Regulation of the BH3-only protein NOXA in mantle cell lymphoma: relevance for novel treatment concepts

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Hiermit erkläre ich, dass ich die vorliegende Dissertation selbstständig und unter ausschließlicher Verwendung der angegebenen Hilfsmittel und der Ratschläge von jeweils namentlich aufgeführten Personen angefertigt habe.

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Contents

List of abbreviations

3'-UTR	3' untranslated region
ALCL	anaplastic large cell lymphoma
ATL	adult T-cell leukemia
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
APS	ammonium persulfate
BCR	B cell receptor
BH3	Bcl-2 homology domain 3
BSA	bovine serum albumin
°C	degree Celsius
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CLL	chronic lymphoid leukemia
CML	chronic myelogenous leukemia
CRL	Cullin-containing RING finger ligases
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DUB	deubiquitinating enzyme
EDTA	ethylenediaminetetraacetic acid
e.g.	*"exempli gratia"*, for example
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FACS	fluorescence-activated cell sorter
FA	fatty acid
FASN	fatty acid synthase
FBS	fetal bovine serum

FITC	fluorescein isothiocyanate
g	gravitational acceleration
h	hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	horse radish peroxidase
Ig	immunoglobulin
IGH	immunoglobulin heavy chain complex
kDa	kilo Dalton
µF	microfarad
µg	microgram
µl	microliter
µM	micromolar
M	molar
mA	milliampere
MCL	mantle cell lymphoma
mg	milligram
min	minute
miRNA	micro ribonucleic acid
ml	milliliter
MOMP	mitochondrial outer membrane permeabilization
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
ng	nanogram
nm	nanometer
nM	nanomolar
OMM	outer mitochondrial membrane
PBS	phosphate-buffered saline
PCD	programmed cell death
PCR	polymerase chain reaction

PI	propidium iodide
PI3K	phoshatidyl-inositol-3 kinase
PKC	protein kinase C
PPW	pentose phosphate pathway
qPCR	quantitative real time polymerase chain reaction
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	second
siRNA	small interfering ribonucleic acid
$T_{½}$	half-life
TAG	triacylgyceride
TBST	TRIS-buffered saline with Tween 20
TCA	tricarboxylic acid
TCR	T cell receptor
TEMED	tetramethylethylenediamine
TGCT	testicular germ cell tumor
TRIS	tris(hydroxymethyl)aminomethane
UPS	ubiquitin-proteasome system
V	volt
v/v	volume/volume
W	watt
w/v	weight/volume

Summary

Mantle cell lymphoma (MCL) is an aggressive form of non-Hodgkin lymphoma and characterized by only transient responses to chemotherapy and relatively short survival. Extensive research is ongoing to improve current therapy and identify novel targets for treatment of this B cell malignancy. The central role of BH3-only proteins in mediating the response of tumor cells to anticancer drugs has been shown by many studies. The present work aimed at the investigation of the role of the BH3-only protein NOXA in the decision between life and death in MCL. Astonishingly, basal *NOXA* mRNA and NOXA protein expression levels were found in this study to be extremely discrepant in MCL cell lines as well as primary cells from MCL patients. The malignant B cells express very high constitutive levels of *NOXA* mRNA. In strong contrast, NOXA protein levels are very low in MCL cell lines and even hardly detectable in primary MCL cells. It could be demonstrated in this study that chronic active B-cell receptor (BCR)-signaling and Cyclin D1 overexpression, the hallmark of MCL, contribute to the maintenance of the high constitutive transcript levels of the pro-apoptotic Bcl-2 family member. Furthermore, the phoshatidyl-inositol-3 kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway was determined as the major mediator responsible for the high *NOXA* mRNA expression downstream of the BCR and surprisingly also Cyclin D1. Overexpression of the cell cycle regulatory protein Cyclin D1 contributes to enhanced *NOXA* transcription by exerting a positive feedback loop on the PI3K/AKT/mTOR signaling.

Increased expression of the pro-apoptotic Bcl-2 family member *NOXA* is generally implicated in induction of cell death. Intriguingly, the high basal transcript levels of the BH3-only protein do not impair viability of MCL cells. The malignant B cells adapt to this permanent pro-apoptotic signal by extensive polyubiquitination and rapid proteasome-mediated degradation of NOXA ($T_{½} < 30$ min). Importantly, the resulting phenotype of high *NOXA* mRNA and low NOXA protein expression appears to constitute a druggable Achilles heel of MCL. Exposure of the cells to the proteasome inhibitor Bortezomib accumulates NOXA protein and efficiently induces apoptosis. In addition to Bortezomib, the neddylation inhibitor MLN4924 and the fatty acid synthase inhibitor Orlistat were identified in the present study as effective inducers of NOXA protein and NOXA-dependent apoptosis in MCL. Such as Bortezomib, both inhibitors target rapid NOXA protein turnover thereby stabilizing the pre-existing pool of the pro-apoptotic protein. Interestingly, in contrast to the proteasome

inhibitor, MLN4924 as well as Orlistat interfere with ubiquitination of NOXA and stabilize the BH3-only protein in a proteasome independent manner. Therefore, these substances not only represent alternatives to Bortezomib treatment but may also offer new approaches to treat Bortezomib resistant MCL cells. Indeed, results from the present work demonstrate that Bortezomib resistant MCL cells were still sensitive to Orlistat and MLN4924.

Remarkably, active PI3K/AKT/mTOR signaling is needed for effective accumulation of NOXA and induction of cell death upon treatment with Bortezomib, Orlistat or MLN4924 indicating that the high constitutive *NOXA* mRNA levels are essential for the sensitivity of MCL cells to inhibitors targeting NOXA turnover.

Especially fatty acid metabolism appears to represent a very promising target for treatment of MCL. Constitutive Cyclin D1 overexpression in MCL was found to have an inhibitory effect on cellular metabolism thereby rendering MCL cells susceptible to drugs targeting the fatty acid synthase such as Orlistat. Furthermore, inhibition of fatty acid metabolism may not only constitute a new and selective strategy to kill MCL cells but also sensitize the malignant B cells to other anticancer drugs.

In summary, the present study highlights a critical role of the BH3-only protein NOXA in MCL and provides evidence that the regulation of the pro-apoptotic protein might represent an Achilles heel of this aggressive tumor. Targeting the phenotype of high *NOXA* mRNA/low NOXA protein expression by inhibitors inferring with different steps of the ubiquitin-proteasome system may provide a novel therapeutic approach to kill MCL cells and improve clinical outcome of this lymphoid neoplasm.

Zusammenfassung

Das Mantelzell-Lymphom (MCL) zählt zu den Non-Hodgkin Lymphomen und ist gekennzeichnet durch einen aggressiven klinischen Verlauf. MCL-Patienten sprechen häufig nur kurzeitig auf konventionelle Chemotherapie an und haben eine sehr schlechte klinische Prognose. Um eine effizientere Therapie zu ermöglichen und neue Behandlungsansätze zu identifizieren werden verstärkt Forschungsbemühungen unternommen. Die zentrale Rolle der „BH3-only"-Proteine für das Ansprechen von Tumorzellen auf Chemotherapeutika wurde in verschiedenen Arbeiten gezeigt. Ziel der vorliegenden Arbeit war es, die Rolle des „BH3-only"-Proteins NOXA bei der Entscheidung zwischen Überleben und Tod im Mantelzell-Lymphom zu erforschen. Im Rahmen dieser Arbeit wurde eine extreme Diskrepanz zwischen der basalen Expression an *NOXA*-mRNA und NOXA-Protein in MCL-Zelllinien sowie in primären Zellen von MCL-Pateinten beobachtet. Die malignen B-Zellen sind gekennzeichnet durch eine sehr hohe Expression an *NOXA*-mRNA. Im Gegensatz dazu sind die basalen NOXA-Proteinlevel nur in geringem Maße in den MCL-Zelllinien bzw. kaum in primären MCL-Zellen nachzuweisen. Es konnte gezeigt werden, dass sowohl die chronische Aktivierung des B-Zell-Rezeptor (BCR)-Signalwegs sowie die für das MCL charakteristische Überexpression an Cyclin D1 zum Erhalt der konstitutiv hohen mRNA-Expression des pro-apoptotischen Bcl-2-Familienmitglieds beitragen. Darüber hinaus wurde der Phoshatidyl-inositol-3 Kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR)-Signalweg als wichtigster dem BCR nachgeschalteten Signalweg, der für den hohen Level an *NOXA*-Transkript in den MCL-Zellen verantwortlich ist, identifiziert. Die Überexpression von Cyclin D1 scheint überraschenderweise durch eine positive Rückkopplungsschleife auf den PI3K/AKT/mTOR-Signalweg zu der hohen *NOXA*-mRNA-Expression beizutragen.

Eine erhöhte Expression von *NOXA* wird gewöhnlich mit der Induktion von Zelltod in Verbindung gebracht. Erstaunlich ist, dass die erhöhte Transkription von *NOXA* keinen negativen Einfluss auf die Viabilität der MCL-Zellen zu haben scheint. Die malignen B-Zellen passen sich durch extensive Polyubiquitinierung und verstärkten proteasomalen Abbau von NOXA-Protein an das konstitutive pro-apoptotische Signal an ($T_{½} < 30$ min). Dieser Phänotyp einer hohen *NOXA*-mRNA-Expression und einem geringen NOXA-Proteinlevel scheint eine durch Medikamente angreifbare Achilles-Sehne des MCLs darzustellen. Setzt man die Zellen dem Proteasomeninhibitor Bortezomib aus, führt dies zu einer raschen und effizienten Anreicherung an NOXA-Protein und der Induktion von Zelltod. Neben

Bortezomib konnten im Rahmen dieser Arbeit mit dem Neddylierengsinhibitor MLN4924 und dem Fettsäure-Synthaseinhibitor Orlistat zwei weitere Induktoren des NOXA-Proteins und des NOXA-abhängigen Zelltods in MCL-Zellen identifiziert werden. Ebenso wie Bortezomib sind beide Inhibitoren in der Lage, den rapiden Abbau von NOXA-Protein zu stoppen und den bereits existierenden Pool des pro-apoptotischen Proteins zu stabilisieren. Im Gegensatz zu Bortezomib beeinträchtigen MLN4924 sowie Orlistat jedoch die Ubiqitinierung von NOXA und stabilisieren das „BH3-only"-Protein unabhängig vom Proteasom. Wie in dieser Arbeit gezeigt werden konnte stellen diese Substanzen somit nicht nur Alternativen zu Proteasomeninhibitoren dar, sondern eröffnen auch neue Möglichkeiten für die Behandlung von Bortezomib-resistenten MCL-Zellen.

Bemerkenswert ist, dass ein aktiver PI3K/AKT/mTOR-Signalweg für eine effiziente Akkumulierung des NOXA-Proteins und Zelltodinduktion nach Behandlung der malignen B-Zellen mit den Inhibitoren des Ubiquitin-Proteasom-Systems notwendig ist. Dies deutet darauf hin, dass die Sensitivität der MCL-Zellen gegenüber diesen Inhibitoren von der konstitutiv hohen *NOXA*-mRNA Expression abhängig ist.

Der zelluläre Fettsäurestoffwechsel in MCL-Zellen könnte ein sehr erfolgsversprechendes Ziel für neue Behandlungsansätze zu sein. Wie in der vorliegenden Arbeit beschrieben werden konnte, hat die konstitutive Überexpression von Cyclin D1 in MCL einen hemmenden Einfluss auf die zelluläre Stoffwechselaktivität und macht die Zellen somit angreifbarer für Inhibitoren des Fettsäuremetabolismus wie beispielsweise Orlistat. Darüber hinaus könnten Inhibitoren des Fettsäuremetabolismus nicht nur eine neue und sehr selektive Möglichkeit darstellen, um MCL-Zellen zu töten, sondern auch dazu beitragen, dieses aggressive Lymphom gegenüber anderen Krebsmedikamenten sensitiver zu machen.

Zusammenfassend konnte im Rahmen der vorliegenden Arbeit gezeigt werden, dass das „BH3-only"-Protein NOXA eine kritische Rolle im MCL spielt und, dass dessen Regulation möglicherweise eine Achilles-Sehne dieses aggressiven Tumors darstellt. Das gezielte Eingreifen in die diskrepante Expression von *NOXA*-mRNA und NOXA-Protein durch verschiedene Inhibitoren des Ubiquitin-Proteasom-Systems könnte eine neuer Ansatz für ein gezieltes Angreifen von MCL-Zellen sein und zur Verbesserung des Therapieerfolges beitragen.

1 Introduction

1.1 Mantle cell lymphoma

1.1.1 Biology and clinical features of MCL

Mantle cell lymphoma (MCL) is a B cell neoplasm and accounts for approximately 6% of all cases of non-Hodgkin lymphoma (Pérez-Galán et al., 2011a). MCL is more than twice as common among men (male-to-female ratio ~2.5:1) and median age of diagnosis is approximately 60 years (Jares and Campo, 2008; Smedby and Hjalgrim, 2011).

MCL is a very aggressive type of tumor with relatively short response to conventional chemotherapy, frequent relapses and a median survival of 5–7 years (Jares and Campo, 2008; Pérez-Galán et al., 2011a). MCL is characterized by an abnormal proliferation of mature B-lymphocytes mainly infiltrating the lymphoid system but also frequently found in the bone marrow, peripheral blood and extranodal sites. Morphologically, MCL is sub-classified in the classical, blastoid and pleomorphic cytological variant (Campo et al., 1999; Jares et al., 2007). The neoplastic cells have the tendency to colonize the mantle zone of the lymphoid follicles and express the B cell markers CD19, CD20, CD22 and CD79A. They are also positive for surface immunoglobulins (IgM/IgD) and the T cell associated antigen CD5 (Jares et al., 2007). Their similarity in distribution and phenotype to normal CD5-positive B cells, a rare subpopulation of naïve B cells, indicates that these cells are the normal counterpart of MCL cells (Jares and Campo, 2008). However, recent studies suggest that the scenario is more complex. A subset of MCL was found to carry IGHV hypermutations indicating that at least a small percentage of tumors experienced antigen selection (Hadzidimitriou et al., 2011; Jares et al., 2012).

1.1.2 Oncogenic mechanisms

Cyclin D1

The initial oncogenic event in pathogenesis of MCL is thought to be the t(11;14)(q13;q32) translocation. This alteration is the genetic hallmark of MCL and leads to juxtaposition of *CCND1* (*Cyclin D1*) to the immunoglobulin heavy chain complex (IGH). The result of this translocation, which occurs already at the pre-B stage of differentiation in bone marrow is the

constitutive overexpression of Cyclin D1 that is generally not expressed in normal B cells (Campo et al., 1999; Fernàndez et al., 2005). Cyclin D1 is involved in cell cycle progression and plays a crucial role in G_1-S transition. It promotes cell proliferation by binding and activating the cyclin-dependent kinases CDK4 and CDK6. Assembly of the Cyclin D1/CDK complexes leads to phosphorylation and inactivation of retinoblastoma protein 1 (RB1). The tumor suppressor RB1 thereby loses its inhibitory effects on the cell cycle and releases the transcription factor E2F that promotes expression of genes involved in DNA replication and S phase entry. The resulting accumulation of Cyclin E, which in turn binds and activates CDK2 leads to the irreversible inactivation of RB1 and finally promotes cell cycle progression into S phase (Jares and Campo, 2008). Furthermore, increased amounts of Cyclin D1/CDK complexes is thought to sequester p27 and titrate away the CDK inhibitor from Cyclin E/CDK2 thereby increasing the activity of the complexes and further promoting S phase entry (Fernàndez et al., 2005). Together, Cyclin D1 overexpression contributes to the pathogenesis of MCL by deregulating the cell cycle at the G1-S transition thereby facilitating cell cycle progression and tumor proliferation.

In addition to its role in cell cycle progression, Cyclin D1 has several CDK independent functions, which affect different important cellular processes. Cyclin D1 overexpression might therefore have additional oncogenic roles in MCL (Jares et al., 2012). It was proposed that Cyclin D1 has effects on migration and differentiation by binding several transcription factors and interacting with histone-modifying enzymes (Fu et al., 2004; Musgrove et al., 2011). A recent study screening for direct protein partners also revealed that Cyclin D1 binds to RAD51 thereby promoting homologous recombination-mediated DNA repair (Jirawatnotai et al., 2011). On contrary, Cyclin D1 seems to facilitate chromosome instability by directly interacting with genes involved in chromosomal segregation and chromatin remodeling (Casimiro et al., 2012). Furthermore, Cyclin D1 expression influences cellular metabolism. It was proposed that Cyclin D1 inhibits mitochondrial activity in B cells (Tchakarska et al., 2011) and decreases the expression of many metabolic enzymes thereby affecting aerobic glycolysis and fatty acid synthesis in tumor cells (Buchakjian and Kornbluth, 2010; Sakamaki et al., 2006). The exact role of these additional functions of Cyclin D1 in the pathogenesis of MCL is still unclear.

Interestingly, a rare subset of MCL lacks the t(11;14)(q13;q32) translocation and consequently Cyclin D1 overexpression. Morphology and phenotype of these tumors, however, show the typical features of MCL (Jares et al., 2012). Recent studies have shown that rearrangements of *Cyclin D2* are the most frequent genetic events in these cells, which

might substitute Cyclin D1 and contribute to the pathogenesis of these MCL cases (Salaverria et al., 2013).

Secondary genetic alterations

Several experiments suggest that Cyclin D1 overexpression alone is not sufficient for complete transformation and the aggressive phenotype of MCL (Jares and Campo, 2008; Jares et al., 2007). Aberrant expression of Cyclin D1 in transgenic mice, for example, did not develop spontaneous lymphomas and required other oncogenic events such as MYC expression for tumorigenesis (Lovec et al., 1994). The observation that the t(11;14)(q13;q32) translocation is observed in peripheral blood cells of a small percentage (1 - 2 %) of healthy individuals further supports this hypothesis (Hirt et al., 2004).

MCL is one of the lymphoid tumors with the highest degree of genomic instability. The secondary genetic alterations include losses, gains and amplifications of certain chromosomal regions (Jares et al., 2007). The underlying mechanism of the genomic instability is not well understood. Cyclin D1/CDK4 mediated initiation of re-replication during S phase was proposed to contribute to this phenomenon (Kim and Diehl, 2009). Most recurrent chromosomal alterations found in MCL affect genes involved in processes, which are commonly deregulated in tumor cells (Pérez-Galán et al., 2011a). Two important pathways, which are frequently targeted by secondary alterations are the INK4a/CDK/RB1 and ARF/MDM2/p53 pathway leading to further deregulation of the cell cycle in MCL cells (Jares et al., 2012). The central role of the cell cycle in pathogenesis of MCL is highlighted by the finding that the proliferation gene expression signature is a predictive marker of MCL patient survival (Rosenwald et al., 2003). Other secondary chromosomal aberrations that may contribute to the development of this aggressive disease involve the DNA damage response. The *ATM* gene, a central mediator of responses to DNA double strand breaks (Kitagawa and Kastan, 2005), is frequently deleted or mutated in MCL cases that show a high degree of genetic instability and complex karyotypes. Additionally, deregulation of ATM downstream targets CHK1 and CHK2 were found in this tumor (Royo et al., 2011). Aberrant expression of apoptosis related proteins leading to defects in cell death induction such as amplification and overexpression of the anti-apoptotic protein Bcl-2 (Beà et al., 2009) or deletion of the pro-apoptotic gene *BIM* (Tagawa et al., 2005) are also recurrent features of MCL and contribute to the onset of the lymphoma.

Other deregulated oncogenic signaling pathways

As previously described, MCL cells are genetically instable and carry several secondary alterations likely contributing to the aggressive phenotype and pathogenesis of MCL. In addition to that, several signaling pathways involved in cellular survival and proliferation are deregulated in MCL further promoting the complexity of this disease (Jares et al., 2012).

The phoshatidyl-inositol-3 kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) signaling pathway plays a key role in multiple cellular processes and is deregulated in many types of cancers. The oncogenic pathway controls expression of many cell cycle and apoptosis related proteins and is also involved in regulation of cellular metabolism, differentiation, migration and angiogenesis (Engelman, 2009). Recent work demonstrated constitutive activation of AKT and mTOR as well as increased gene expression of related downstream targets in MCL (Col et al., 2008; Rizzatti et al., 2005). The mechanism how the PI3K/AKT/mTOR pathway is activated in this lymphoma, however, remains elusive so far. A possible role of the B cell receptor (BCR) in activation of this oncogenic signaling pathway was proposed (Jares et al., 2012). BCR signaling was recently found to be constitutively active in MCL cells (Young and Staudt, 2013). The BCR pathway engages multiple signaling components. Activation of the BCR leads to the recruitment and activation of several kinases such as SYK or the Bruton's tyrosine kinase (BTK). This results in activation of downstream pathways including the PI3K/AKT/mTOR, MAP kinase (MAPK) and NF-κB signaling pathway (Rickert, 2013). Chronic active and tonic BCR signaling that differ in involvement of downstream signaling pathways were described in B cell malignancies. Both types of BCR signaling lead to constitutive activation of the PI3K/AKT/mTOR pathway. MCL is thought to have a chronic active BCR pathway. In contrast to tonic BCR activation this type most likely needs antigen stimulation. The putative foreign or self-antigen, however, is unknown (Young and Staudt, 2013). Recent studies targeting the BCR receptor and PI3K/AKT/mTOR pathway suggest that this signaling axis plays a major role in pathogenesis of MCL (Kim et al., 2012; Wang et al., 2013).

Other oncogenic signaling pathways reported to be active in MCL include NF-κB, JAK/STAT and WNT signaling (Jares et al., 2012). Constitutive activation of the NF-κB signaling pathway leading to overexpression of several anti-apoptotic genes in MCL (Pham et al., 2003) might also be a result of chronic BCR signaling.

