 or overexpression of BCL-2.(96). Our Western blot analysis from EμMyc NR4A1 -/- and EμMyc NR4A1 +/+ lymphomas revealed that NR4A1 loss increased the protein levels of MDM2 and Bcl-xL. MDM2, a member of the Arf-Mdm2-p53 pathway is a known oncogene and interacts with p53.(106) It has been reported that NR4A1 represses the expression of MDM2 at both transcriptional and post-transcriptional level.(107, 108) Since p53 expression levels were equally in Myc driven tumors with and without NR4A1 loss it might be presumed that MDM2 contributes to Myc driven lymphomagenesis in a p53-independent way. To the best of our knowledge, repression of the antiapoptotic/oncogenic Bcl-xL by NR4A1 has never been described before. Taken together our data, we suggest that NR4A1 is a repressor of the known oncogenes MDM2 and Bcl-xL.(109, 110) Thus, their over-expression caused by NR4A1 loss significantly augments Myc driven lymphomagenesis.

Different studies have already shown that agonists directly binding NR4A1 induce apoptosis *in vitro*. (64, 77, 111) We could demonstrate that treating primary lymphoma cell lines with different NR4A1 agonists reduces cell viability, demonstrating the potential of NR4A1 as a promising novel target for the development of new therapeutic drugs. However, further investigation especially *in vivo* testing like application of the NR4A1 agonist in EμMyc NR4A1+/+ transgenic mice are needed to demonstrate that NR4A1 is suitable as drug target.

Outlook

In this study, we investigated the role of NR4A1 in an *in vivo* lymphoma mouse model for the first time. We could show that NR4A1 loss accelerates lymphomagenesis in Myc induced lymphomas. Furthermore, this study revealed that the loss of NR4A1 might have a significant impact regarding dissemination of tumor cells.

Taken all of our findings together, our data indicates that NR4A1 functions as a tumor suppressor of Myc driven lymphomagenesis. However, there is still some work left in order to fully elucidate the function of NR4A1:

First, comparing gene expressions profiles of EμMyc NR4A1 -/- to EμMyc NR4A1 +/+ - lymphomas to determine different alterations in both genetic pathways. Additionally, genes need to be validated by qPCR and Western Blot analysis. These data can provide valuable insight on how NR4A1 is regulated and about NR4A1 targets genes.

Second, to demonstrate the potential of NR4A1 as drug target more *in vivo* experiments like application of NR4A1 in EμMyc NR4A1 +/+ mice will be performed as already mentioned above.

Supplementary

Table 7 Primer sequences for the mouse genotyping

Mouse Strain	Primer Sequence	Primer Type
B6.Cg-Tg(IghMyc)22Bri/J	TTA GAC GTC AGG TGG CAC TT	Transgene Forward
	TGA GCA AAA ACA GGA AGG CA	Transgene Reverse
	CTA GGC CAC AGA ATT GAA AGA TCT	Internal Positive Control Forward
	GTA GGT GGA AAT TCT AGC ATC ATC C	Internal Positive Control Reverse
B6;129S2-Nr4a1tm1Jmi/J	CAC GAG ACT AGT GAG ACG TG	Mutant
	CCA CGT CTT CTT CCT CAT CC	Common
	TGA GCA GGG ACT GCC ATA GT	Wild type Reverse

Master Mix for genotyping

NR4A1 master mix:

6µl KAPA2G Fast Hot Start Genotyping Mix

1,2µl Common primer [10pmol/µl]

1,8µl Wild type Reverse primer [10pmol/µl]

1,8µl Mutant primer [10pmol/µl]

2µl DNA

Myc internal control master mix:

6µl KAPA2G Fast Hot Start Genotyping Mix

0,6µl Internal Positive Control Forward [10pmol/µl]

0,6µl Internal Positive Control Reverse [10pmol/µl]

2,8µl H_2O

2µl DNA

Myc transgene master mix:

6µl KAPA2G Fast Hot Start Genotyping Mix

0,6µl Transgene Forward [10pmol/µl]

0,6µl Transgene Reverse [10pmol/µl]