1.2 Treatment

The biological and clinical heterogeneity of MCL makes this tumor one of the most difficult to treat B cell lymphoma. Only few patients are considered to be cured and there is no standard therapy available for MCL (Smith, 2011). First line treatment regimens are usually based on conventional chemotherapeutic combination protocols such as CHOP (Cyclophosphamide, Doxorubicin, Vincristine and Prednisone) or hyper-CVAD (Cyclophosphamide, Doxorubicin, Vincristine, Dexamethasone; alternated with high-dose Methotrexate and Cytarabine). The intensive chemotherapy regimens initially induce good responses, but the tumor relapses in most patients. Combination of high dose combination chemotherapy with Retuximab, a monoclonal antibody against CD20, slightly increased complete response rate and overall survival (Dreyling and Hiddemann, 2009; Jares et al., 2007). Nevertheless, the development of new treatment strategies for MCL is urgently needed.

The progress that has been made in understanding the complex oncogenic alterations in MCL provides new promising targets such as the BCR and PI3K/AKT/mTOR pathway or the apoptosis regulating Bcl-2 family proteins (Pérez-Galán et al., 2011a). As mentioned before, recent studies showed significant activity of the BTK inhibitor Ibrutinib in relapsed or refractory MCL cells indicating that inhibition of the BCR signaling may provide a reasonable target for therapy of MCL in the future (Advani et al., 2013; Wang et al., 2013). Approaches inhibiting the PI3K/AKT/mTOR pathway downstream of the BCR were also promising. PI3K inhibitors showed good clinical response rates and Temsirolimus, an inhibitor of mTOR, was even approved for relapsed or refractory cases of MCL (Pérez-Galán et al., 2011a). Inhibitors of the ubiquitin-proteasome system (UPS) also recently entered the clinic for treatment of MCL (Holkova and Grant, 2012) and the proteasome inhibitor Bortezomib (*Velcade*) has also been successfully approved for relapsed or refractory MCL (Chen et al., 2011). Bortezomib is thought to trigger apoptosis in the B cell neoplasm by inducing the pro-apoptotic Bcl-2 family member NOXA (Pérez-Galán et al., 2006). Direct targeting of the anti-apoptotic members of the Bcl-2 family, which are frequently overexpressed in MCL, is another promising option for future treatment strategies. Several BH3-mimetics, a new class of anti-cancer drugs antagonizing the pro-survival member of the Bcl-2 family, have shown activity both as single agent as well as in combination with other drugs (Pérez-Galán et al., 2011a).

1.3 Bcl-2 family

1.3.1 Apoptosis

Programmed cell death (PCD) plays a major role in development, maintenance of tissue homeostasis and immunity of multi-cellular organisms. Defects in this critical mechanism have been linked to the development of numerous diseases including cancer (Strasser et al., 2011). It was recently shown that different forms of PCD exist (Yuan and Kroemer, 2010). However, the best-studied and most prominent type of PCD is known as apoptosis. The term apoptosis was first used by Kerr *et al*. to describe the morphological features of "controlled cell deletion" (Kerr et al., 1972). The process of apoptosis is accompanied by characteristic morphological changes such as cell-shrinkage, fragmentation of the cell into apoptotic bodies and rapid phagocytosis by neighboring cells. Biochemical hallmarks of apoptosis are condensation and inter-nucleosomal fragmentation of nuclear DNA (Saraste and Pulkki, 2000). The apoptotic morphology and the typical "DNA laddering" are a result of activation of so-called caspases. These cysteine proteases mediate cellular destruction by cleaving multiple cellular proteins and activating specific DNases (Cory and Adams, 2002).

Two distinct apoptotic signaling pathways can induce caspase activation. The extrinsic or "death-receptor pathway" is activated upon stimulation of death receptors of the tumor necrosis factor (TNF) family such as Fas and TNF-R1. Specific ligand binding leads to the formation of the "death-inducing signaling complex (DISC)" and subsequent activation of caspases 8 and effector caspases. The Bcl-2 family controlled intrinsic or "mitochondrial pathway" is activated in response to stress stimuli such as growth-factor withdrawal or severe DNA damage. The intracellular stress signals activate pro-apoptotic members of the Bcl-2 family that triggers mitochondrial outer membrane permeabilization (MOMP) and the release of cytochrome c and other proteins such as SMAC/DIABLO from the mitochondrial outer membrane. This triggers activation of caspase 9 that in turn activates effector caspases (Strasser et al., 2011).

Although both types of apoptotic signaling seem to be largely independent a crosstalk of the extrinsic and intrinsic pathway was observed in certain cell types. Caspase 8, a key player in death receptor mediated apoptosis, can process and activate the pro-apoptotic Bcl-2 family Bid and boost caspase activation via the intrinsic pathway (Cory and Adams, 2002).

1.3.2 The Bcl-2 family and their function in apoptosis

The Bcl-2 family is functionally divided into two groups, the anti-apoptotic and the pro-apoptotic proteins. Bcl-2, Bcl-x_L, Bcl-w, Mcl-1 and A1 are the major members of the anti-apoptotic Bcl-2 family subgroup. The pro-apoptotic proteins are further subdivided into the apoptosis initiators, the so-called Bcl-2 homology 3 (BH3)-only proteins (NOXA, PUMA, Bim, Bmf, Hrk, Bad, Bik and Bid) and into effectors (Bax and Bak) which are able to oligomerize and form pores in the outer mitochondrial membrane (OMM) thereby promoting MOMP and subsequent caspase activation. Complex interactions of the Bcl-2 family members tightly control the activation of Bax and Bak (Chipuk et al., 2010). The pro-apoptotic members of both subgroups can bind with high affinity to their anti-apoptotic relatives. The interactions between the proteins are very specific. The effector protein Bax, for example, binds all pro-survival Bcl-2 family members, whereas Bak only interacts with Bcl-x_L, Mcl-1 and A1. A similar difference in selectivity was also observed for the BH3-only proteins. Bim, PUMA and the cleaved form of Bid are thought to bind all anti-apoptotic proteins. In contrast, Bad binds not to Mcl-1 and A1, whereas NOXA only shows high affinity to these two anti-apoptotic Bcl-2 family members (Chen et al., 2005; Willis et al., 2005).

Stress-induced apoptosis via the intrinsic pathway requires both types of pro-apoptotic proteins. The BH3-only proteins are activated (transcriptional or post-translational) in response to various stress stimuli and act upstream of Bax and Bak (Adams and Cory, 2007). Two major models have been proposed to explain how they activate the pro-apoptotic effector proteins and finally induce MOMP. The “direct activation” model suggests that certain BH3-only proteins, so-called “activators” including Bim, PUMA and tBid transiently bind and activate Bax and Bak by inducing conformational changes. In healthy cells the anti-apoptotic proteins prevent induction of cell death by binding and sequestering the activator proteins. The other BH3-only proteins such as NOXA and Bad, termed “sensitizers” in this model, are not able to directly activate Bax and Bak and only bind to their anti-apoptotic partner. Upon intracellular stress, the sensitizers compete with the activators for binding with the anti-apoptotic proteins thereby leading to the release of the activators, which then directly activate Bax and Bak (Strasser et al., 2011). Another scenario in this model is possible. The “sensitizers” lower the threshold for apoptosis induction by sequestering the anti-apoptotic proteins. Stress-induced activators cannot be inhibited by the bound pro-survival proteins and can directly activate the pro-apoptotic effector proteins (Chipuk et al., 2010). In contrast, the “indirect activation” model proposes that none of the BH3-only proteins directly binds and

activates the pro-apoptotic effectors. In healthy cells the anti-apoptotic Bcl-2 family members sequester Bax and Bak and prevent their activation. The BH3-only proteins provoke apoptosis by targeting and neutralizing the anti-apoptotic proteins thereby releasing the effectors and allowing them to oligomerize and induce MOMP (Strasser et al., 2011). However, work from Mérino *et al*. suggests that induction of apoptosis via the intrinsic pathway requires aspects of both models (Mérino et al., 2009).

1.3.3 Bcl-2 family and cancer

Resistance to programmed cell death is a hallmark of cancer (Hanahan and Weinberg, 2011). Deregulation of the Bcl-2 family is one way of tumor cells to circumvent apoptosis induction thereby promoting tumor development and proliferation (Kelly and Strasser, 2011). The pro-survival protein Bcl-2 was the first member of the Bcl-2 family, which was implicated in cancer. It was found that overexpression of Bcl-2, such as caused by the t(11;14) translocation in follicular lymphoma, has oncogenic potential and cooperates with the oncogene *MYC* to immortalize lymphoid cells (Vaux et al., 1988). Now it is known that not only overexpression of pro-survival proteins but also loss of their pro-apoptotic relatives is present in many human cancers and can promote malignant transformation (Adams and Cory, 2007). As mentioned before, high levels of Bcl-2 protein and deletions of *BIM* are frequently observed in MCL (Beà et al., 2009; Tagawa et al., 2005). Aberrant expression of Bcl-2 family is not always caused by direct genetic alterations. Several pro-survival members such as Bcl-2 or Bcl-x_L are transcriptional targets of central oncogenic pathways. For instance, expression of the oncogenic tyrosine kinase Bcr/Abl resulting in activation of multiple downstream pathways transcriptionally induces *Bcl-x_L* expression that is thought to promote development of CML (Horita et al., 2000; Oetzel et al., 2000). Levels of Bcl-2 family members can also be affected by deregulation of proteins involved in protein stability. Loss of FBW7, an essential protein for UPS- mediated Mcl-1 degradation, leads to enhanced stability and accumulation of this unstable anti-apoptotic protein and is frequently found in many cancers (Inuzuka et al., 2011). In contrast, active MAPK signaling can promote enhanced turnover of the pro-apoptotic protein BIM thereby reducing its protein levels and contributing to tumorigenesis and metastasis (Akiyama et al., 2009; Ley et al., 2003).

In addition to their role in tumor development and maintenance, the constitutive and stress induced Bcl-2 family protein levels significantly influence the response to anticancer drugs. Overexpression of several anti-apoptotic members renders tumor cells refractory to

chemotherapeutics and often correlates with drug resistance (Strasser et al., 2011). Up-regulation of Bcl-2, for example, is involved in chemoresistance of human small cell lung cancer cell lines (Sartorius and Krammer, 2002). On the contrary, the BH3-only proteins are essential to induce apoptosis in response to many agents used in cancer therapy. Stress signals mediated by the cytotoxic agents lead to the activation of distinct BH3-only proteins which initiate the apoptotic program (Adams and Cory, 2007). For example, Bim and Puma are crucial for the response of ALL cells to glucocorticoids and apoptosis following DNA damaging agents relies on p53 mediated induction of PUMA and/or NOXA in p53 wild-type cancer cells (Bouillet et al., 1999; Villunger et al., 2003). Apoptosis induction upon treatment with the proteasome inhibitor Bortezomib seems to depend on the cellular background. In myeloma, CLL or MCL NOXA was found to be the major mediator of the cytotoxic effects (Baou et al., 2010; Gomez-Bougie et al., 2007; Pérez-Galán et al., 2006), whereas Bik and Bim were implicated in cell death upon Bortezomib treatment in various other cancer types (Li et al., 2008; Nikrad et al., 2005).

Moreover, the constitutive levels of the BH3-only proteins were also implicated to influence response to chemotherapy. It was proposed that the balance between the pro- and anti-apoptotic Bcl-2 family members determines the proximity of a cancer cell to the apoptotic threshold. A so-called “primed” cell in which most anti-apoptotic proteins are constitutively occupied by their pro-apoptotic binding partners is closer to this point and likely more sensitive to an apoptotic stimulus through treatment with cytotoxic agents (Davids and Letai, 2012). For example, recent work showed that high constitutive NOXA levels prime testicular germ cell tumors to undergo rapid and massive apoptosis in response to Cisplatin treatment (Gutekunst et al., 2013).

1.3.4 *NOXA* (*PMAIP1*)

As described above, the BH3-only protein NOXA was implicated in cell death induction of tumor cells upon treatment with certain anticancer drugs such as Bortezomib or Cisplatin. However, NOXA appears not only to play a crucial role in fine-tuning life/death decisions in response chemotherapy but also in normal physiology and tumor development (Ploner et al., 2008). For example, NOXA is involved in maintenance of memory T cell homeostasis and selection of high affinity clones upon T cell activation (Wensveen et al., 2010; Yamashita et al., 2008). Similar observations have been made for B cells. *NOXA* was found to be transcriptionally induced upon B cell activation and represents a critical factor for apoptosis in

activated B cells and selection of high affinity clones during B cell expansion (Bretz et al., 2011; Wensveen et al., 2012). NOXA-induced cell death may therefore be important to prevent oncogenic transformation of lymphoid cells and/or autoimmunity. As previously described, NOXA protein is a so-called "sensitizer" BH-only protein, which promotes apoptosis induction by selectively sequestering its anti-apoptotic partners Mcl-1 and A1 (Strasser et al., 2011). In addition to binding and neutralizing the pro-survival proteins, NOXA promotes the UPS-mediated degradation of Mcl-1 through triggering an enhanced interaction of Mcl-1 with the ubiquitin-ligase MULE (Gomez-Bougie et al., 2011). Interestingly, recent reports proposed that NOXA might also be a direct activator of Bax and Bak (Du et al., 2011). Beside their important function in apoptosis, NOXA and its binding partner Mcl-1 were also implicated in regulation of other forms of PCD. Ras-induced autophagic cell death was associated with NOXA-mediated displacement of Mcl-1 from autophagy regulator Beclin-1 (Elgendy et al., 2011).

NOXA was first identified as a phorbol-12-myristate-13-acetate (PMA)-responsive gene that is highly expressed in adult T-cell leukemia (ATL) and therefore first named *APR* (ATL-derived PMA-responsive gene) (Hijikata et al., 1990). Later it was renamed *PMAIP1* (PMA-induced protein 1) before it was rediscovered as a pro-apoptotic gene which is induced upon x-ray irradiation in a p53-dependent manner and found to encode for a BH3-only member of the Bcl-2 family (Oda et al., 2000). Cellular *NOXA* mRNA and NOXA protein expression is tightly controlled and not only regulated by p53. *NOXA* was found to be induced by several other transcription factors including the p53 relative p73 (Flinterman et al., 2005), HIF1α (Kim et al., 2004) or E2F1 (Hershko and Ginsberg, 2004). In contrast, Bmi1, which belongs to a group of chromatin remodeling and histone modifying enzymes, negatively regulates the BH3-only gene and represses *NOXA* transcription (Yamashita et al., 2008). Furthermore, *NOXA* expression is regulated at post-transcriptional levels. As its main binding partner Mcl-1, the BH3-only protein is target of ubiquitination and proteasomal degradation (Baou et al., 2010). The NOXA-specific E3 ubiquitin ligase, however, is yet unknown. SAG, a subunit of SCF E3 ligases was implicated in the regulation of NOXA protein stability (Jia et al., 2010). The deubiquitinating enzyme UCH-L1 was identified to remove ubiquitin residues from NOXA, thereby protecting NOXA protein from degradation. Loss of UCH-L1 can promote enhanced degradation of NOXA and contribute to chemoresistance (Brinkmann et al., 2013). Interestingly, recent studies revealed that NOXA can be degraded by the proteasome also in absence of ubiquitination (Craxton et al., 2012). A second mechanism of posttranscriptional

regulation of *NOXA* expression involves miRNAs. MiRNA-200c targets the 3'-UTR of *NOXA* mRNA and can represses its basal expression (Lerner et al., 2012).

1.4 The ubiquitin-proteasome system (UPS)

The ubiquitin-proteasome system (UPS) is the central pathway responsible for selective degradation of many proteins in eukaryotic cells and essential for sustaining cellular protein homeostasis (Hershko and Ciechanover, 1998). The UPS is also an important regulator of critical cellular processes by controlling the turnover of many proteins involved in cell cycle, stress response, signal transduction and apoptosis. Deregulation of the UPS can contribute to the development of disease including malignant transformation (Hershko, 2005).

Degradation of a protein by the UPS involves two steps. Unwanted, damaged or misfolded proteins are first targeted by attachment of multiple ubiquitin residues. The tagged proteins are then directed to the multisubunit 26S proteasome complex that mediates degradation of the proteins (Ciechanover, 1998). Ubiquitination of proteins is a strongly regulated sequential process. An ubiquitin-activating enzyme (E1) initially activates ubiquitin. Next, the activated ubiquitin is transferred to an ubiquitin-conjugating enzyme (E2). A substrate specific ubiquitin ligase (E3) finally transfers and conjugates the ubiquitin to the substrate. After the first ubiquitin has been linked to the substrate, the E3 ligase can elongate the ubiquitin chain by attaching additional residues to the previously conjugated ubiquitin molecule leading to polyubuquitination of the substrate. Elongation of the ubiquitin chain can also be carried out by E4 enzymes, a subclass of E3 ligases, which only catalyze chain extension. The resulting polyubiquitination targets the proteins for proteasomal degradation. In contrast, monoubiquitination of proteins does not promote degradation and is thought to have other biological functions (Tai and Schuman, 2008). The E3 ligases play a crucial role in the ubiquitination process through selective binding of the substrates. The approximately 1,000 different E3 ligases can be divided into three classes: HECT (homologous to E6-AP carboxy terminus), U-box and RING (really interesting new gene) E3 ligases (Micel et al., 2013). Cullin-containing RING finger ligases (CRLs) are a subgroup of RING ligases, which targets several substrates implicated in cancer development. Interestingly, CRL ligase activity is positively regulated by conjugation of NEDD8, a ubiquitin-like protein (UBL) that is similarly conjugated to substrates as ubiquitin (Watson et al., 2011). The counterparts of E3 ligases are the deubiquitinases (DUBs), a large group of proteases, which can remove ubiquitin chains and rescue protein from degradation.

The 26S proteasome that mediates degradation of ubiquitin tagged proteins is a cylindrical 2.5 MDa multimeric protein, which consists of two subcomplexes: a catalytic core particle (CP) and one or two regulatory particles (RPs). The CP or 20S proteasome contains catalytic threonine residues (β1, β2 and β5) with caspase-like, trypsin-like or chymotrypsin-like activity and mediates proteolytic activity of the complex. The RP forms the base and the lid of the proteasome and recognizes polyubiquintinated proteins. It also mediates deubiquitination, unfolding and translocation of the substrate proteins into the CP where they get destructed (Tanaka et al., 2012).

The UPS plays a critical role in turnover of both proteins with oncogenic potential and tumor suppressors such as Bcl-2-family members, Cyclin D1 or p53 and deregulation of the UPS can contribute to the development of cancer. Consequently, the UPS became a target of cancer therapy (Micel et al., 2013). The most prominent and clinical successful agent targeting the UPS is the proteasome inhibitor Bortezomib, a reversible inhibitor of the chymotrypsin-like site in the 20S proteasome (Chen et al., 2011). Besides direct targeting the proteasome, inhibition of the ubiquitination process has become an attractive alternative to disrupt the UPS pathways. Inhibitors such as MLN4924, which inhibits neddylation and thereby the activity of CRLs, are currently in clinical development (Jia and Sun, 2011).

1.5 Fatty acid metabolism and cancer

Metabolic reprogramming is another hallmark of cancer (Hanahan and Weinberg, 2011). A well-known metabolic feature of tumor cells is the metabolic state termed aerobic glycolysis or Warburg effect. This phenotype is characterized by a high rate of glycolysis and secretion of lactate even under aerobic conditions (Kim and Dang, 2006). This type of metabolism allows the tumor cells to sustain higher proliferation rates and promotes survival (DeBerardinis et al., 2008). A less known metabolic feature of tumor cells is the increased *de novo* fatty acid (FA) synthesis (Kuhajda, 2000). Interestingly, both metabolic pathways are interconnected. The high level of glycolysis provides precursors and energy in form of citrate and NADPH for *de novo* synthesis of FAs. In contrast to normal cells, cancer cells seem to depend on *de novo* FA synthesis to maintain constant supply of lipids, which are essential in highly proliferating cells. Consequently, increased activity and expression of several enzymes involved in FA synthesis and lipogenesis has been observed in tumor cells (Menendez and Lupu, 2007). The key player in FA synthesis is the fatty acid synthase (FASN). This metabolic enzyme synthesizes the 16-carbon FA palmitate and is the only human protein

capable of *de novo* long chain FA synthesis (Kuhajda, 2006). Overexpression of FASN is a frequent alteration in cancer cells and often an indicator of poor prognosis (Gansler et al., 1997; Visca et al., 2004). The underlying mechanisms leading to overexpression of FASN in many tumor cells are unclear. Oncogenic signaling pathways such as PI3K/AKT/mTOR and MAPK signaling have been implicated in enhanced transcription of *FASN* and gene amplifications have also been observed (Menendez and Lupu, 2007). Furthermore, FASN protein levels are posttranslationally regulated by the UPS and high expression levels of the ubiquitin-removing enzyme USP2 have been found to stabilize FASN and contribute to increased protein levels of the metabolic enzyme (Graner et al., 2004).

FASN is not only a downstream target of oncogenic signaling pathways. It has also been shown that the metabolic enzyme has oncogenic function itself. Several studies revealed that enhanced levels of FASN promote proliferation and survival of cancer cells likely by positively regulating cell cycle progression (Liu et al., 2010). Beside its metabolic function, FASN has been suggested to play a role in regulating gene expression of various important cellular processes such as DNA replication, proliferation and apoptosis (Knowles and Smith, 2007a). Based on these findings, FASN became a target for cancer therapy. Several pre-clinical studies using FASN inhibitors such as Cerulenin or Orlistat revealed cytotoxic effects of FASN inhibition in a variety of cancer cells (Liu et al., 2010). Interestingly, recent work showed that FASN inhibition also induces apoptosis in MCL indicating that the metabolic enzymes might also play a significant role in pathogenesis of this lymphoid neoplasm and represent an interesting new target for therapy (Gelebart et al., 2012).

1.6 Aims

As described in the previous chapters, mantle cell lymphoma (MCL) is one of the most hard to treat lymphoid neoplasms and new treatment approaches are urgently needed.

The BH3-only protein NOXA is thought to play an essential role in fine-tuning the apoptotic response upon a variety of intracellular stress signals. Interestingly, NOXA was implicated in proteasome inhibitor induced cell death in several B cell malignancies including MCL. Furthermore, NOXA-mediated apoptosis has also physiological functions at certain stages of B cell development. Therefore, NOXA might be an important player in life/death decisions in certain B cell neoplasms and represent a central apoptotic switch in MCL cells.

The aim of this work was to investigate the constitutive expression and regulation of the pro-apoptotic BH3-only protein NOXA in MCL and its potential role as a therapeutic target of this aggressive malignancy.

In detail, gene expression databases, MCL cells lines and primary samples from MCL patients were used to

(I) explore the constitutive *NOXA* gene and NOXA protein expression levels in MCL and compare them to tumors derived from other entities.

(II) investigate how *NOXA* mRNA and NOXA protein levels are regulated in MCL and to what extent the multiple altered oncogenic pathways influence expression of *NOXA*.

(III) determine whether the pathways regulating *NOXA* gene and NOXA protein expression represent potential targets for treatment of MCL.

(IV) screen for drugs or drug combinations that induce/activate NOXA and selectively induce cell death in MCL.

2 Material and methods

2.1 Cell culture

2.1.1 Cell culture medium

All eukaryotic cell lines were cultured in RPMI 1640. The medium was supplemented as indicated below.

RPMI 1640 medium (500 ml)	Biochrom
500 ml medium were supplemented with:	
10% (v/v) FBS	Gibco
0,1 g/l Penicillin/Streptomycin	Gibco
10 mM HEPES (2-(4-Hydroxyethyl)-1-piperazinyl)ethanolsulfonsäure); pH 7,4	Merck
2 mM L-glutamine	Biochrom
0,13 mM L-asparagine	Serva
0,05 mM ß-mercaptoethanol	Merck
1 mM Natriumpyruvat	Gibco
3 ml 100x non-essential amino acids	Biochrom

2.1.2 Cell lines

MCL cell lines used in this study are listed in Table 1. Cell lines derived from other entities used in this study are listed in Table 2.

Table 1: MCL cell lines used in this study

cell line	p53 status	obtained from
Mino	mutant	MCL cell lines were a kind gifts from Andreas Rosenwald, University of Würzburg
Rec1	wild type	
Jeko1	mutant	
Granta519	wild type	
Jvm2	wild type	

Table 2: Cancer cell lines used in this study

cell line	source	obtained from
2102EP	embryonal carcinoma	was kindly provided by Thomas Mueller, University of Halle
NTERA2/D1	embryonal carcinoma	LGC Standards
H460	non-small cell lung cancer	ATCC
A549	lung adenocarcinoma	ATCC
H23	non-small cell lung cancer	ATCC
OVCAR5	ovarian carcinoma	NCI-60 panel
SKOV3	ovarian carcinoma	NCI-60 panel
A2780	ovarian carcinoma	NCI-60 panel

2.1.3 Cell cultivation

All cell lines were cultured in sterile cell culture flasks at 37 °C with 5 % CO_2 and passaged every 2-3 days. Adherent cell lines were sub-cultivated using Trypsin/EDTA.

Cell culture flasks	Sarstedt
Serological pipettes	Corning
6-, 12-, 24-, 96-well-plates	Greiner Bio-One
15 ml tubes	Sarstedt
50 ml tubes	Sarstedt
Trypsin/EDTA	Gibco
Laminar flow hood	Heraeus
CO_2-Incubator	Heraeus

2.1.4 Trypan blue staining

Cell viability and cell count was determined by staining of the cells with trypan blue and subsequent microscopy using a hemocytometer. The vital stain trypan blue selectively colors dead cells and can therefore be used to assess cell count of viable cells.

Trypan Blau 0,5% (w/v)	Biochrom
Neubauber hemocytometer	Roth
Mikroskop	Zeiss

2.1.5 Cryopreservation and thawing of cell lines

Cultured cell lines were replaced by cryopreserved stocks every 3-4 months to prevent genetic alterations.

For cryopreservation, 1-2 x 10^6 cells were resuspended in ice-cold freezing medium (10 % DMSO in FBS) and transferred to a cryo-tube. Cells were then transferred to a special freezing container to gradually cool down the cells to -80 °C. Cryopreserved cells were stored at -196 °C in liquid nitrogen. To re-cultivate, cryopreserved cells were thawed at 37 °C and immediately transferred to pre-warmed culture medium. Subsequently, cells were centrifuged (5 min, 1400 rpm, RT) and resuspended in fresh medium to get rid of DMSO and then cultivated under standard cultivation condition.

Dimethyl Sulfoxid (DMSO)	Sigma
FCS	Gibco
1,8 ml CryoTube™	Nalgene Nunc
5100 Cryo 1°C Einfrierbox ‚Mr. Frosty'	Nalgene Nunc

2.2 Primary cell culture

The local ethics committee approved the collection of patient and healthy donor samples (project number 159/2011BO2) and informed consent was obtained from the patients in accordance with the Declaration of Helsinki.

2.2.1 Isolation of primary cells from lymph nodes

Lymph node samples from biopsies of MCL patients were transferred to a cell strainer and squeezed with a sterile syringe head to obtain a single cell suspension. Primary cells were then centrifuged (5 min, 1400 rpm, RT) and washed in 10 ml PBS. Cell count and cell viability was assessed by trypan blue staining and the cells then cryopreserved or further cultivated and directly used for experiments.

Cell strainer	BD Falcon
PBS	Biochrom

2.2.2 Isolation of peripheral blood mononuclear cells (PBMCs) from blood samples

Blood samples from MCL patients or healthy donors were diluted with sterile PBS (1:1). To separate PBMCs from other blood components, 25 ml Ficoll was added to a 50 ml tube and the blood suspension slowly layered over the Ficoll without mixing both layers. After centrifugation (25 min, 1400 rpm, without brake, RT), the PBMCs form the interface between both layers. The interface was transferred to a new tube, diluted with PBS and centrifuged again (5 min, 1400 rpm, RT). Cells were then resuspended in PBS, counted and cryopreserved or further cultivated and directly used for experiments.

BIOCOLL Separating Solution (Ficoll)	Biochro

2.2.3 Cultivation of primary cells

Extracted primary MCL cells obtained from lymph node or blood samples were cultivated in X-VIVO™ 10 medium supplemented with 10% human serum at a concentration of 2 x 10^6 cells/ml. PBMCs of healthy donors were cultivated in RPMI 1640 supplemented with 10 % FBS. Both media were further supplemented with the additives described in 2.1.1. PBMCs of healthy donors were stimulated by addition of 2 µg/ml phytohemagglutinin-L (PHA-L) to the culture medium.

EDTA-S-Monovette	Sarstedt
X-VIVO™ 10 medium	Lonza
Phytohämagglutinin-L (PHA-L)	Roche

2.3 Reagents

2.3.1 DNA damaging agents

Genotoxic stress was induced by treatment of the cells with the DNA damaging agents Cisplatin (*cis*-Diaminodichloroplatinum (II)) and Doxorubicin. Cisplatin and Doxorubicin were used at concentrations of 10 µM and 100 nM, respectively.

Cisplatin	Robert Bosch Hospital, Stuttgart
Docorubicin	Robert Bosch Hospital, Stuttgart

2.3.2 Proteasome inhibitors

To study the effect of proteasome inhibition, cells were treated with the proteasome inhibitors MG132, Lactacystin and the clinically approved agent Bortezomib (*Velcade*). The inhibitors were used at 1 µM (MG132), 5 µM (Lactacystin) and 10 nM (Bortezomib).

MG132	Selleckchem
Lactacystin	Calbiochem
Bortezomib (*Velacade*)	Selleckchem

2.3.3 PI3K/AKT/mTOR pathway inhibitors

The influence of inhibition of the PI3K/AKT/mTOR pathway on cellular viability and *NOXA* expression was investigated using a panel of inhibitors targeting different components of the signaling pathway. Cells were treated with the PI3K/mTOR dual inhibitor Bez235, the PI3K inhibitors LY29004 and BKM120 (3µM), and the mTOR inhibitor Temsirolimus. Bez235 and Temsirolimus were used at a concentration of 1 µM. LY29004 and BKM120 were used at 10 µM and 3 µM, respectively.

Bez235	Selleckchem
LY29002	Selleckchem
BKM120	Selleckchem
Temsirolimus	Sigma

2.3.4 BCR signaling inhibitor

The inhibitor Ibrutinib was used to disrupt chronic active BCR signaling in MCL cells. Ibrutinib selectively targets Bruton's tyrosine kinase (BTK), which is a downstream kinase of the BCR. Ibrutinib was used at a concentration of 1 µM.

Ibrutinib	Selleckchem

2.3.5 MAPK pathway inhibitors

To inhibit MAPK signaling, cells were treated with PD0325901. This inhibitor specifically targets MEK1 and MEK2. PD0325901 was used at a concentration of 1 μM.

PD0325901	Selleckchem

2.3.6 Fatty acid synthase inhibitors

Cellular fatty acid metabolism was targeted using the fatty acid synthase (FASN) inhibitors Orlistat, Cerulenin and C75. The irreversible FASN inhibitor Orlistat targets the thioesterase domain of FASN and was used at a concentration of 15 μM. The natural compound Cerulenin and its chemically synthesized analogue C75 are both targeting the ketoacyl synthase domain of FASN and were used at 10 μM.

Orlistat	Sigma
Cerulenin	Sigma
C75	Sigma

2.3.7 Neddylation inhibitor

The inhibitor MLN4924 was used to inhibit activity of CRL E3 ubiquitin ligases. MLN4924 is a small molecule inhibitor of the NEDD8 activating enzyme (NAE) and disrupts neddylation and activation of the CRL ligase family. MLN4924 was used at a concentration of 0.5 μM.

MLN4924	ChemieTek

2.4 Analysis of cell viability

2.4.1 Annexin V-FITC/propidium iodide (PI) apoptosis assay

Annexin V/PI staining of the cells followed by flow cytometry was used in this study to measure induction of apoptosis.

Cells in early apoptosis show characteristic changes in their cell membrane. In this stage, the cells expose phosphatidylserine on their cell surface, which is usually located on the cytosolic site of the membrane in viable cells. The phospholipid can be bound by the Ca^{2+}-dependent phospholipid binding protein Annexin V. This interaction can be used to detect apoptotic cells by flow cytometry through labeling Annexin V with a fluorophor such as fluorescein-isothiocyanat (FITC).

During late apoptosis or necrosis the cell membrane becomes permeable and Annexin V can enter the cells and also bind phosphatitylserine on the inner leaflet of the membrane. To distinguish between early and late apoptotic cells, Annexin V-FITC staining was combined with propidium iodide (PI) staining. PI is an intercalating fluorescent agent, which is excluded from viable cells but can penetrate cells with damaged or already perforated membranes in late apoptosis or necrosis. Double staining of the cells with Annexin V-FITC and PI is therefore a sign for late apoptosis or necrosis whereas single staining of the cells with Annexin V-FITC indicates early stages of apoptosis.

For Annexin V-FITC/PI staining cells were harvested and washed with PBS. Then cells were washed with Annexin V binding buffer (10 mM HEPES, 140 mM sodium chloride, 25 mM, calcium chloride, pH 7.4) before they were resuspended in 100 µl Annexin V/PI staining solultion (5 µl AnnexinV-FITC, 2,5 µl PI (stock solution: 50 µg/ml)) and incubated for 10 min at room temperature. After addition of additional 400 µl Annexin V binding buffer, cells were briefly vortexed and directly analyzed by flow cytometry using CELLQuest software.

Annexin V-FITC	BD Pharmingen
Propidium iodide stock solution	Sigma
Annexin V binding buffer (10x)	BD Pharmingen
Fluorescence Activated Cell Analyzer “FACScan”	Becton Dickinson
CELLQuest software	Becton Dickinson

2.4.2 MTT assay

The MTT assay (Mosmann, 1983) is based on the mitochondrial activity of viable and proliferating cells and was used in this study to measure cytotoxicity of drugs and to generate dose-response curves. The yellow tetrazolium dye MTT is reduced to the purple formazan by a mitochondrial dehydrogenase that is only active in live cells. The formazan staining can be quantified by spectrophotometry and is an indicator of cell viability and proliferation.

For analysis, 7-8 x 10^3 cells per well were seeded in a volume of 200 µl in a 96-well-plate and cultivated in absence or presence of different inhibitors concentrations. After 48 h of treatment, 10 µl MTT solution (10 mg/ml MTT in PBS) were added to each well and the cells incubated at 37 °C for 2 h. The cells were then lysed by addition 90 µl MTT lysis buffer (15% SDS in DMF:H_2O (1:1); pH 4,5) and incubated over night at room temperature on a shaker. Subsequently, formazan staining was analyzed using a spectrophotometer and dose-response curves were generated using GraphPad Prism 5.0 software.

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid))	Sigma
DMF	Sigma
SDS	Sigma
ELISA-Reader ‚Wallac'	SLT Labinstruments
GraphPad Prism 5.0 software	GraphPad Software, Inc.

2.5 Drug combination screening

Screening for combinatory effects of fatty acid synthase inhibitors with other substances was performed using high-throughput fluorescence microscopy.

Cells were seeded in black 96-well-plates with µClear® bottom in medium containing NucView™ 488 caspase-3 substrate. The cells were left untreated or were treated with 15 µM Orlistat or 10 µM Cerulenin. To study effects of combination treatment, a panel of inhibitors (Table 3) was immediately added to the either untreated or FASN-inhibitor treated cells. The cells were then incubated for 24 h at 37 °C and then stained with 5 µM propidium iodide (PI) and 10 µM Hoechst 33342. Cells were analyzed using the InCell Analyzer high throughput microscope and the image analysis software InCell investigator.

The fluorescence dye Hoechst 33342 was used to stain nuclei for automated cell determination and analysis of cell count. As described above (2.4.2), PI stains late apoptotic and necrotic cells. The NucView™ 488 caspase-3 substrate, which was added before treatment, can rapidly cross the cell membrane and enters the cytoplasm. Upon caspase activation in response to an apoptotic stimulus the caspase-3 substrate is cleaved by the effector caspase-3, which leads to the release of a high-affinity DNA dye. The released DNA dye stains the nucleus and is another indicator of apoptotic cells or can be used in combination with PI to distinguish between apoptosis and necrosis.

Table 3: Substances used for combination screening

substance	mode of action/target	used concentration	company
Cisplatin	DNA damage	10 µM	Robert Bosch Hospital, Stuttgart
Etoposid	DNA damage	10 µM	Robert Bosch Hospital, Stuttgart
RITA	p53 activator	1 µM	Cayman chemicals
Nutlin-3	p53 activator	10 µM	Sigma
Metformin	AMPK agonist	1 mM	Selleckchem
2-deoxy-D-glucose (2DG)	inhibitor of glycolysis	4 mM	Sigma
PD0325901	MEK1/2 inhibitor	1 µM	Selleckchem
Lenalidomide	diverse	10 µM	Selleckchem
5-Fuorouracil (5-FU)	thymidylate synthase inhibitor	100 µM	Selleckchem
Methotrexate	dihydrofolate reductase inhibitor	20 µM	Robert Bosch Hospital, Stuttgart
Fenofibrate	PPARα agonist	30 µM	Sigma
Rosiglitazone	PPARγ agonist	50 µM	Sigma
ABT737	BH3-mimetic	1 µM	Toronto Research Chemicals

Hoechst 33342	Cell Signaling
NucView™ 488 caspase-3 substrate	Biotinum
InCell Analyzer 1000	GE healthcare
InCell investigator analysis software	GE healthcare

2.6 Protein analysis

2.6.1 Total protein extraction

Cells were harvested and collected by centrifugation (1400rpm, 5 min, 4 °C). The pellet was then washed with 1 ml ice-cold PBS, centrifuged again (1400rpm, 5 min, 4 °C) and immediately quick-frozen in liquid nitrogen. Cells were lysed by resuspending the frozen pellet in an appropriate volume of lysis buffer (50 mM TRIS, 250 mM sodium chloride, 0.1 % Triton X-100, 5 mM EDTA, pH 7.6) followed by sonification for 20 sec. To protect proteins from cleavage and de-phosphorylation, protease and phosphatase inhibitor cocktail was added to the cell lysis buffer. After cell disruption, cellular debris was pelleted by centrifugation

(13.000 rpm, 15 min, 4 °C) and the supernatant containing the total protein lysate transferred to a new tube. Afterwards, protein concentration of the lysates was measured by Bradford assay. For further analysis of the protein lysates by SDS-PAGE and subsequent Western blot, 1x Laemmli buffer (62.5 mM TRIS, 20 % (v/v) glycerol, 5 % (v/v) β-mercaptoethanol, 2 % (w/v) SDS, 1 % bromophenol blue, pH 6.8) was added to equal amounts of total protein (20-40 µg) and samples boiled at 95 °C for 5 min.

Complete protease inhibitor cocktail tablets	Roche
PhosSTOP Phosphatase Inhibitors cocktail tablets	Roche
Ultrasound homogenizator *Sonopuls HD200*; *MS72* titan microtip	Bandelin Elektronik

2.6.2 Separating protein by SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) can be used to separate proteins according to their molecular weight. Discontinuous electrophoresis was performed in this study using gels consisting of a stacking and running (separating) gel. The gels were prepared by mixing acrylamide/bis-acrylamide, SDS and TRIS buffer (stacking gel: 0.5 M, pH 6.8; running gel: 1.5 M, pH 8.8). Polymerization was started by addition of TEMED and ammonium persulfate (APS). Depending on the molecular weight of the protein of interest, different concentrations of acrylamide/bis-acrylamide for preparation of the running gel were added. Low concentrations of acrylamide were used to resolve proteins with high molecular weight, whereas high concentrations of acrylamide were used to resolve proteins with low molecular weight. Gradient running gels (acrylamide concentration: 5-20 %) were used to investigate proteins with a molecular weight below 10 kDa.

For separation, the previous boiled samples and a molecular weight protein marker were loaded on the gel and electrophoresis was started immediately. Gel electrophoresis was performed at 6 mA per gel over night using a vertical electrophoresis system filled with electrophoresis buffer (25 mM TRIS, 0.2 M glycin, 1 % SDS).

Ammonium persulfate (APS)	Bio-Rad
TEMED	Roth
30% acrylamid/Bis-solution (37, 5:1)	Bio-Rad
Vertical electrophoresis system, Protean® II xi Cell	Bio-Rad
Molekulargewicht-Marker	Cell Signaling

2.6.3 Western blot analysis

After gel electrophoresis, the separated proteins were transferred on a nitrocellulose membrane using semi-dry transfer cell.

The nitrocellulose membrane was incubated in transfer buffer (0,025 M TRIS, 0,192 M glycin, 20% (v/v) methanol, 1% (w/v) SDS) and transferred on three layers of filter paper that were also previously soaked in transfer buffer. The SDS gel was then applied to the membrane and covered with another three layers of soaked filter paper. Transfer of proteins on the membrane was carried out at 15 V for 90 min. The membrane was then washed in TBST (137 mM sodium chloride, 2.7mM potassium chloride, 25mM TRIS, 0.1 % (v/v) Tween-20, pH7.4) and blocked with 5 % (w/v) skim milk in TBST for 1 h at room temperature to avoid unspecific binding of antibodies. Incubation of the membranes with primary antibodies was carried out at 4 °C over night. The primary antibodies were diluted in either 5 % (w/v) skim milk in TBST, 5 % (w/v) BSA in TBST or 1 % (v/v) Roche blocking solution (Table 4).

After incubation with the primary antibody, the membrane was washed 4 x 15 min in TBST before it was incubated with a horseradish peroxidase-coupled secondary antibody. Secondary antibodies were chosen according to the source of the primary antibody (Table 5) and diluted in 5 % (w/v) skim milk in TBST. After 1 h at room temperature, the membrane was washed 4 x 15 min in TBST and the protein of interest detected by chemoluminescence. For this, the membrane was incubated with the peroxidase substrate “SuperSignal West Dura Extended Duration Subatrate” for 5 min and the intensity detected by x-ray films according to the manufacturer’s instructions. Densitrometric analysis of protein band intensity was performed using AIDA software.

To reuse the nitrocellulose membranes for analysis of additional proteins, primary and secondary antibodies were removed by incubating the membranes in a stripping solution (62.5 mM Tris-HCl, 2% (w/v) SDS, 100 mM β-mercaptoethanol; pH 6.7) at 52 °C for 20 min. After several washing steps with TBST and re-blocking with 5 % (w/v) skim milk in TBST, the membrane could be used for re-incubation with a different primary antibody.