2,8µl H_2O

2µl DNA

Table 8 Genotyping program for NR4A1 mice

Thermal cycling conditions		
Stage	**Temperatur [°C]**	**Time [min]**
Hold	94	10
Cycle [35 Cycles]	94	0,15
	62	0,15
	72	0,15
Hold	72	2
Hold	4	∞

Table 9 Genotyping program for Myc internal control

Thermal cycling conditions		
Stage	**Temperatur [°C]**	**Time [min]**
Hold	94	10
Cycle [10 Cycles] -0,5°C per Cycle	94	0,2
	65	0,15
	68	0,1
Cycle [28 Cycles]	94	0,15
	60	0,15
	72	0,1
Hold	72	2
Hold	4	∞

Table 10 Genotyping program for Myc transgene

Thermal cycling conditions		
Stage	Temperatur [°C]	Time [min]
Hold	94	10
Cycle [10 Cycles]	94	0,15
	72	0,15
	72	0,15
Cycle [28 Cycles]	94	0,15
	65	0,15
	72	0,15
Hold	72	1
Hold	4	∞

qPCR Master Mix

Master mix for all genes:

5 µl Kapa Probe FAST ABI Prism
1 µl ready to use Primer mix form
4 µl cDNA

Table 11 Program for qPCR

Thermal cycling conditions		
Stage	**Temperatur [°C]**	**Time [min]**
Hold	95	1
Cycle [40 Cycles]	95	0,02
	60	0,2

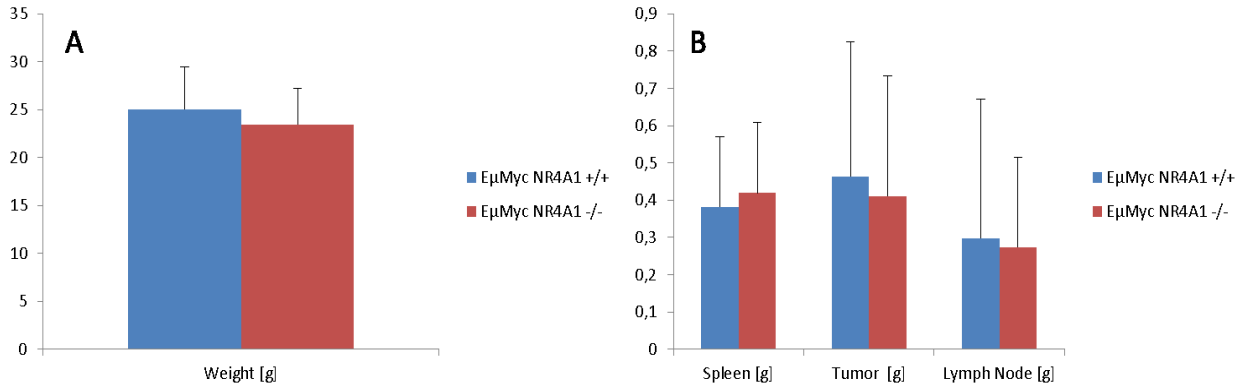

Figure 14 NR4A1 loss in EμMyc mice does not affect weight or tumor load. A. No difference regarding weight between mice with or without NR4A1. B. NR4A1 loss does not affect tumor load or spleen and lymph node weight between both mice strains.