Table 4: Primary antibodies used in this study

antibody	source	company	dilution
β-actin	rabbit	Cell Signaling	1:5000 in 5 % (w/v) skim milk in TBST
(Ser473)-Akt	rabbit	Cell Signaling	1:1000 in 5 % BSA in TBST
Bak	rabbit	Cell Signaling	1:500 in 5 % BSA in TBST
Bax	rabbit	Cell Signaling	1:1000 in 5 % BSA in TBST
Bcl-2	rabbit	Cell Signaling	1:1000 in 5 % BSA in TBST
Bcl-xL	rabbit	Cell Signaling	1:1000 in 5 % BSA in TBST
CD79A	rabbit	Cell Signaling	1:1000 in 5 % BSA in TBST
Cyclin D1	mouse	Cell Signaling	1:2000 in 5 % (w/v) skim milk in TBST
(Thr202/Tyr204)-Erk1/2	rabbit	Cell Signaling	1:1000 in 5 % BSA in TBST
FASN	rabbit	Cell Signaling	1:500 in 1 % Roche blocking solution in TBS
GAPDH	rabbit	Cell Signaling	1:5000 in 5 % (w/v) skim milk in TBST
(Ser32/36)-IκB	mouse	Cell Signaling	1:500 in 1 % Roche blocking solution in TBS
Mcl-1	rabbit	Cell Signaling	1:500 in 5 % BSA in TBST
NOXA	mouse	Calbiochem	1:500 in 1 % Roche blocking solution in TBS
p65	rabbit	Cell Signaling	1:1000 in 5 % BSA in TBST
Puma	rabbit	Cell Signaling	1:500 in 5 % BSA in TBST
(Ser235/236)-S6	rabbit	Cell Signaling	1:2000 in 1 % Roche blocking solution in TBS

Table 5: Secondary antibodies used in this study

antibody	source	company	dilution
anti-rabbit-IgG-HRP	goat	Cell Signaling	1:2000 in 5 % (w/v) skim milk in TBST
anti-mouse-IgG-HRP	goat	Cell Signaling	1:2000 in 5 % (w/v) skim milk in TBST

Skim milk powder	Fluka
Western Blocking Reagent	Roche
Protran nitrocellulose membrane	Boehringer
Filter paper, Gel-blotting paper	Schleicher & Schuell
Trans-Blot® semi-dry transfer cell	Bio-Rad
SuperSignal® West Dura Extended Duration Substrate	Pierce Biotechnology
Chemo luminescence detection film, Lumi-Film	Roche
Autoradiography cassette	Amersham Biosciences
Film processor, Curix60	AGFA

2.6.4 Pull-down of polyubiqutinated proteins

Cells were harvested and collected by centrifugation (1400rpm, 5 min, 4 °C). The pellet was then washed with 1 ml ice-cold PBS and the cells immediately lysed by addition of 500 µl lysis buffer (50 mM TRIS, 250 mM sodium chloride, 1 % Triton X-100, 5 mM EDTA, pH 7.6) and severe vortexing. Lysate was cleared by centrifugation (13.000 rpm, 15 min, 4 °C) and total protein concentration determined by Bradford assay. Pull-down of polyubiquitinated proteins was performed using agarose coupled TUBE2 (tandem ubiquitin binding entities) beads. For this, 1-2 mg of total protein was added to 15 µl of previously equilibrated agarose-TUBEs and incubated over night at 4 °C on a rocker platform. Beads were then washed three times with 1 ml TBST and supernatant removed without disturbing the beads. Polyubiquitinated proteins were eluted from agarose-TUBEs by addition of 1x Laemmli buffer (62.5 mM TRIS, 20 % (v/v) glycerol, 5 % (v/v) β-mercaptoethanol, 2 % (w/v) SDS, 1 % bromophenol blue, pH 6.8), boiling at 95 °C for 5 min and subsequent centrifugation (13.000 rpm, 5 min, RT). The cleared sample containing polyubiqutinated proteins was directly analyzed by Western blot.

Agarose-TUBE2	Tebu-Bio

2.6.5 Analysis of protein half-life

Protein stability was analyzed by cycloheximide pulse-chase experiments. Cells were exposed to 20 µg/m cycloheximide (CHX) and samples harvested after 15, 30, 45, 60 and 90 minutes. The cell pellet was washed in 1 ml ice-cold PBS and immediately frozen in liquid nitrogen. Samples were then lysed and expression of the protein of interest analyzed by Western blot. Treatment of the cells with CHX blocks protein biosynthesis and can therefore be used in time course experiments to determine protein half-life.

Cycloheximide	Sigma

2.7 RNA interference

For gene silencing a mixture of four siRNAs was used. Sequences of SMARTpool siRNAs are described in Table 6. Non-targeting siRNA was used as a control in all experiments. For transfection, cells were set to a density of 3.2×10^6 ml. 800 µl of the cell suspension were then mixed with 12.8 µl siRNA mixture (stock: 50 µM) in a 4 mm electroporation cuvette and transfected by electroporation using a single-pulse protocol (250 V, 1800 µF, ∞ W). 16 to 48 hours upon electroporation cells were treated according to requirements. To evaluate knockdown efficacy samples were harvested, lysed and analyzed by Western Blot.

Table 6: Sequences of siGenome SMARTpool siRNAs

target gene	sequences of SMARTpool siRNAs	
CD79A	CAUAGGAGAUGUCCAGCUG	CAAGGUCCCAGCAUCAUUG
	GGAAACGAUGGCAGAACGA	UCCAAUGCCCGCACAAUAG
p65	GGAUUGAGGAGAAACGUAA	CUCAAGAUCUGCCGAGUGA
	GGCUAUAACUCGCCUAGUG	GAUUGAGGAGAAACGUAAA
Cyclin D1	GUUCGUGGCCUCUAAGAUG	CCGAGAAGCUGUGCAUCUA
	GAACAGAAGUGCGAGGAGG	ACAACUUCCUGUCCUACUA

siGenome SMARTpool siRNA	Dharmacon
Non-targeting siRNA#1	Dharmacon
4 mm electroporation cuvette	Peqlab
EquiBio Easyject Plus electroporation unit	Peqlab

2.8 RNA analysis

2.8.1 RNA isolation

Total RNA from cell lines and primary samples was isolated using RNeasy kit according to the manufacturer's instructions.

RNeasy kit	Qiagen

2.8.2 cDNA synthesis

For cDNA synthesis 500 ng of total RNA were mixed with 200 ng oligo(dT) primer in a total volume of 15 µl H_2O. The sample was then heated to 70 °C for 5 min and immediately placed on ice for 1 min. A reaction mix (10 µl per sample) containing first strand-buffer, dNTPs, RNasin (25 Units) and the Reverse transcriptase M-MLV (200 Units) was added and the sample incubated at 42 °C for 60 min and 70 °C for 10 min. The cDNA was directly used for further analysis or stored by -20 °C.

M-MLV Reverse transcriptase	Promega
RNasin Ribunuclease inhibitor	Promega
Oligo(dT) primer	Promega
dNTPs	Fermentas
5x first strand buffer	Promega

2.8.3 Gene expression analysis

Cancer Cell Line Encyclopedia (CCLE)

The Cancer Cell Line Encyclopedia (CCLE) database (Barretina et al., 2012) was used to compare mRNA expression in 1036 cancer cell lines (www.broadinstitute.org/ccle). To compare *NOXA* gene expression patterns, RMA-normalized mRNA expression data for *NOXA* mRNA levels were downloaded from CCLE and analyzed using GraphPad Prism 5.0 software. For analysis of relative gene expression levels of the pro-apoptotic Bcl-2 family members, raw Affymetrix CEL files were downloaded from the CCLE database and analyzed using GeneSpring GX 12.0 software.

GeneSpring GX 12.0 software	Agilent

7900HT Fast Real-Time PCR System

NOXA mRNA expression in MCL cell lines and primary samples was analyzed using TaqMan-based real-time PCR. *NOXA* gene expression was normalized to the housekeeping gene *GAPDH*. TaqMan assays are depicted in Table 7. For gene expression analysis, a reaction mix consisting of 1 µl of cDNA, 2 µl 2x TaqMan Gene Expression Master Mix and 0.25 µl TaqMan assay in a total volume of 5 µl was prepared in a 384-well-plate. Gene

expression was then analyzed by the 7900HT Fast Real-Time PCR System using following thermal cycling conditions:

50 °C for 2 min
95 °C for 10 min
40 cycles of:
    95 °C for 15 sec
    60 °C for 60 sec

Table 7: TaqMan assays used for 7900HT Fast Real-Time PCR System

gene name	TaqMan assay number
NOXA/*PMAIP1*	Hs00560402_m1
GAPDH	Hs02758991_g1

7900HT Fast Real-Time PCR System	Applied Biosystems
MicroAmp® Optical 384-well reaction plate	Applied Biosystems
Optical Adhesive Covers	Applied Biosystems
TaqMan assays (Table 7)	Applied Biosystems
2x TaqMan Gene Expression Master Mix	Applied Biosystems

BioMark HD System

NOXA mRNA expression in the panel of different cancer cell lines was analyzed using TaqMan assays and the high-throughput BioMark HD System according to the manufacturers's instructions. TaqMan assays used for BioMark HD System are listed in Table 8.

Table 8: TaqMan assays used for BioMark HD System

gene name	TaqMan assay number
NOXA/*PMAIP1*	Hs00560402_m1
TBP	Hs00427620_m1

BioMark HD System	Fluidigm
TaqMan assays (Table 8)	Applied Biosystems

2.9 Metabolomics

2.9.1 Extraction of intracellular metabolites

Intracellular metabolites were obtained by methanol/chloroform extraction. 10 x 10^6 cells were harvested and centrifuged (1400 rpm, 5 min, 4 °C). The pellet was washed once with ice-cold PBS and quick-frozen in liquid nitrogen. The frozen pellet was homogenized in 160 µl ice-cold methanol and 50 µl H_2O and sonificated for 20 sec. In the next step, 160 µl chloroform and 190 µl H_2O were added and the sample mixed by vortexing for 1 min. The sample was then placed on ice for 10 min for phase separation. After 10 min of centrifugation (2000 g, 10 min, 4 °C) the upper aqueous phase and the lower organic were separated and the extracts dried at 40 °C under nitrogen and frozen at -20 °C.

Chloroform	Roth
Methanol	Roth

2.9.2 Analysis of intracellular metabolite concentrations

Analysis of intracellular metabolites was performed by the Analytical Department of the Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology. The concentrations of pyruvate, fumarate, malate, citrate and ketoglutarate were analyzed by GC-MS as described by Hofmann *et al.* and Maier *et al.* (Hofmann et al., 2008; Maier et al., 2009). The phosphorylated metabolites hexose-6-phosphate (hexose-6P), fructose-6-phosphate (fructose-5P), phosphoenolpyruvate (PEP), 6-phosphoglycerate, sedoheptulose-7-phosphate (sedoheptulose-7P), ribose-5-phosphate (ribose-5P) and ribulose-5-phosphate (ribulose-5P) were analyzed by LC-MS-MS as described by Maier *et al.* (Maier et al., 2010). Triglycerides (TAGs) were analyzed by LC-MS as described by Niklas *et al.* (Niklas et al., 2012).

2.10 Statistics

All data represent means ±SD. Two-sided paired *t*-Test was used for statistical analysis of differences in paired samples. Differences with a *P*-value < 0.05 were considered to be statistically significant.

3 Results

3.1 MCL cells are characterized by high constitutive *NOXA* mRNA expression but low levels of NOXA protein

The BH3-only protein NOXA has been shown to play an essential role in fine-tuning the apoptotic response upon various cellular stresses (Ploner et al., 2008) and its constitutive expression levels can influence the response to chemotherapy (Brinkmann et al., 2013; Gutekunst et al., 2013).

Recent studies highlighted an important role of NOXA protein in B cell development mediating apoptosis in cycling and activated, low affinity B cells (Bretz et al., 2011; Wensveen et al., 2011). NOXA was also proposed to be an important player in Bortezomib-induced apoptosis in MCL (Pérez-Galán et al., 2006). A better understanding of the role of this pro-apoptotic Bcl-2 family member in MCL and the mechanism of how *NOXA* is regulated may offer new and more effective strategies for therapy of this aggressive B cell malignancy. Consequently, the first part of this study was aimed at the elucidation of the phenotype of constitutive expression of *NOXA* transcript and protein in MCL.

3.1.1 *NOXA* (*PMAIP1*) gene expression levels

To compare *NOXA* transcript levels in a broad range of cancer entities and to get an idea of the heterogeneity of *NOXA* gene expression the public available datasets from the Cancer Cell Line Encyclopedia (CCLE) were used (Barretina et al., 2012). CCLE is a database providing whole genome gene expression profiles of a large collection of cancer cell lines (www.broadinstitute.org/ccle).

Interestingly, *NOXA* gene expression levels were highly variable not only between the different cancer entities but also between tumors of the same origin (Figure 1A). Nevertheless, median *NOXA* mRNA expression in cell lines derived from hematologic malignancies was the second highest of all tumor entities (Figure 1A). Among blood cancers, *NOXA* expression was very heterogeneous. Notably, MCL cell lines together with anaplastic large cell lymphoma (ALCL) and chronic lymphocytic leukemia (CLL) expressed the highest amounts of *NOXA* transcript (Figure 1B). Three of five MCL cell lines were among the twenty cell lines with highest *NOXA* mRNA levels and the MCL cell line Granta519 even

showed the highest *NOXA* expression in the panel of more than 1000 cell lines available from CCLE (Figure 1C).

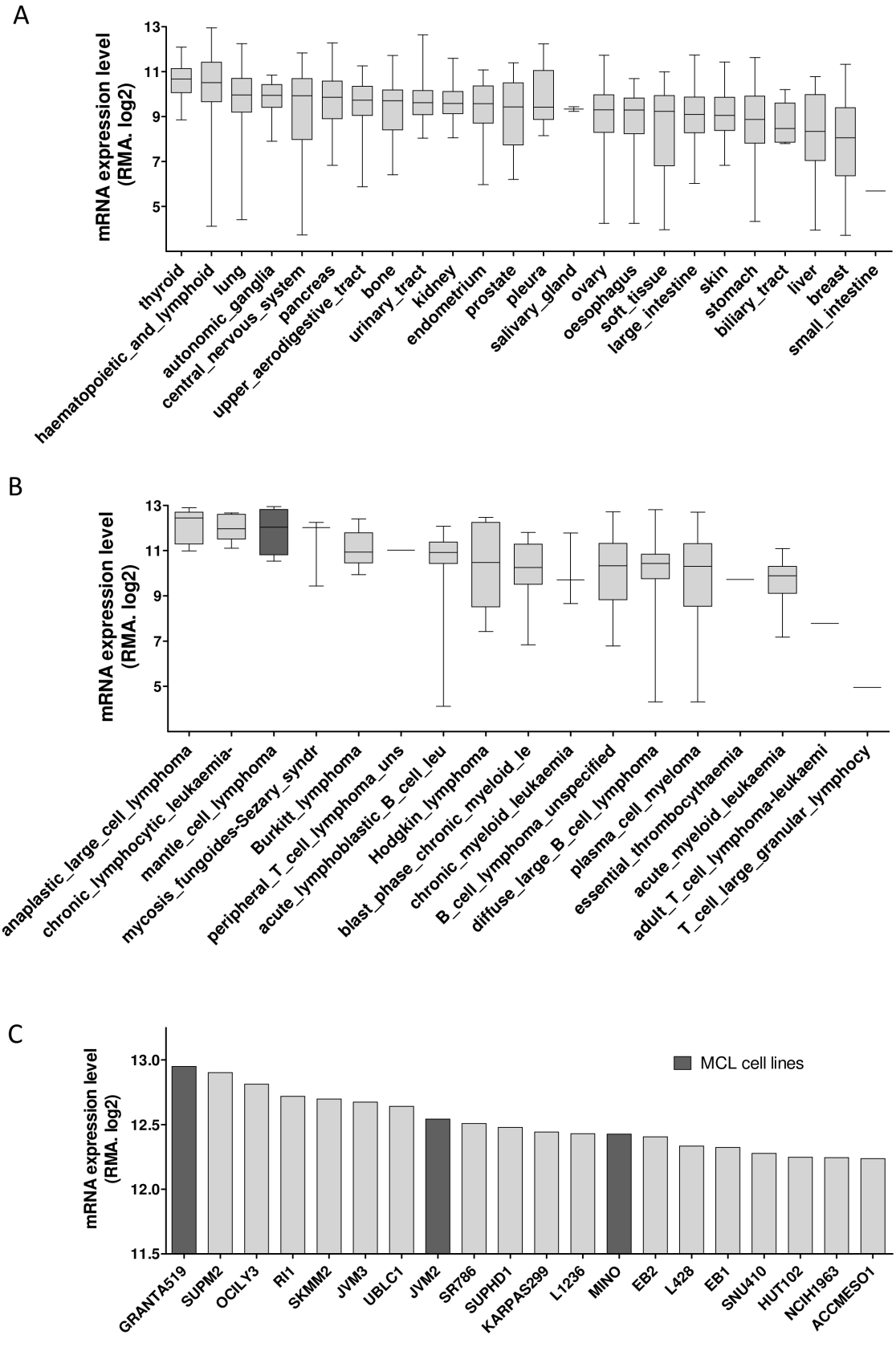


Figure 1: Heterogeneity of *NOXA* gene expression in cancers cell lines derived from different entities.
NOXA mRNA expression profile in a broad range of cancer cell lines was compared using gene expression data available from CCLE database. (A) Comparison of *NOXA* transcript levels across the range of on CCLE available cancer entities. Log2 transformed RMA values derived from CCLE database. (B) Constitutive *NOXA* transcript levels in cell lines of hematologic and lymphoid malignancies. Log2 transformed RMA values derived from CCLE database. (C) The twenty cell lines with highest *NOXA* mRNA expression in the panel of cancer cell lines available on CCLE. Log2 transformed RMA values derived from CCLE database.

To better assess the significance of the observed high constitutive *NOXA* transcript levels in MCL cells the gene expression profile of the whole set of the pro-apoptotic Bcl-2 family members was evaluated. Astonishingly, *NOXA* transcript was found to be the most abundant mRNA among this panel in MCL cells (Figure 2) indicating a central role of this BH3-only gene in MCL.

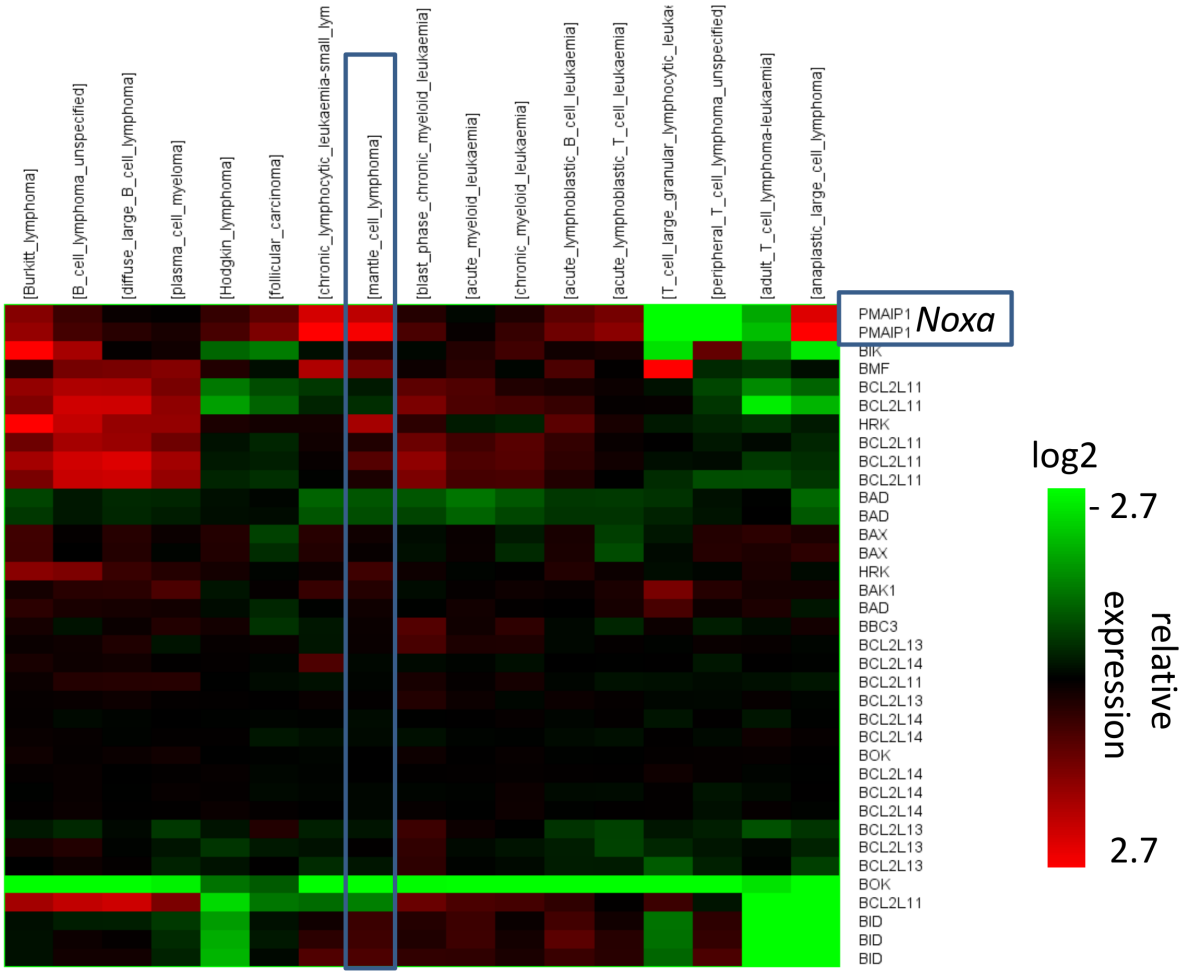


Figure 2: *NOXA* transcript is the most abundant mRNA of pro-apoptotic Bcl-2 family members in MCL cell lines.
Comparison of relative gene expression levels of pro-apoptotic Bcl-2 family members in hematopoietic and lymphoid malignancies. Heat map represents log2 transformed RMA normalized values. Gene expression data were obtained from CCLE and analyzed using Genespring gene expression analysis software.