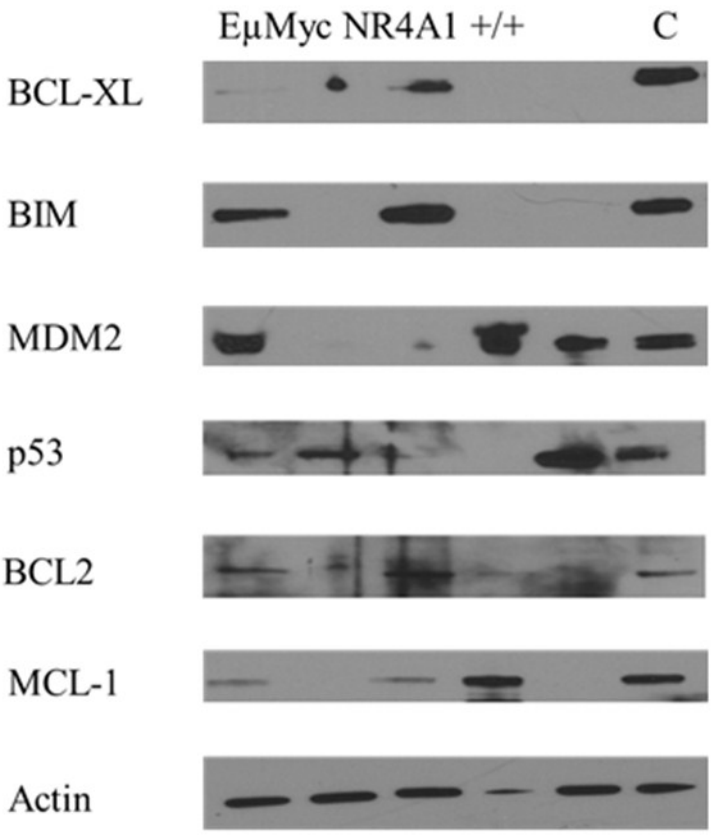

Figure 15 Immunoblotting analysis of EμMyc NR4A1 +/+ tumors. Representative blots for Bcl-xL, Bim, MDM2 p53 BCL2, P19Arf and MCL-1 protein expression. Actin was used as a loading control on all blots. As antibody control (C) a wildtype mouse spleen was used.

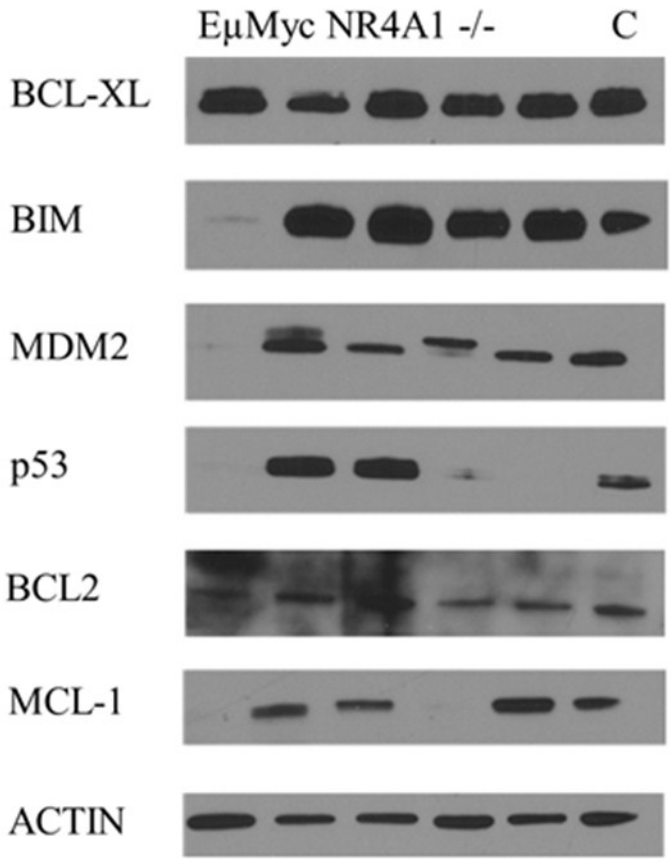

Figure 16 Immunoblotting analysis of EμMyc NR4A1 -/- tumors. Representative blots for Bcl-xL, Bim, MDM2 p53 BCL2, $P19^{Arf}$ and MCL-1 protein expression. Actin was used as a loading control on all blots. As antibody control (C) a wildtype mouse spleen was used.