3.1.2 Discrepancy between *NOXA* transcript and NOXA protein levels in MCL

In order to confirm the observation made with data from the CCLE, *NOXA* gene expression was measured in our own collection of cancer cell lines consisting of MCL cell lines (Mino, Rec1), embryonal carcinoma cell lines (NTERA2/D1, 2102EP) lung cancer cell lines (A549, NCI-H23, NCI-H460) and ovarian cancer cell lines (OVCAR5, SKOV3, A2780). *NOXA* mRNA levels were significantly higher in the MCL cell lines when compared to the other cancer cell lines derived from different entities and PBMCs of healthy donors (Figure 3A).
Since it has recently been shown that *NOXA* expression is regulated both at transcriptional (Ploner et al., 2008) as well as post-transcriptional levels (Baou et al., 2010; Brinkmann et al., 2013), NOXA protein levels were measured in the same panel of cell lines by Western blot analysis to investigate whether transcript and protein levels are correlated in these cells (Figure 3B). Remarkably, the pattern of NOXA protein expression was not correlated to mRNA expression. Some cell lines exhibited higher proteins levels than expected from the gene expression data (2102EP, H460) whereas others expressed much lower NOXA protein compared the transcript level. Especially in the MCL cell lines Mino and Rec1 NOXA protein was found to be relatively low contrasting the high transcript levels detected in these cells.

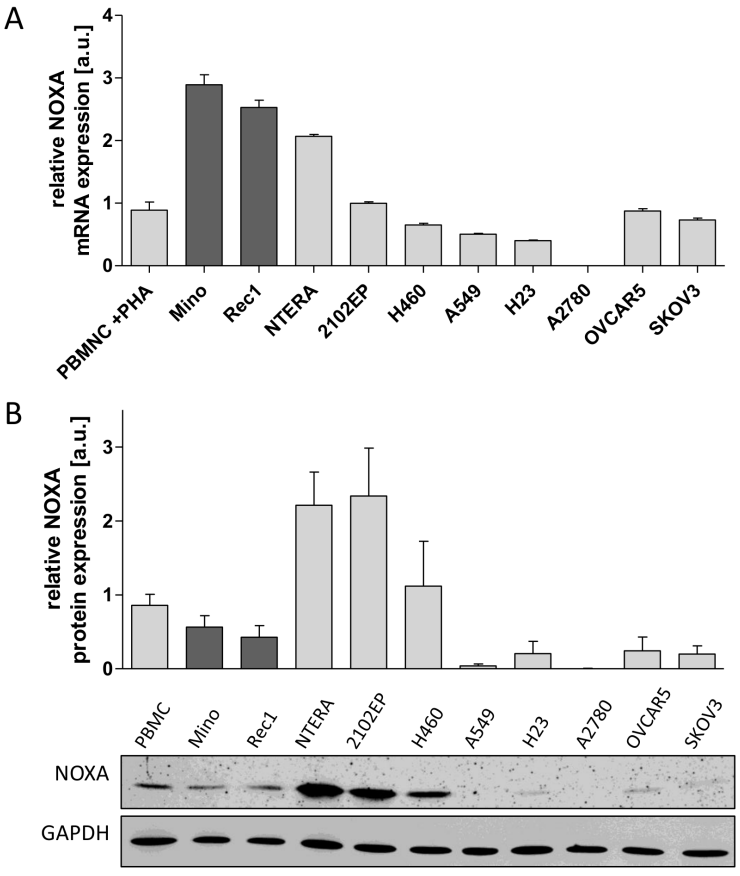
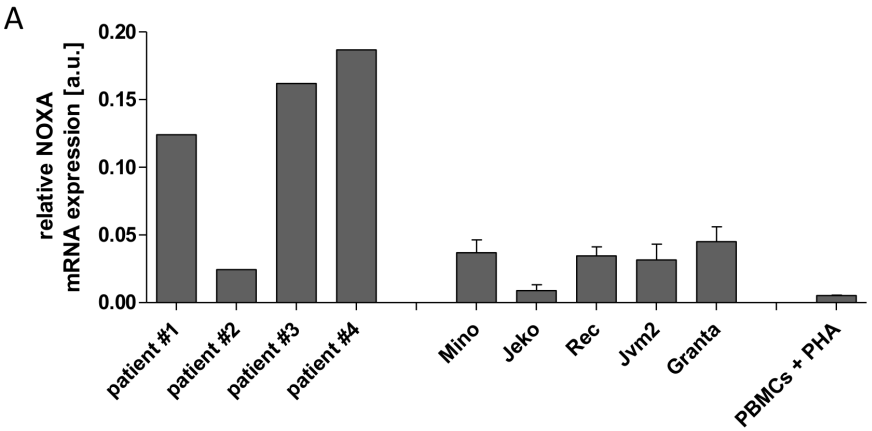


Figure 3: *NOXA* mRNA and protein expression is not correlated in most cancer cell lines.
NOXA mRNA and protein expression was analyzed in a panel of cancer cell lines derived from different entities (A) Analysis of *NOXA* gene expression in MCL cell lines Mino and Rec1 compared to embryonal carcinoma cell lines (NTERA2/D1, 2102EP) lung cancer cell lines (A549, NCI-H23, NCI-H460), ovarian cancer cell lines (OVCAR5, SKOV3, A2780) and PHA stimulated PBMCs of healthy donors using high-throughput real-time PCR BioMark HD System. *NOXA* mRNA expression was normalized to *TBP*. Data represent means ±SD from three technical replicates. (B) Comparison of NOXA protein expression levels in MCL cell lines Mino and Rec1, embryonal carcinoma cell lines NTERA2/D1 and 2102EP, lung cancer cell lines A549, NCI-H23 and NCI-H460, ovarian cancer cell lines OVCAR5, SKOV3 and A2780, and PHA stimulated PBMCs of healthy donors. Upper panel: Densitometric evaluation of NOXA protein expression analyzed by Western blot analysis. NOXA protein expression was normalized to GAPDH levels. Data reflect means ±SD from 3 experiments. Lower panel: Representative Western blot of NOXA protein expression in the panel of cell lines.

To determine if the observed phenotype of high *NOXA* mRNA/low NOXA protein expression is also present *in vivo, NOXA* transcript and protein levels of the MCL cell lines Mino, Rec1, Jvm2, Granta519 and Jeko1 were compared with primary samples from MCL patients and PBMCs of healthy donors. Except Jeko1, all MCL cell lines expressed substantially higher *NOXA* mRNA levels than control cells and confirmed the previously observations made for Mino and Rec1. The primary samples derived from MCL patients displayed similar or even higher constitutive *NOXA* mRNA levels as compared with the MCL cell lines (Figure 4A). As expected, NOXA protein was only expressed at relatively low levels in MCL cell lines. NOXA protein was hardly detectable in primary MCL cells and the discrepancy between RNA and protein levels was even more pronounced (Figure 4B).
These data support the view that *NOXA* expression is highly regulated on various levels. Particularly in MCL cells a strong discrepancy between *NOXA* mRNA and NOXA protein was observed.



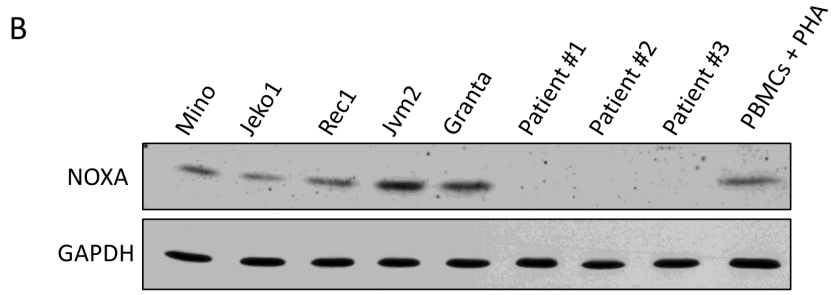


Figure 4: MCL cells have the phenotype of high *NOXA* mRNA/low NOXA protein levels.
Comparison of *NOXA* mRNA and protein expression in a panel of MCL cell lines, primary samples from MCL patients and PBMCs of healthy donors (A) *NOXA* mRNA levels in MCL cell lines Mino, Jeko1, Rec1, Jvm2, and Granta519, four samples from MCL patients, and PBMCs of healthy donors measured by conventional quantitative real-time PCR. *NOXA* mRNA expression was normalized to *GAPDH*. Data of MCL cell lines and PBMCs represent means ±SD from 3 experiments. (B) Constitutive NOXA protein expression in MCL cell lines Mino, Jeko1, Rec1, Jvm2, and Granta519, primary MCL samples and PHA stimulated PBMCs of healthy donors analyzed by Western blot.

3.2 High *NOXA* mRNA in MCL cells is mediated by chronic BCR signaling and Cyclin D1 overexpression

The previous results have shown that MCL cells express surprisingly high transcript levels of the BH-only gene *NOXA*. This pro-apoptotic Bcl-2 family member is probably best known as a transcriptional target of p53 in response to cellular stress (Ploner et al., 2008). However, more recently it has been shown that *NOXA* can also be induced independently of p53 (Hershko and Ginsberg, 2004; Pérez-Galán et al., 2006). The high *NOXA* mRNA levels observed in MCL cells seem also to be mediated in a p53 independent manner since high *NOXA* gene expression levels were also observed in MCL cell lines with mutant p53 (Figure 4). All further experiments aiming the investigation of how the high *NOXA* mRNA levels are mediated in MCL cells were conducted using a p53 wild type cell line (Rec1) and a p53 mutant cell line (Mino).

3.2.1 High *NOXA* mRNA is dependent on active BCR signaling

Various oncogenic signaling pathways are constitutively activated in MCL cells (Pérez-Galán et al., 2011a). One feature of MCL is the chronic activation of the BCR signaling pathway (Young and Staudt, 2013). Studies in normal B cells revealed that *NOXA* mRNA expression is induced upon BCR activation (Wensveen et al., 2012). To elucidate whether chronic signaling

of the BCR pathway mediates the high *NOXA* mRNA levels in MCL cells *CD79A*, an essential part of the BCR, was silenced by RNAi. Disturbance of chronic BCR activation reduced downstream signaling as monitored by measuring the phosphorylation status of BCR downstream effector kinases ERK, AKT, IκB and S6 (Figure 5A, upper panel). Inhibition of BCR signaling was accompanied by a significant decrease in *NOXA* gene expression (Figure 5A, lower panel). These results indicate that chronic active BCR signaling is indeed responsible for maintaining the high *NOXA* mRNA levels in MCL. This hypothesis was further supported by the observation of similar effects upon pharmacological inhibition of the BCR pathway using Ibrutinib, an inhibitor of the BCR associated kinase BTK (Figure 5B).

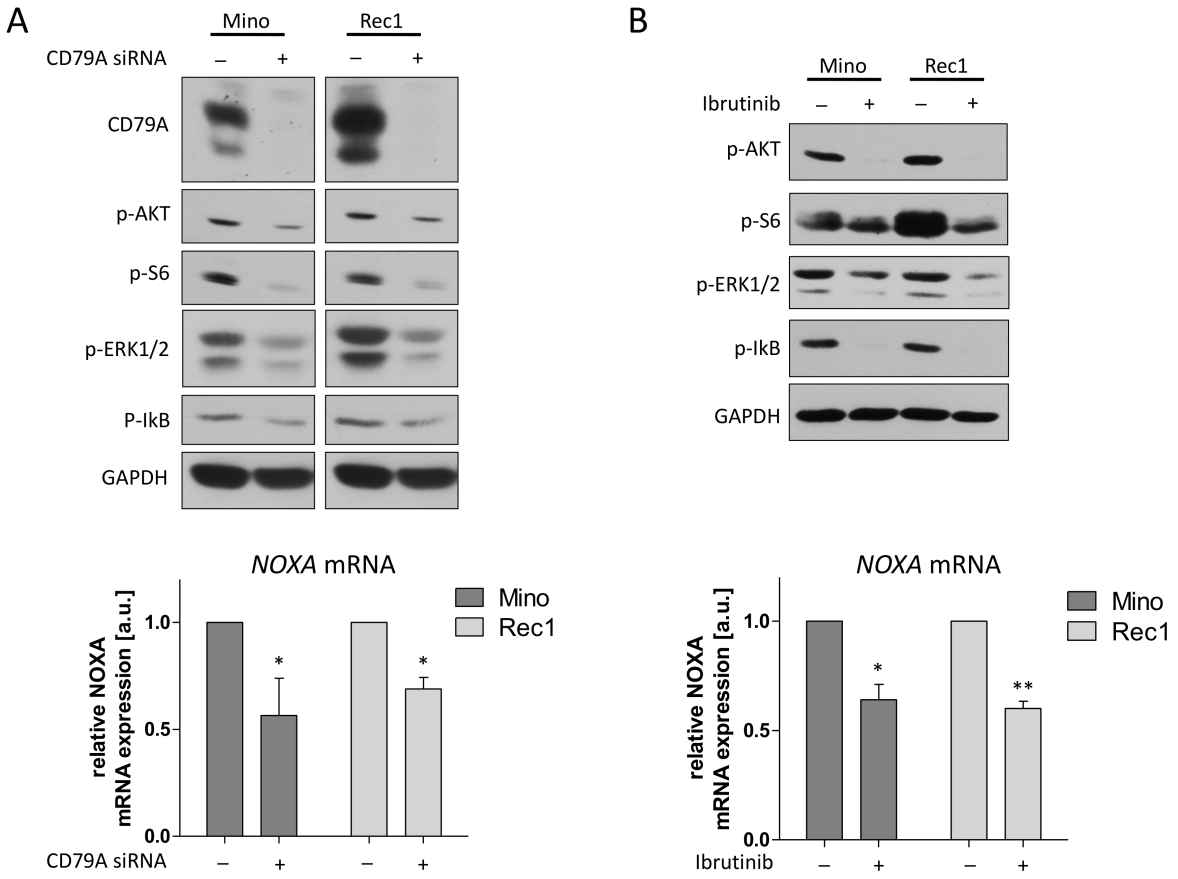


Figure 5: Chronic BCR signaling mediates the high constitutive *NOXA* mRNA levels in MCL cells.

(A) Effect of RNAi mediated silencing of *CD79A* on phosphorylation of BCR downstream effector kinases (upper panel) and constitutive expression of *NOXA* mRNA (lower panel; *: $P < 0.05$). Cells were transfected with non-targeting control siRNA or siRNA targeting *CD79A* and incubated for 24 hours. Knockdown efficacy and phosphorylation status was determined by Western blot analysis and of *NOXA* mRNA levels quantified by real-time PCR. *NOXA* mRNA expression was normalized to *GAPDH*. Data represent means ±SD from 3 experiments. (B) Effect of BTK inhibition on phosphorylation status of BCR downstream effector kinases (upper panel) and constitutive expression of *NOXA* mRNA (lower panel; *: $P < 0.05$, **: $P < 0.01$). Cells were treated with 1 µM Ibrutinib for 6 hours then harvested for Western blot analysis and quantification of *NOXA* mRNA levels. *NOXA* mRNA expression was normalized to *GAPDH*. Data represent means ±SD from 3 experiments.

3.2.2 PI3K pathway is the major mediator of *NOXA* expression downstream of the BCR

As mentioned before, chronic active BCR signaling engages multiple oncogenic downstream pathways including the MAP kinase (MAPK), NF-κB or PI3K/AKT/mTOR signaling pathway (Young and Staudt, 2013). To test which of the BCR downstream pathways mediates the high *NOXA* transcript levels in MCL, each signaling pathway was targeted individually using selective inhibitors or RNAi. Interestingly, inhibition of the MAPK pathway by PD0325901, a small molecule inhibitor targeting MEK1/2, slightly enhanced *NOXA* transcription in both MCL cell lines (Figure 6A). Moreover, knockdown of the transcription factor p65 (RELA), the main downstream effector of NF-κB signaling, did not significantly influence the high levels of *NOXA* mRNA (Figure 6B) indicating that both signaling pathway are not involved in the maintenance of the high *NOXA* transcript levels downstream of the BCR. In contrast, inhibition of the PI3K/AKT/mTOR pathway by the dual PI3K/mTOR inhibitor Bez235 strongly reduced *NOXA* transcripts (Figure 6C).

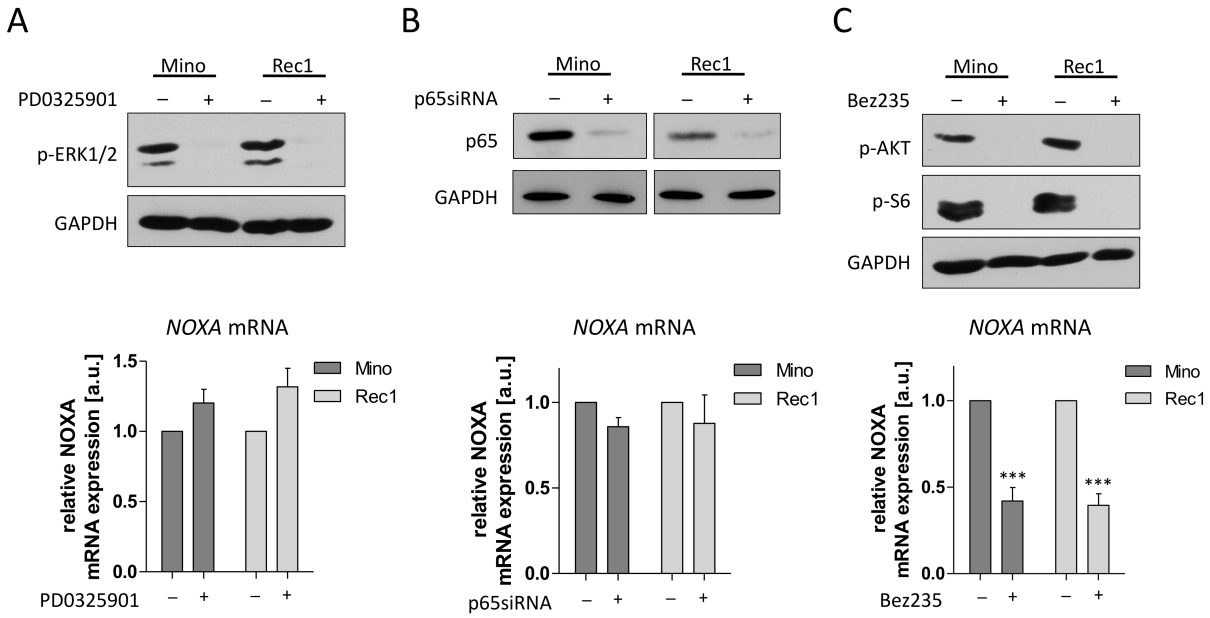


Figure 6: The PI3K/AKT/mTOR pathway is the major mediator of high *NOXA* mRNA levels downstream of the BCR. Impact of inhibition of BCR downstream pathways on constitutive *NOXA* expression. (A) Inhibition of the MAPK pathway. Cell lines Mino and Rec1 were treated with 1 µM of PD0325901 for 6 hours then harvested for Western blot analysis and quantification of *NOXA* mRNA. (B) Effect of p65 knockdown. MCL cell lines Mino and Rec1 were transfected with control siRNA or siRNA targeting p65 and incubated for 48 hours. Knockdown efficacy was measured by Western blot analysis and *NOXA* mRNA levels quantified by real-time PCR. (C) Inhibition of PI3K/AKT/mTOR pathway. Cell lines Mino and Rec1 were treated with 1 µM of Bez235 for 6 hours then harvested for Western blot analysis and quantification of *NOXA* mRNA levels (***: $P < 0.001$).

NOXA mRNA expression was normalized to *GAPDH*, respectively. All data represent means ±SD from 3 experiments.

Further experiments using different inhibitors of the PI3/AKT/mTOR pathway including the PI3K inhibitors LY294002 and BKM120 and the mTOR inhibitor Temsirolimus supported this finding as all of the inhibitors were able to reduce *NOXA* transcript levels. Interestingly, highest efficacy was observed when PI3K and mTOR inhibitors were combined, indicating that both PI3K and mTOR are essential for maintaining high *NOXA* transcription (Figure 7).

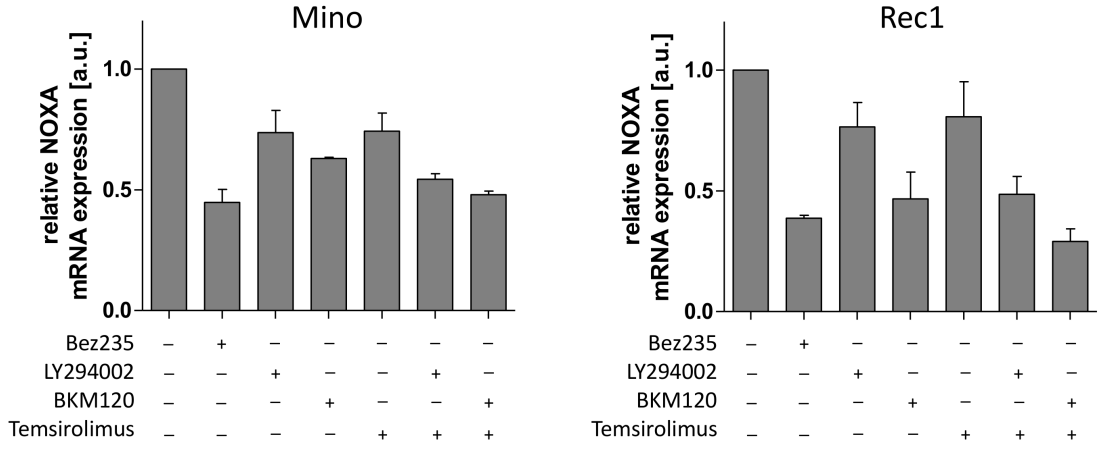


Figure 7: PI3K and mTOR signaling are needed for maintenance of high *NOXA* transcript levels in MCL.
Effects of different inhibitors of the PI3/AKT/mTOR pathway alone and in combination on *NOXA* mRNA expression. MCL cell lines Mino and Rec1 were treated with the PI3K/mTOR dual inhibitor Bez235 (1 μM), the PI3K inhibitor LY29004 (10 μM), the PI3K inhibitor BKM120 (3μM) and the mTOR inhibitor Temsirolimus (1 μM) or with combinations of LY29004 and Temsirolimus or BKM120 and Temsirolimus. 6 hours upon treatment cells were harvested for quantification of *NOXA* mRNA levels by real-time PCR. *NOXA* mRNA expression was normalized to *GAPDH*. Data represent means ±SD from 2 experiments.