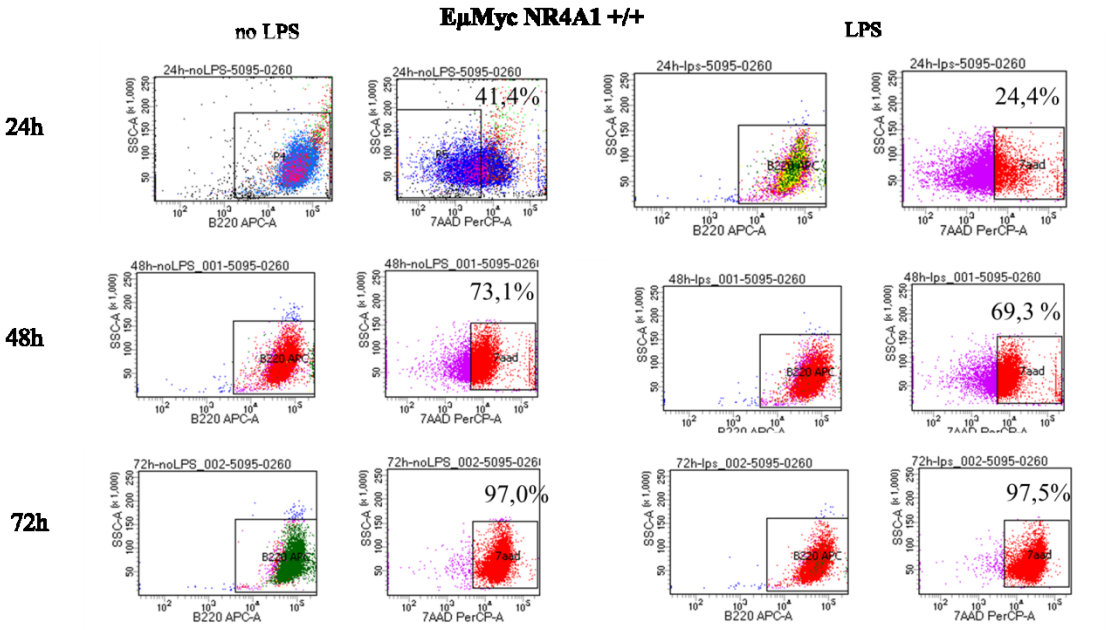

Figure 17 Representative FACS analysis for the viability study of B220$^+$ EμMyc NR4A1 +/+ lymphoma cells treated with or without LPS. Viable cells were defined as B220$^+$ and 7-AAD negative cells. Plots represent B220$^+$ and 7-AAD negative cells.

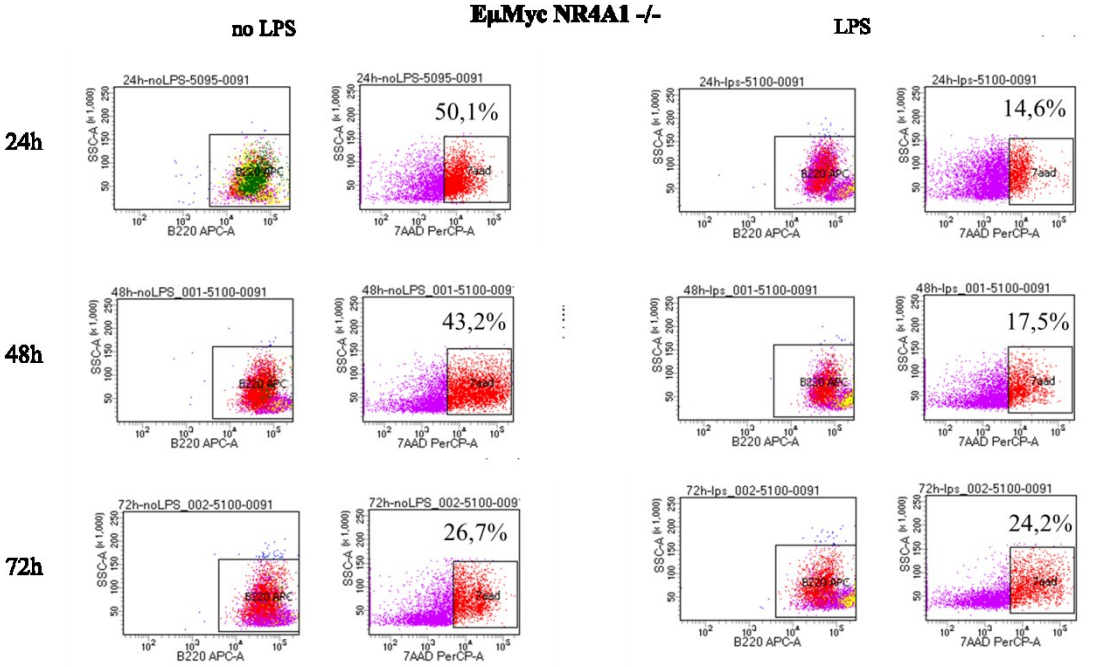

Figure 18 Representative FACS analysis for the viability study of B220$^+$ EμMyc NR4A1 -/- lymphoma cells treated with or without LPS. Viable cells were defined as B220$^+$ and 7-AAD negative cells. Plots represent B220$^+$ and 7-AAD negative cells.

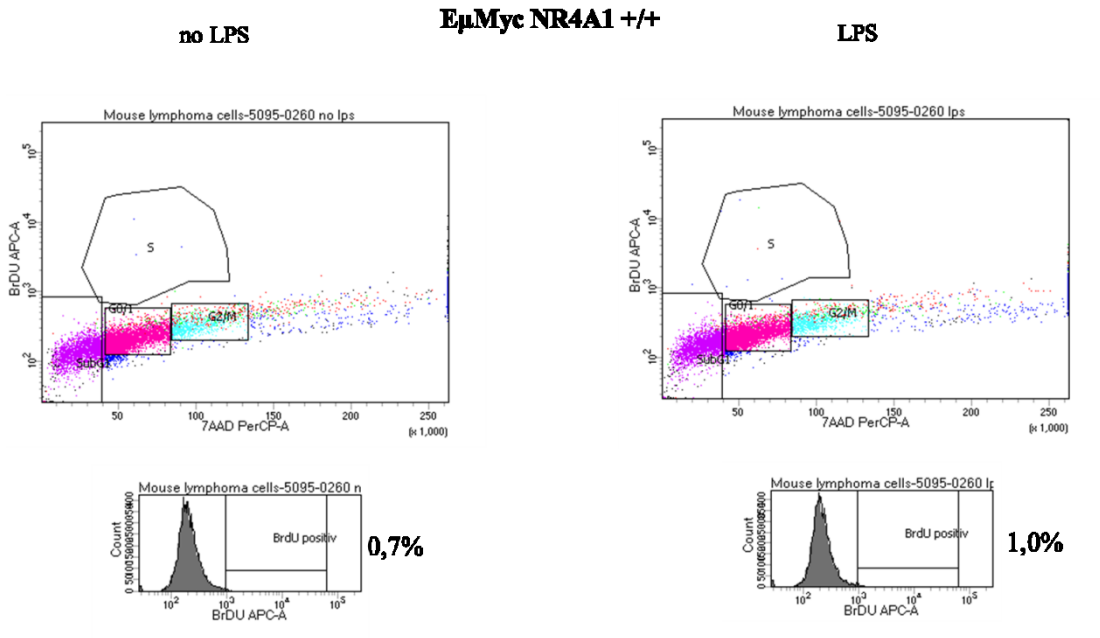

Figure 19 Representative plot for cell proliferation of B220^{+} EμMyc NR4A1 +/+ lymphoma cells after 72h. Cell proliferation was determined by BrdU incorporation.