The phenotype of high *NOXA* mRNA expression was also observed in primary MCL cells (Figure 4). To elucidate whether constitutive activation of the PI3K/AKT/mTOR pathway is also present in primary MCL cells and responsible for the high transcript levels of the BH3-only gene *NOXA*, PI3K and mTOR activity of primary MCL samples was compared to MCL cell lines and stimulated PBMCs of healthy donors. As shown in Figure 8A, cell lines as well as primary MCL cells showed significant phosphorylation of AKT and/or S6, the downstream targets of PI3K and mTOR (Figure 8A). Inhibition of the PI3K/AKT/mTOR pathway by treatment of primary tumor cells with Bez235 led to a strong reduction of *NOXA* mRNA expression (Figure 8B) further supporting the previous findings of the PI3K/AKT/mTOR pathway being the major player in the maintenance of the high *NOXA* gene expression levels in MCL.

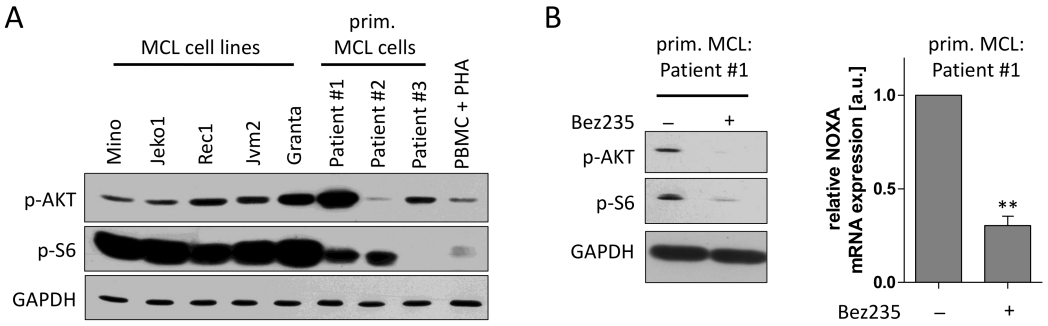


Figure 8: PI3K/AKT/mTOR signaling is also mediating the high *NOXA* transcript levels in primary MCL cells.
(A) Comparison of constitutive PI3K/AKT/mTOR signaling in MCL cell lines, primary MCL samples and PBMCs of healthy donors. Intensity of PI3K/AKT/mTOR signaling was assessed by analyzing the phosphorylation status of PI3K downstream kinases AKT and S6 by Western blot analysis. (B) Effect of PI3K/AKT/mTOR pathway inhibition on constitutive *NOXA* mRNA expression in MCL patients. Primary MCL cells were treated with 1 µM of Bez235 for 6 hours then harvested for Western blot analysis and quantification of *NOXA* mRNA levels (**: $P < 0.01$). *NOXA* mRNA expression was normalized to *GAPDH*. Data represent means ±SD from 3 technical replicates.

3.2.3 Cyclin D1 overexpression contributes to the high *NOXA* mRNA levels by exerting an positive feedback loop an on the PI3K/AKT/mTOR pathway

Cyclin D1 overexpression constitutes the central oncogenic mechanism in MCL and is considered to be the primary event in the pathogenesis of this B cell malignancy (Jares et al., 2007, 2012). Consequently, it was investigated whether the aberrant expression of Cyclin D1 also influences *NOXA* gene expression in MCL. Interestingly, RNAi mediated down-regulation of *Cyclin D1* showed similar results as previously observed for inhibition of BCR signaling although not that pronounced (Figure 9). Remarkably, Western blot analysis revealed that reduced Cyclin D1 expression led to a partial inhibition of the PI3K/AKT/mTOR pathway in MCL cells pointing to an existing positive feedback loop on the PI3K/AKT/mTOR pathway (Figure 9, left panel). This unexpected effect might be responsible for the decrease in *NOXA* mRNA levels upon silencing of *Cyclin D1*. The hypothesis was supported by the finding that RNAi mediated knockdown of *Cyclin D1* did not further reduce *NOXA* mRNA in cells which were treated with the PI3K/AKT/mTOR pathway inhibitor Bez235 indicating that both mechanisms target the same pathway (Figure 10).

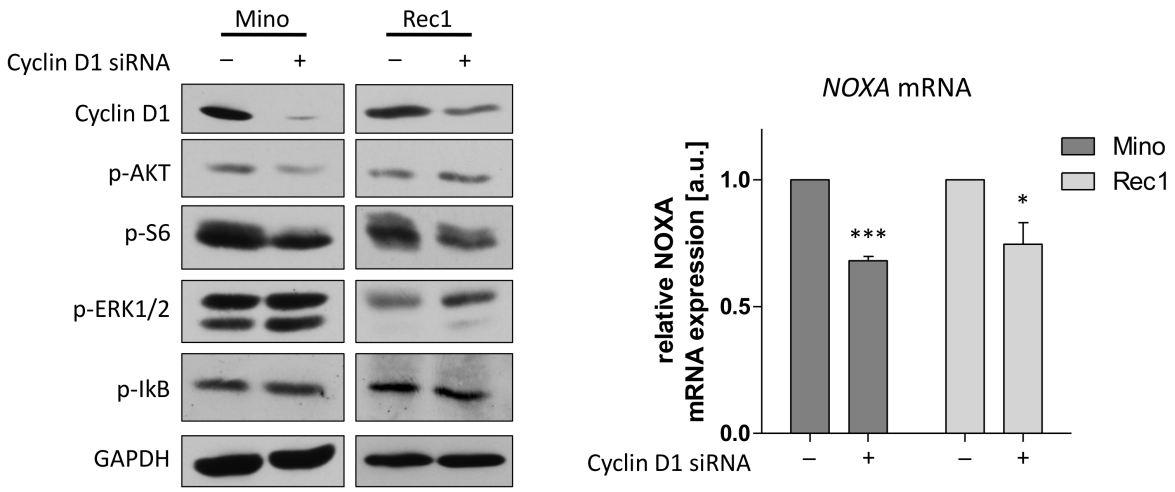


Figure 9: Cyclin D1 overexpression in MCL contributes to high *NOXA* mRNA levels in MCL.
Knockdown of *Cyclin D1* attenuates PI3K/AKT/mTOR signaling and reduces constitutive *NOXA* mRNA levels in MCL cell lines Mino and Rec1. Cells were transfected with control siRNA or siRNA targeting *Cyclin D1* and incubated for 16 hours. Knockdown efficacy and effect on the main signaling pathways was analyzed by Western blot analysis (left panel). *NOXA* mRNA levels were quantified by real-time PCR (lower panel; *: $P < 0.05$, ***: $P < 0.001$). *NOXA* mRNA expression was normalized to *GAPDH*. Data represent means ±SD from 3 experiments.

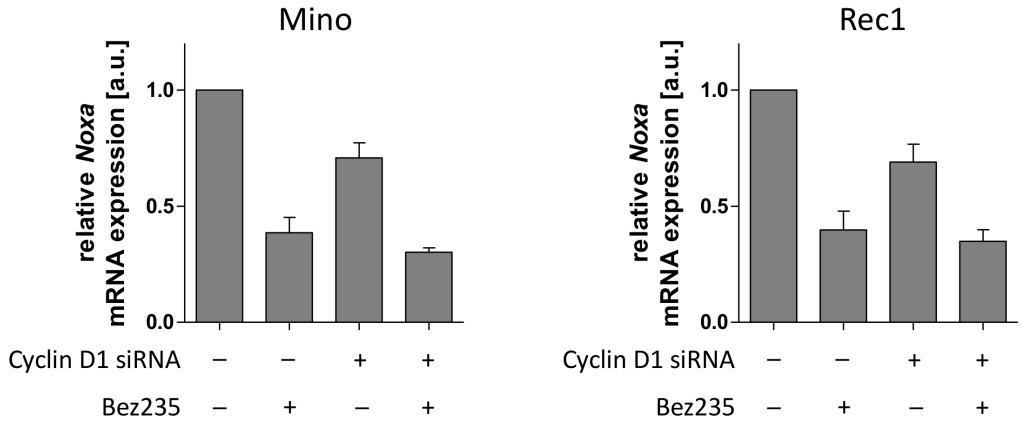


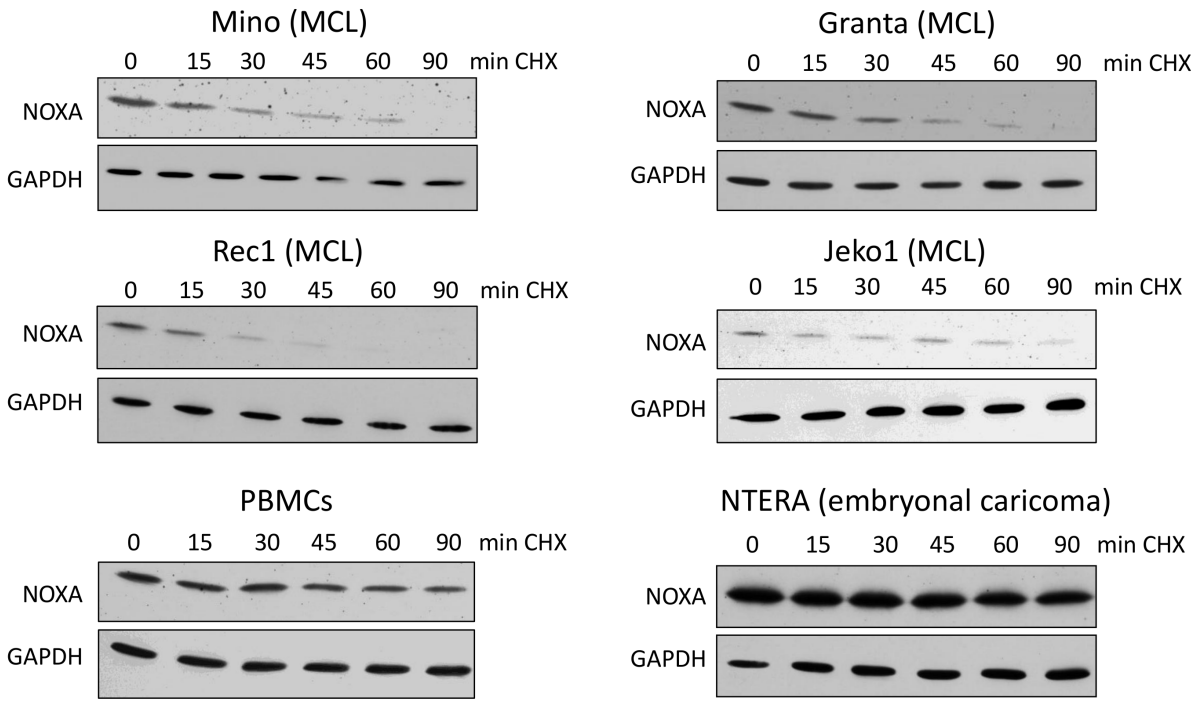
Figure 10: Cyclin D1 contributes to the high constitutive *NOXA* mRNA expression via the PI3/AKT/mTOR pathway.
MCL cells were transfected with control siRNA or siRNA targeting *Cyclin D1*. After 16 hours cells were treated with 1 µM Bez235 and cultivated for another 6 hours. *NOXA* mRNA expression was then analyzed by real-time PCR. *NOXA* mRNA expression was normalized to *GAPDH*. Data represent means ±SD from 3 experiments.

3.3 NOXA protein is rapidly degraded by the ubiquitin-proteasome system in MCL

The observation that BCR- and Cyclin D1-mediated high *NOXA* mRNA levels in MCL do not lead to significant NOXA protein raised the question of the cause of this strong discrepancy between *NOXA* mRNA and NOXA protein level.

3.3.1 NOXA protein has a very short half-life in MCL cells

To determine whether the lack of correlation between *NOXA* transcript and protein is due to increased NOXA protein turnover in MCL cells, NOXA protein stability was analyzed in MCL cells and compared to that of a panel of cells by cycloheximide pulse-chase experiments and subsequent Western blot analysis (Figure 11). Compared to PBMCs of healthy donors and the panel of cancer cell lines NOXA protein turnover was found to be significantly enhanced in MCL cells. The half-life of NOXA was 15 - 30 minutes in the MCL cell lines Mino, Rec1, Granta519 and Jeko1. In contrast, NOXA protein was significantly more stable in PBMCs and the cancer cell lines NTERA, H460 and OVCAR5. These data indicate that MCL cells adapt to the constitutive high levels of *NOXA* mRNA by rapidly degrading NOXA protein thereby keeping protein levels low in the cell.



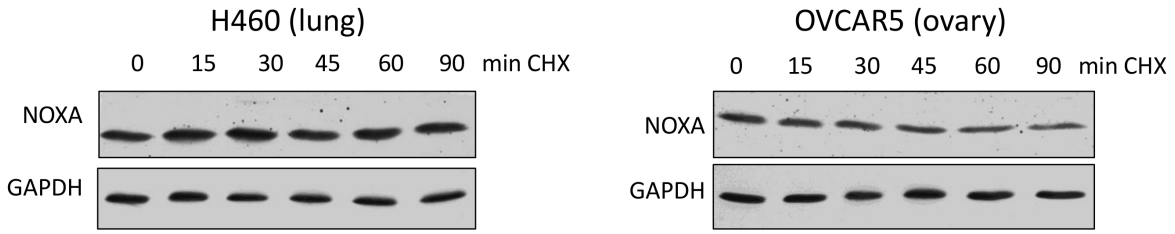


Figure 11: NOXA protein is rapidly degraded in MCL cells.

NOXA protein stability in MCL cell lines (Mino, Rec1, Granta519 and Jeko1), PHA stimulated PBMCs of healthy donors and the cancer cell lines NTERA (embryonal), H460 (lung) and OVCAR5 (ovary) was measured by cycloheximide pulse-chase experiments. Cells were exposed to 20 µg/ml cycloheximide (CHX) and samples harvested after 15, 30, 45, 60 and 90 minutes. Half-life of NOXA was then determined by Western blot analysis.

3.3.2 NOXA protein is extensively polyubiquitinated and degraded by the proteasome

Recent studies using different cell systems have shown that NOXA protein levels are post-translationally regulated by the ubiquitin-proteasome system (UPS) (Baou et al., 2010; Brinkmann et al., 2013). To further explore the mechanism underlying the short NOXA protein half-life in MCL, it was investigated whether the rapid NOXA protein turnover is mediated by polyubiquitination and proteasomal degradation. As shown in Figure 12, pull-down of polyubiqutinated proteins using agarose coupled Tandem Ubiquitin Binding Entities (TUBEs) followed by Western blot analysis revealed that NOXA protein is extensively ubiquitinated and degraded by the proteasome. Inhibition of the proteasome by treatment of the cells with the proteasome inhibitors MG132 or Lactacystin led to accumulation of NOXA and its slower migrating polyubiquitinated forms in both MCL cell lines. These results demonstrate that although *NOXA* is transcriptionally highly expressed in MCL cells, NOXA protein expression is low due to rapid UPS-mediated turnover.

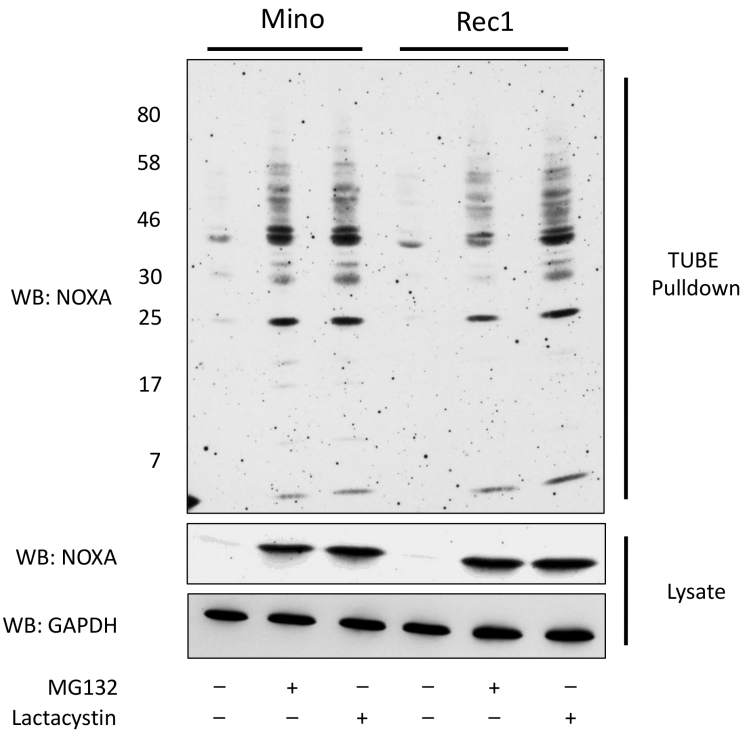


Figure 12: NOXA protein is polyubiquitinated and target of proteasomal degradation.
Cells were cultivated in absence or presence of the proteasome inhibitors MG132 (1 µM) or Lactacystin (5 µM) for 8 hours and then harvest and lysed. Total polyubiquitinated proteins were isolated from cell lysates using agarose-TUBE2 beads. Protein expression and polyubiquitination state of NOXA protein was analyzed by Western blot analysis using a NOXA specific antibody.

3.3.3 Rapid NOXA turnover is independent of the major oncogenic signaling pathways

The previous results have shown that NOXA protein turnover rate is variable and depends on the cellular background. Moreover, NOXA degradation in MCL was found to be a highly regulated process, which is mediated by the UPS. Chronic BCR signaling and Cyclin D1 overexpression was shown to play a major role in maintenance of high *NOXA* mRNA levels. However, these oncogenic pathways might mediate a dual signal and also influence NOXA protein stability. To test this possibility, changes in NOXA protein expression upon inhibition of BCR and Cyclin D1 signaling were investigated by Western blot analysis. As shown in Figure 13, both RNAi mediated knockdown of *CD79A* (left panel) and *Cyclin D1* (right panel) led to a further reduction of the already low NOXA protein levels in MCL. Changes in *NOXA* mRNA expression upon inhibition of these two major oncogenic pathways in MCL were apparently mirrored on protein levels indicating that rapid NOXA protein turnover and

therefore the cause of the strong discrepancy between *NOXA* mRNA and protein expression is not mediated by a dual signal through chronic BCR activation or Cyclin D1 overexpression.

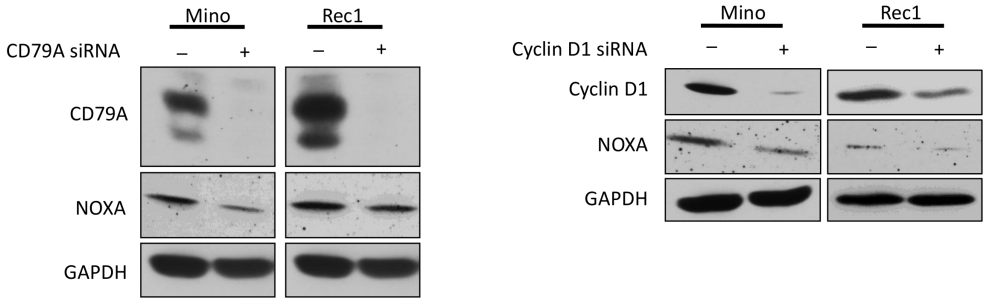


Figure 13: Knockdown of CD79A or Cyclin D1 reduces constitutive expression levels of NOXA protein.
MCL cell lines Mino and Rec1 were transfected with control siRNA and siRNA targeting *CD79A* (left panel) or *Cyclin D1* (right panel) incubated for 24 and 16 hours, respectively. Knockdown efficacy and effect on constitutive NOXA protein levels was analyzed by Western blot analysis.

Nevertheless, from these results it could not be completely excluded that chronic BCR signaling mediates a dual signal in the MCL cells since BCR activation engages multiple downstream pathways and effects on *NOXA* mRNA and NOXA protein stability upon upstream inhibition might interfere with each other. Therefore, NOXA protein turnover was measured upon individual pharmacological inhibition of BCR signaling and the respective downstream pathways. As depicted in Figure 14, targeting BCR signaling upstream with the BTK inhibitor Ibrutinib had no effect on the short NOXA protein half-life in both MCL cell lines suggesting that BCR signaling is indeed not the mediator of enhanced NOXA protein turnover. This was confirmed by further experiments inhibiting BCR downstream pathways since inhibition of these pathways had no stabilizing effect on rapid NOXA protein turnover: Targeting MAPK signaling by treatment of the cells with PD0325901 as well as disturbance of NF-κB signaling using IMD0354, an inhibitor of the kinase IκB, had no influence protein stability of the BH3-only protein in MCL cell. NOXA was even hardly detectable upon inhibition of the PI3K/AKT/mTOR pathway using Bez235 (Figure 14).