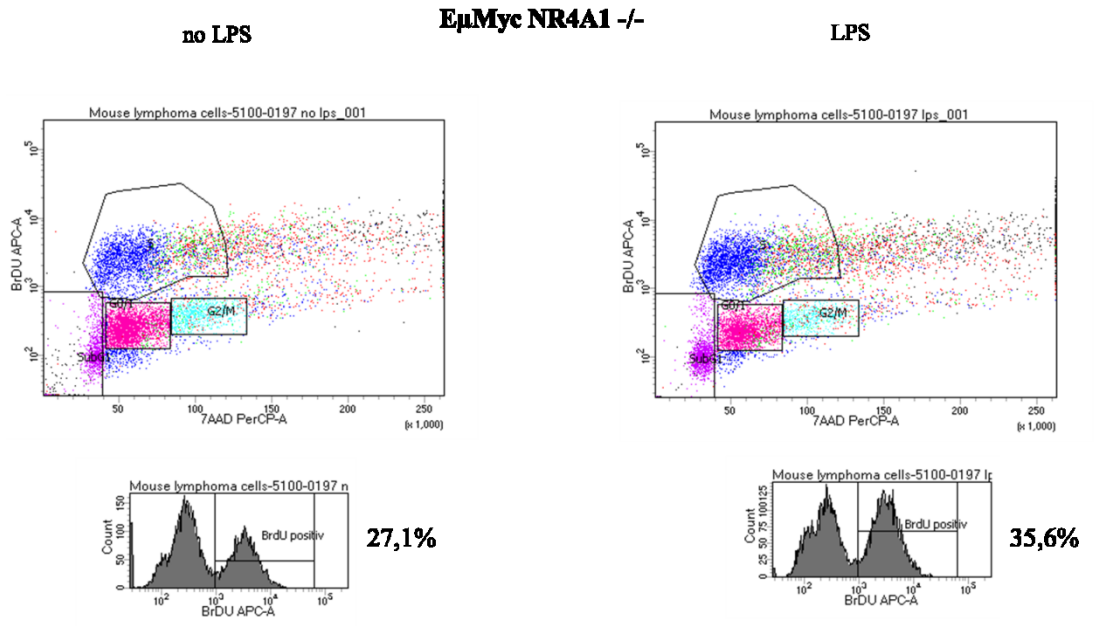

Figure 20 Representative plot for cell proliferation of B220^{+} EμMyc NR4A1 -/- lymphoma cells after 72h. Cell proliferation was determined by BrdU incorporation.

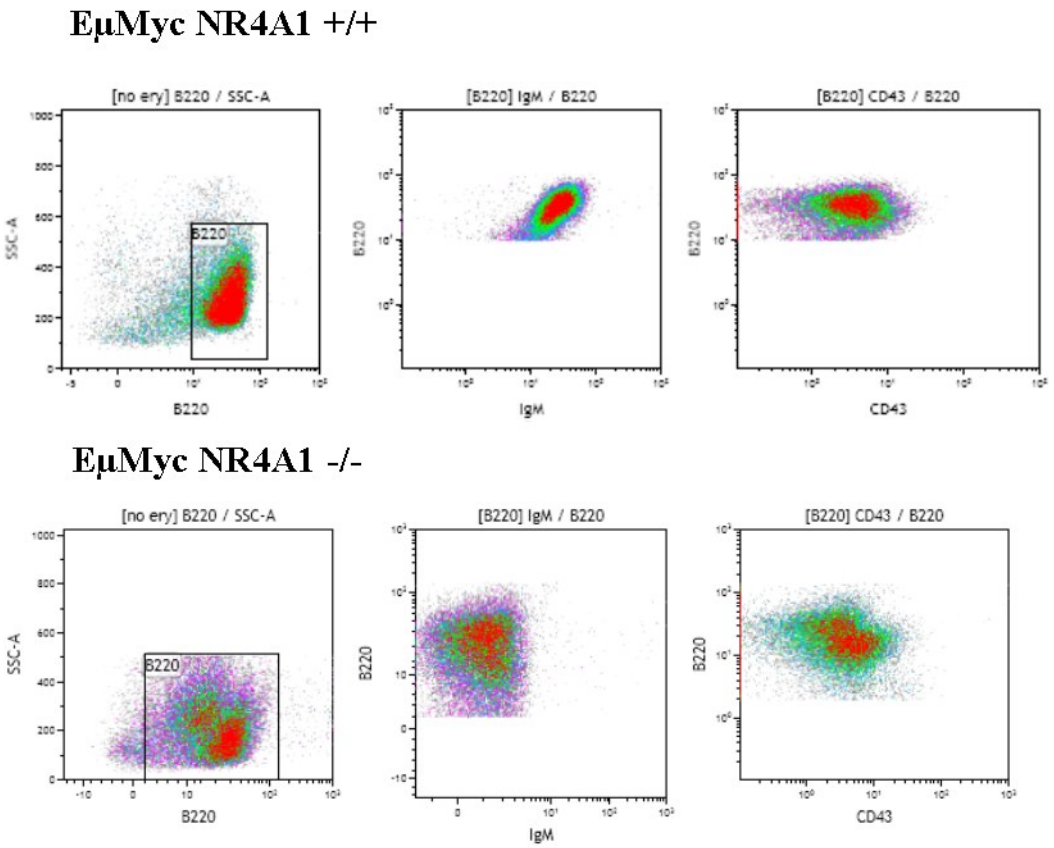

Figure 21 Representative FACS plots for the immunophenotyping of the EμMycNR4A1 +/+ and. EμMycNR4A1 -/- lymphoma. Lymphomas were defined as pro, pre or B-cell lymphoma regarding their expression of B220, IgM and CD43.

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