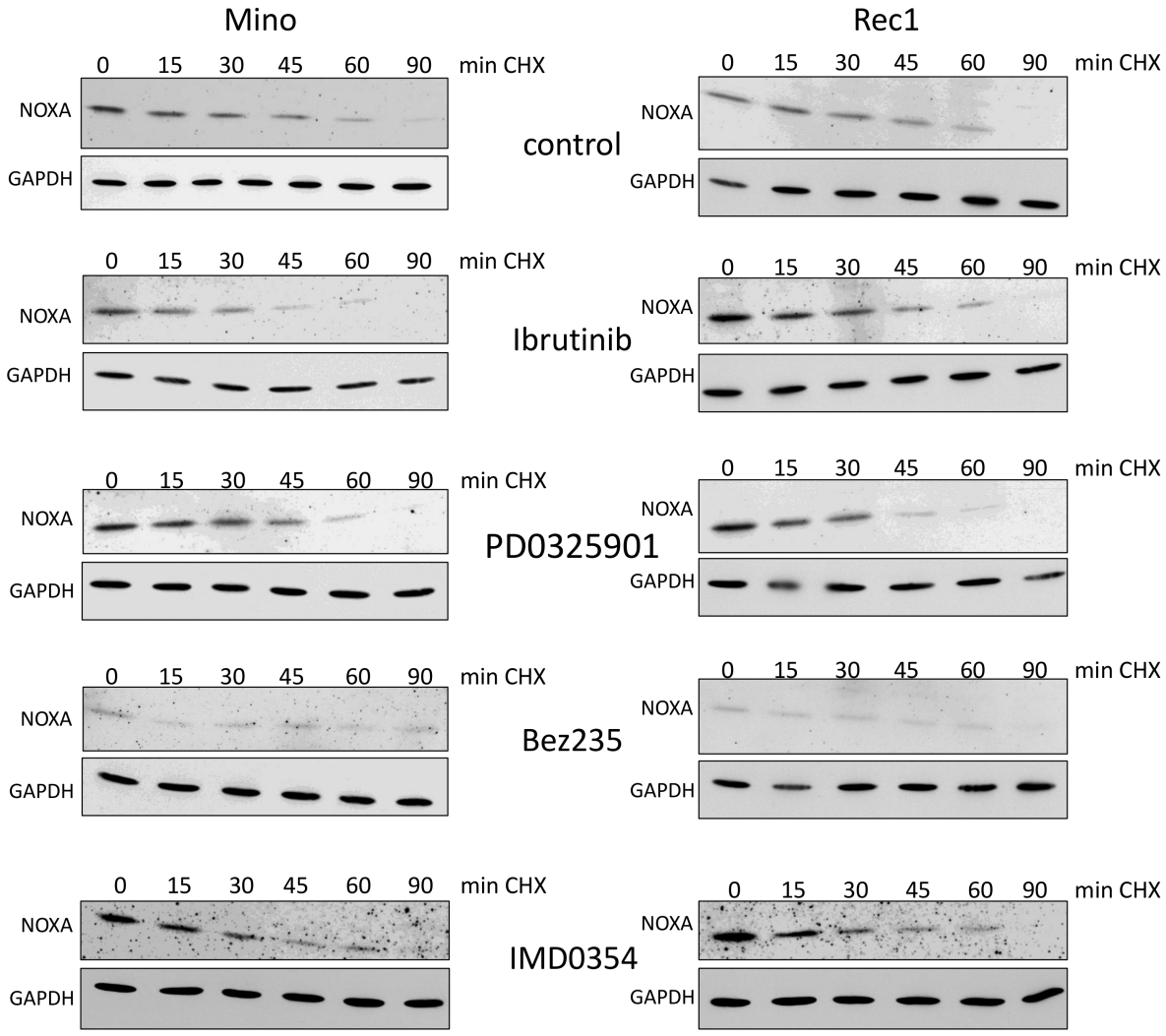


Figure 14: Inhibition of BCR signaling pathways in MCL has no effect in NOXA protein stability.

MCL cell lines Mino and Rec1 were treated with the BCR inhibitor Ibrutinib (1 µM), the MAPK pathway inhibitor PD032590 (1 µM), the PI3K/mTOR dual inhibitor Bez235 (1 µM) and the NF-κB signaling inhibitor IMD0354 (0.5 µM) for 6 hours. The cells were then exposed to 20 µg/ml cycloheximide (CHX) and samples harvested after 15, 30, 45, 60 and 90 minutes. Half-life of NOXA was determined by Western blot analysis.

3.4 Targeting NOXA protein turnover on different levels efficiently induces cell death in MCL cells

The preceding experiments have shown that MCL cells express BCR signaling and Cyclin D1 mediated high transcript levels of the pro-apoptotic Bcl-2 family member *NOXA*. Intriguingly, the high constitutive gene expression of this BH3-only gene does not impair cell viability. MCL cells adapt to this constitutive pro-apoptotic signal by extensive ubiquitination and rapid proteasomal degradation of NOXA protein, which was found to be independent of the main oncogenic pathways in MCL and seems to be regulated by other cellular signals. Nevertheless, targeting the enhanced NOXA protein turnover thereby stabilizing the pro-apoptotic protein and releasing the putative block in execution of cell death might represent an effective strategy to kill MCL cells. Therefore, the identification of substances that are able to accumulate NOXA protein could improve treatment of MCL and might help to uncover cellular processes involved in NOXA protein turnover.

3.4.1 Substances accumulating NOXA protein efficiently kill MCL cells

To identify compounds able to accumulate NOXA protein in MCL cells, known and potential NOXA inducers as well as substances, which have been shown to be effective in MCL, were screened for their capability to induce NOXA protein in the cell lines Mino and Rec1. As expected from our previous experiments using MG132 and Lactacystin and in accordance to recently published data (Holkova and Grant, 2012; Pérez-Galán et al., 2006) the proteasome inhibitor Bortezomib was a potent NOXA protein inducer in both MCL lines (Figure 15). Induction of NOXA protein expression, although not that pronounced, was also observed upon treatment with MLN4924, an inhibitor of neddylation, which is required by several E3 enzymes for ubiquitin ligase activity. Unexpectedly, the fatty acid synthase (FASN) inhibitor Orlistat was also identified to be able to accumulate NOXA protein levels. Orlistat has previously been shown to be effective against MCL (Gelebart et al., 2012), however, the mechanism of how the FASN inhibitor induces cell death remained unclear. Surprisingly, known transcriptional NOXA inducers such as the DNA damaging agents Cisplatin or Doxorubicin (Gutekunst et al., 2011; Kurata et al., 2008) were not able to induce NOXA protein in both p53 mutant (Mino) and wild-type (Rec1) MCL lines (Figure 15).

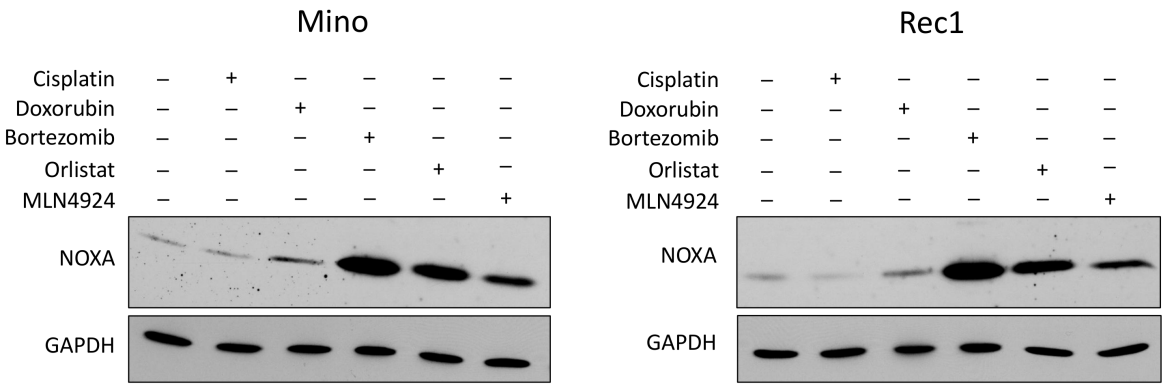


Figure 15: Treatment of MCL cells with Bortezomib, Orlistat and MLN4924 effectively accumulates NOXA protein.
MCL cell lines Mino and Rec1 were treated with the potential NOXA protein inducers Cisplatin (10 µM), Doxorubicin (100 nM), Bortezomib (10 nM), Orlistat (15 µM) or MLN4924 (0.5 µM) for 16 hours and NOXA protein expression was analyzed by Western blot.

To investigate if NOXA protein accumulation has any impact on cellular viability, induction of cell death upon treatment with the panel of substances was measured by Annexin V/PI staining and FACS analysis (Figure 16). In contrast to Doxorubicin, Cisplatin, although also lacking the ability to accumulate NOXA protein in MCL cells, was found to induce cell death in both cell lines independent of their p53 status. However, cell death was most efficiently induced by compounds, which accumulate NOXA protein in MCL cells. These data indicate that the discrepancy between *NOXA* mRNA and NOXA protein levels might represent a druggable Achilles heel of this aggressive malignancy.

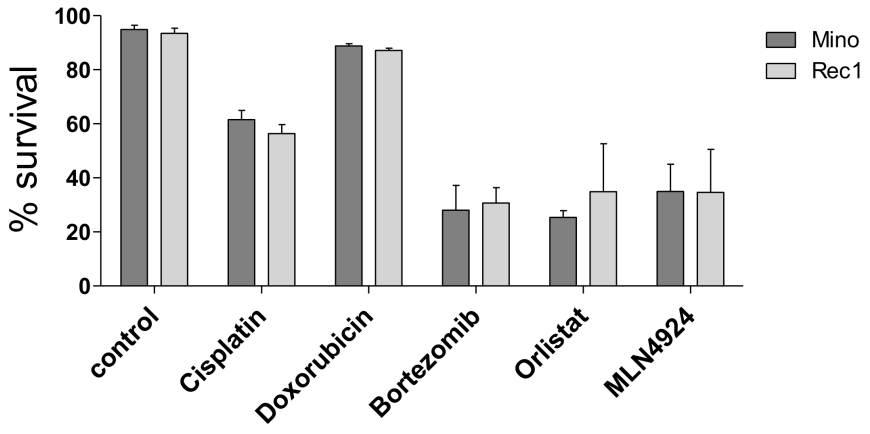
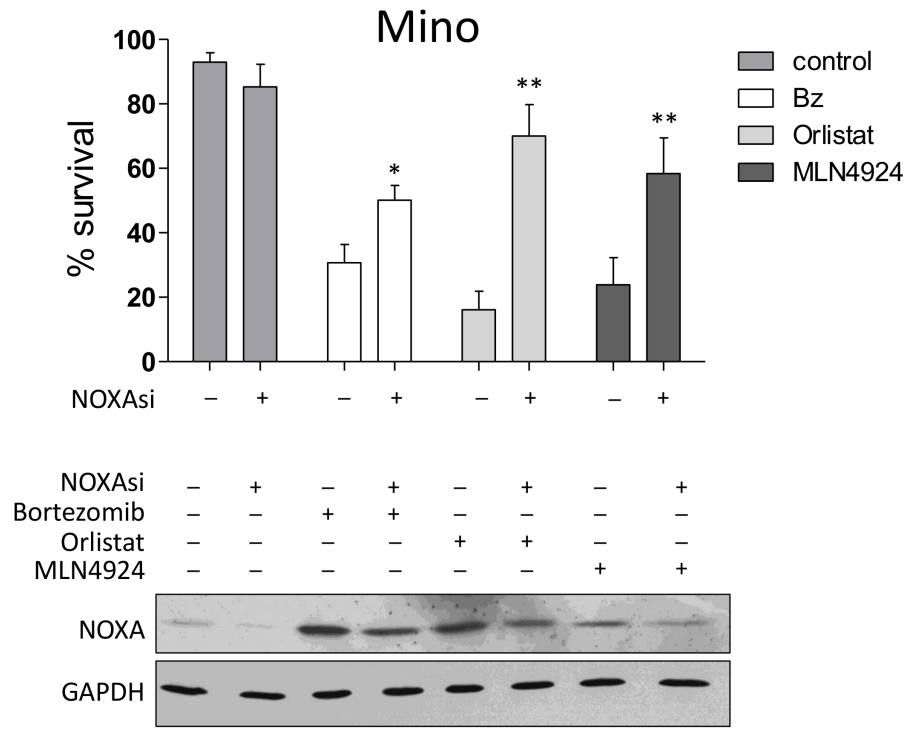


Figure 16: Accumulation of NOXA efficiently induces cell death.
MCL cells were treated with Cisplatin (10 µM), Doxorubicin (100 nM), Bortezomib (10 nM), Orlistat (15 µM) or MLN4924 (0.5 µM) for 24 hours and cell viability was analyzed by Annexin V/PI staining and subsequent flow cytometry. Data represent means ±SD from 3 experiments.

To rule out the possibility that the substances identified to induce NOXA protein accumulation in MCL cells kill independently of NOXA and to determine the role of the BH3-only protein in induction of cell death upon Bortezomib, Orlistat and MLN4924, *NOXA* was silenced prior to treatment. As shown in Figure 17, Western blot and FACS-based cell death analysis revealed that NOXA indeed plays a central role in induction of cell death following treatment with Bortezomib, Orlistat and MLN4924. RNAi mediated knockdown of *NOXA* significantly rescued both cell lines from apoptosis and reduced NOXA protein levels. Interestingly, induction of NOXA-dependent cell death upon MLN4924 treatment was as efficient as observed upon Bortezomib or Orlistat although NOXA protein induction was less pronounced in response to MLN4924 (Figure 17). As the Bcl-2 family member Mcl-1 is the main antagonist of NOXA and its cellular levels determine susceptibility to NOXA-induced cell death (Ploner et al., 2008), Mcl-1 expression was analyzed upon treatment with the NOXA accumulating agents. Western Blot analysis of Mcl-1 expression revealed that Mcl-1 is also induced upon treatment with Bortezomib and Orlistat whereas MLN4924 rather reduced the expression of the anti-apoptotic protein in MCL cells (Figure 18). This observation indicates that less NOXA protein accumulation is sufficient to efficiently induce apoptosis following treatment with MLN4924.



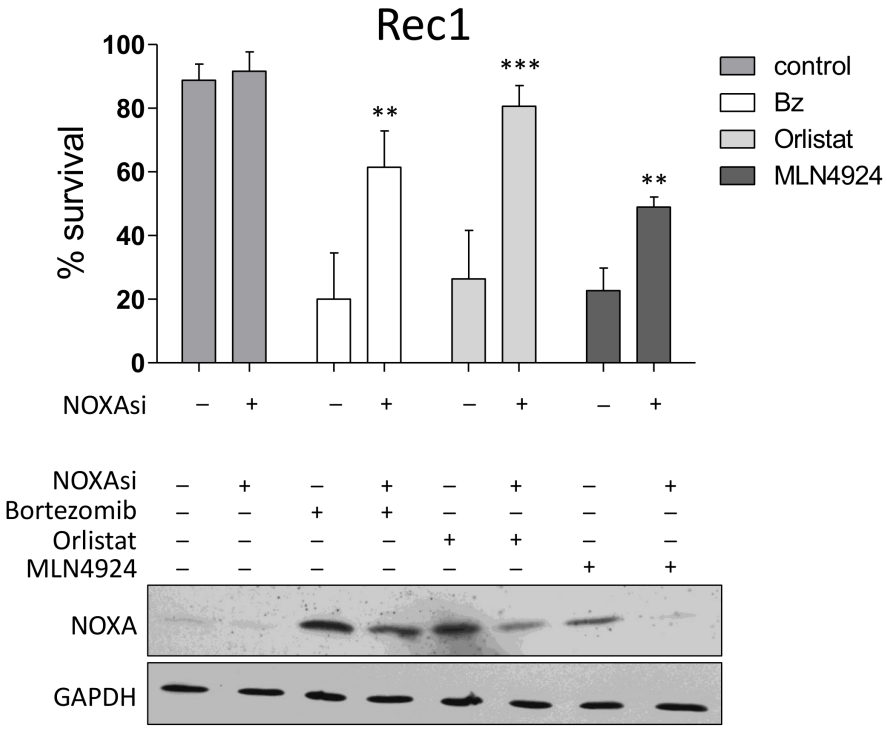


Figure 17: Cell death upon treatment of MCL cells is dependent on NOXA protein accumulation.

MCL cell lines Mino and Rec1 were transfected with control siRNA or siRNA targeting *NOXA*. 24 hours after transfection cells were treated with Bortezomib (10 nM), Orlistat (15 µM) or MLN4924 (0.5 µM) and further cultivated for 24 hours. Cells were then harvested for Western blot analysis and quantification of cell death by Annexin V/PI staining and flow cytometry (**: $P < 0.01$, ***: $P < 0.001$). Data represent means ±SD from 3 experiments.

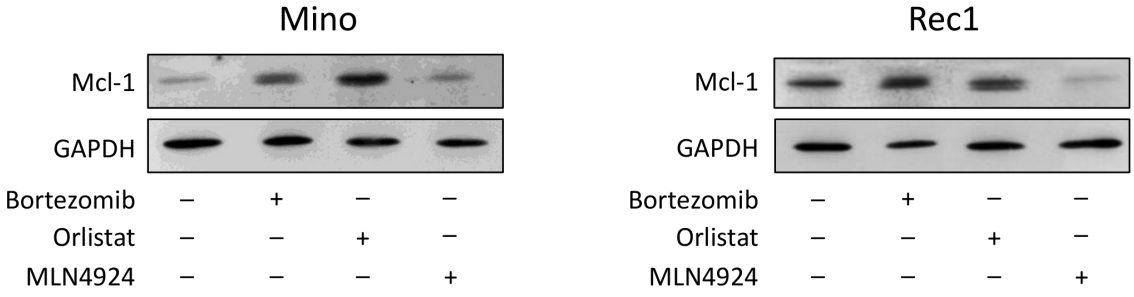
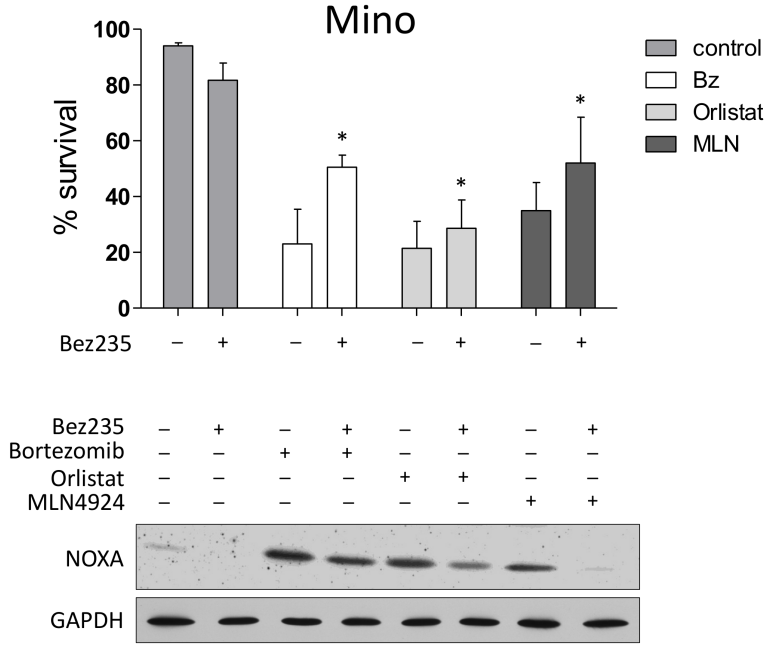


Figure 18: Mcl-1 is induced in response treatment of the cells with Bortezomib and Orlistat.

MCL cell lines Mino and Rec1 were treated for 16 hours with Bortezomib (10 nM), Orlistat (15 µM) and MLN4924 (0.5 µM) and Mcl-1 protein expression was analyzed by Western blot.

3.4.2 PI3K pathway mediated high constitutive *NOXA* mRNA levels are needed to effectively kill MCL cells by Bortezomib, Orlistat and MLN4924

The PI3K/AKT/mTOR pathway was identified as a major player in regulation of constitutive *NOXA* mRNA levels in MCL cells and inhibition of this oncogenic pathway strongly down-regulated *NOXA* mRNA (Figure 6). Since it was previously demonstrated that RNAi mediated silencing of *NOXA* significantly rescues MCL cells from Bortezomib, Orlistat and MLN4924 induced cell death, inhibition of PI3K/AKT/mTOR signaling may also have an impact on induction of NOXA protein and apoptosis by these compounds. Consequently, MCL cell lines were pre-incubated with Bez235 6 hours prior to treatment with the respective inhibitors. Astonishingly, Annexin V/PI staining and Western blot analysis showed a similar picture as previously seen upon RNAi mediated silencing of NOXA: Although not that distinct, inhibition of PI3K/AKT/mTOR pathway also reduced induction of apoptosis and accumulation of NOXA protein upon treatment of the MCL cell lines with Bortezomib, Orlistat or MLN4924 (Figure 19). In line with and further supporting the previous observations were results from experiments performed with Ibrutinib. Inhibition of the main upstream pathway of PI3K/AKT/mTOR prior to treatment with Bortezomib, Orlistat or MLN4924 had also rather antagonistic effects and partially rescued the cell from induction of cell death (Figure 20). These data indicate that a constitutively active PI3K/AKT/mTOR pathway is crucial to effectively kill MCL cells through accumulation of NOXA by these inhibitors.



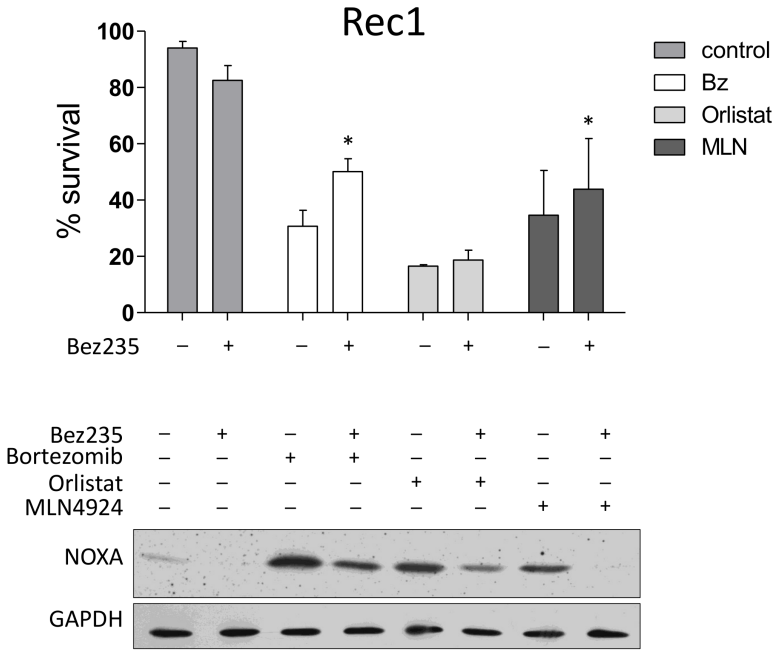


Figure 19: Constitutive active PI3K/AKT/mTOR pathway is crucial for effective accumulation of NOXA protein and induction of cell death upon treatment with Bortezomib, Orlistat or MLN4924.

MCL cell lines Mino and Rec1 were pre-incubated with 1 µM Bez235 (6 hours) before treatment with Bortezomib (10 nM), Orlistat (15 µM) or MLN4924 (0.5 µM). After 16 hours samples were harvested for Western blot analysis. Cell death was quantified by Annexin V/PI staining and flow cytometry 24 hours upon treatment (*: $P < 0.05$). Data represent means ±SD from 3 experiments.

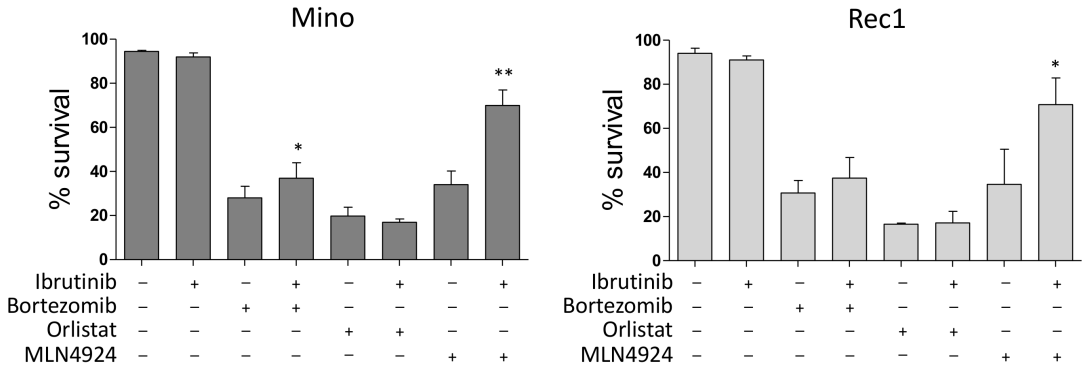


Figure 20: Active BCR signaling is needed for effective accumulation of NOXA protein and induction of cell death upon treatment with Bortezomib, Orlistat or MLN4924.

MCL cell lines Mino and Rec1 were pre-treated with 1 µM Ibrutinib (6 hours) prior to treatment with Bortezomib (10 nM), Orlistat (15 µM) or MLN4924 (0.5 µM). After 24 hours cells were harvested for quantification of cell death by Annexin V/PI staining and flow cytometry (*: $P < 0.05$, **: $P < 0.01$). Data represent means ±SD from 3 experiments.

3.4.3 Bortezomib, Orlistat and MLN4924 accumulate NOXA through interfering with rapid NOXA protein turnover in MCL cells

The previous results showing that high constitutive *NOXA* transcript levels seems to be a prerequisite for effective accumulation of NOXA protein upon treatment with Bortezomib, Orlistat and MLN4924 indicate that these inhibitors rather target the high NOXA protein turnover than transcriptionally activate *NOXA*. As shown in Figure 21, gene expression analysis mostly supported this hypothesis. Treatment of the MCL cell lines Mino and Rec1 with Bortezomib and Orlistat did not significantly change *NOXA* mRNA expression. Surprisingly, treatment with MLN4924, however, considerably further increased *NOXA* transcript levels in MCL cells. In contrast, Cisplatin and Doxorubicin, two DNA damaging agents and classical transcriptional inducers of *NOXA*, had no effect on *NOXA* transcript levels on both cell lines.

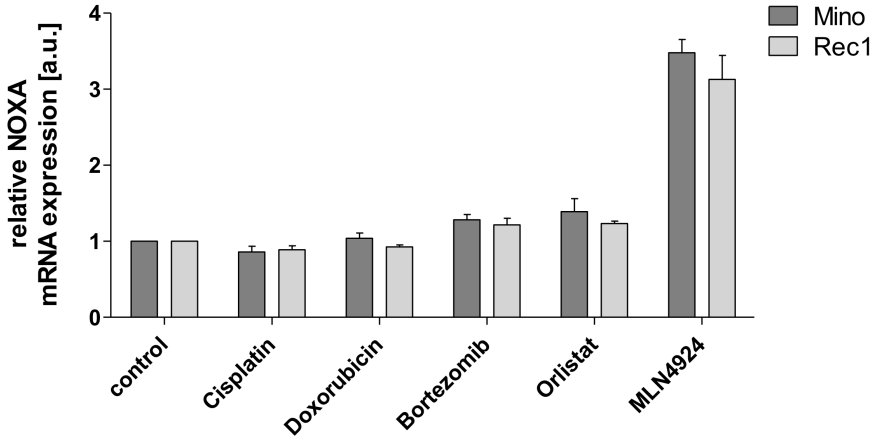


Figure 21: *NOXA* transcript level is not affected by treatment with Bortezomib or Orlistat.
MCL cell lines Mino and Rec1 were treated with Cisplatin (10 µM), Doxorubicin (100 nM), Bortezomib (10 nM), Orlistat (15 µM) or MLN4924 (0.5 µM) for 8 hours and *NOXA* mRNA expression levels analyzed by quantitative real-time PCR. Data represent means ±SD from 3 experiments.

To confirm that Bortezomib and Orlistat target rapid NOXA protein turnover in MCL and to test if MLN4924 not only induces *NOXA* mRNA but also interferes with NOXA degradation, NOXA protein stability in presence of the three inhibitors was analyzed by cycloheximide pulse-chase experiments followed by Western blot analysis. As depicted in Figure 22, treatment of both cell lines with Bortezomib, Orlistat as well as MLN4924 effectively stabilized NOXA protein suggesting that inhibition of rapid NOXA protein turnover is the dominant mechanism by which these inhibitors induce NOXA protein levels.

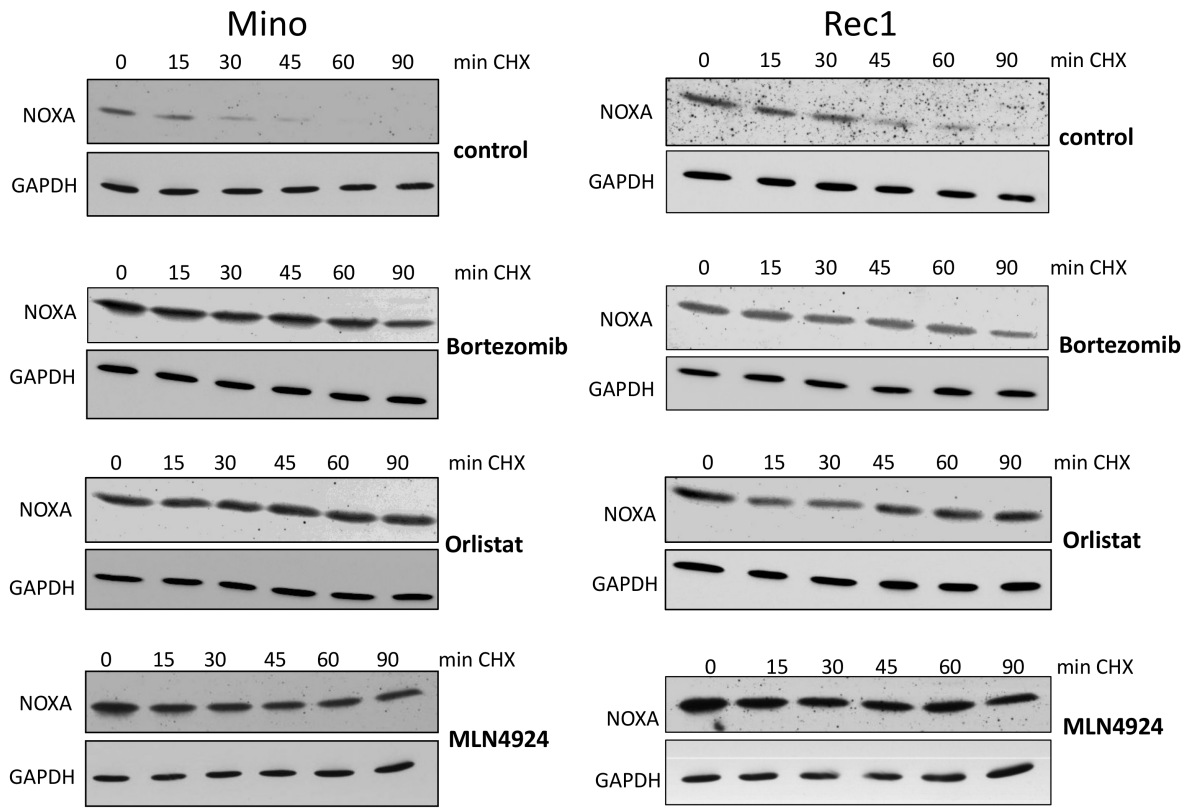


Figure 22: Bortezomib, Orlistat and MLN4924 are inhibiting rapid NOXA protein turnover.
MCL cell lines Mino and Rec1 were treated with Bortezomib (10 nM), Orlistat (15 µM) or MLN4924 (0.5 µM) for 8 hours and then exposed to 20 µg/ml cycloheximide (CHX). Samples were harvested after 15, 30, 45, 60 and 90 minutes and half-life of NOXA protein determined by Western blot analysis.

3.4.4 Orlistat and MLN4924 stabilize NOXA independent of the proteasome and can kill Bortezomib resistant MCL cells

To further explore the mechanism of how Bortezomib, Orlistat or MLN4924 stabilize NOXA protein in MCL cells, pull-down experiments using the agarose-TUBE system followed by Western blot analysis were performed. As a proteasome inhibitor, Bortezomib directly inhibits the degradation step of NOXA protein. Consequently and in agreement with the results obtained with other proteasome inhibitors (Figure 12) Bortezomib treatment resulted in accumulation of polyubiquitinated forms of NOXA (Figure 23). In contrast, both Orlistat and MLN4924 appear to target NOXA turnover in a proteasome independent manner since neither treatment of the cells with Orlistat nor MLN4924 resulted in accumulation of slower migrating polyubiquitinated species of NOXA protein (Figure 23). These results indicate that

Orlistat and MLN4924 target the UPS-mediated NOXA protein turnover upstream of the proteasome by interfering with the process of NOXA ubiquitination.

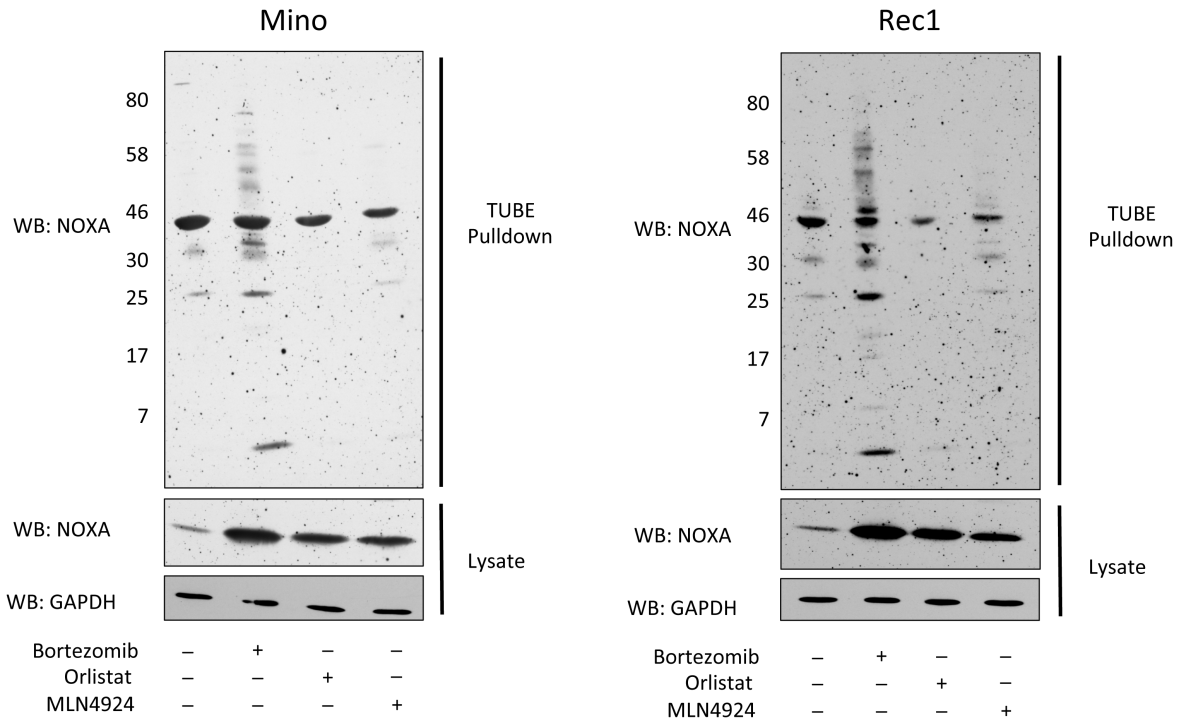


Figure 23: Orlistat and MLN4924 stabilize NOXA by inhibiting ubiquitination of NOXA.

MCL cell lines Mino and Rec1 were cultivated in absence or presence of Bortezomib (10 nM), Orlistat (15 µM) or MLN4924 (0.5 µM) for 8 hours then harvest and lysed. Total polyubiquitinated proteins were isolated from cell lysates using agarose-TUBE2 beads. NOXA protein expression and polyubiquitination state of NOXA were analyzed by Western blot analysis using an NOXA specific antibody.

This observation could be of great clinical interest since resistance to Bortezomib is a frequently observed phenomenon and constitutes a significant problem in MCL patients (Pérez-Galán et al., 2011b). Orlistat and MLN4924 may still be able to induce cell death in Bortetomib resistant cells since their mechanism of NOXA stabilization is independent of the proteasome. To test this hypothesis, Bortezomib sensitive (Jeko1-BS) and Bortezomib resistant MCL cells (Jeko1-BR) were treated with Orlistat and MLN4924 and induction of cell death and NOXA protein accumulation were measured by Annexin V/PI staining and Western blot analysis. As expected, in the Bortezomib sensitive parental cell line Jeko1-BS, NOXA protein and subsequently apoptosis was strongly induced upon Bortezomib treatment. Jeko1-BS cells were also sensitive to Orlistat and MLN4924 and treatment led to the accumulation of NOXA protein (Figure 24, left panel). The Bortezomib resistant clone Jeko1-BR, which was obtained by repeated drug selection showed no signs of cell death when

treated with Bortezomib. Intriguingly, this Bortezomib resistant clone was still sensitive to Orlistat and MLN4924. Compared to the parental cell line Jeko1-BS, Bortezomib induced accumulation of NOXA protein was significantly reduced in the Bortezomib resistant clone. In contrast, NOXA induction by Orlistat and MLN4924 was not altered in Jeko1-BR (Figure 24, right panel). These data suggest that Orlistat and MLN4924 might indeed represent promising alternatives to Bortezomib.

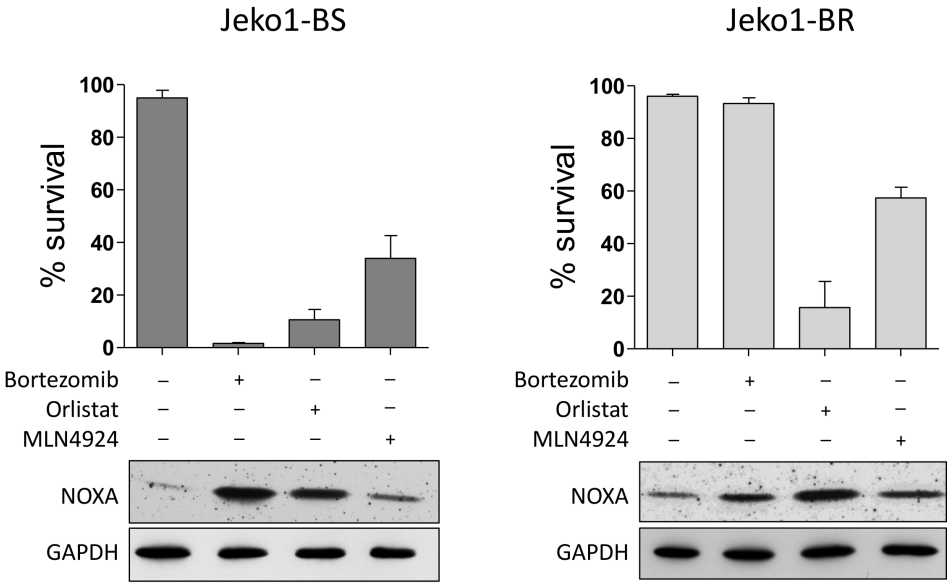


Figure 24: Treatment with Orlistat and MLN4924 can kill Bortezomib resistant MCL cells.
Bortezomib sensitive (Jeko1-BS, left panel) and Bortezomib resistant (Jeko1-BR, right panel) MCL cells were treated for 48h with Bortezomib (10 nM), Orlistat (15 µM) or MLN4924 (0.5 µM) and cell viability measured by Annexin V/PI staining and flow cytometry (upper panel). Data represent means ±SD from 3 experiments. NOXA accumulation was determined after 8 hours of treatment by Western blot analysis (lower panel).

3.4.5 Orlistat and MLN4924 are more selective than Bortezomib in MCL cells

The preceding experiments highlighted that Orlistat and MLN4924 could represent potent alternatives to Bortezomib therapy, especially if treatment with proteasome inhibitors fails. However, selectivity of these compounds has not been investigated so far. Therefore, dose response curves of Bortezomib, Orlistat and MLN4924 in MCL cell lines, and PBMCs and fibroblasts from healthy donors were generated (Figure 25). Interestingly, these experiments revealed that Orlistat and MLN4924 seem to be relatively tumor selective since PBMCs and fibroblasts responded only at highest doses of Orlistat and did even not respond at all to MLN4924. Remarkably, both substances might even be more selective than Bortezomib in

MCL since the proteasome inhibitor also reduced cell viability of control cells at relatively low concentrations (Figure 25, lower panel). These results further support the significance of the previous findings.

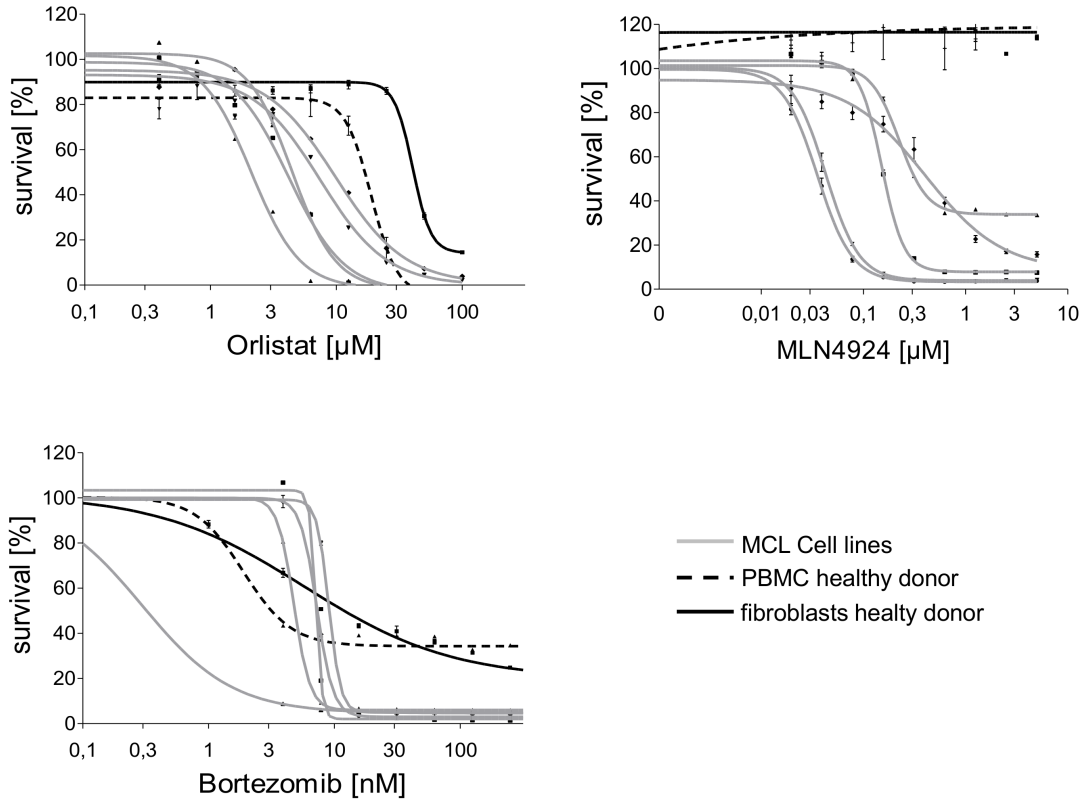


Figure 25: The effect of Orlistat and MLN4924 on cell viability is selective for tumor cells.

MCL cell lines Mino, Jeko1, Rec1, Jvm2, and Granta519 as well as PBMCs and fibroblasts from healthy donors were treated with increasing concentrations of Bortezomib, Orlistat or MLN4924 for 48 hours. Cell viability was then determined by the MTT assay and dose response curves generated using GraphPad Prism 5.0 software.

3.5 Cyclin D1 overexpression renders MCL cells susceptible to FASN inhibitors

Overexpression of FASN has been observed in several types of cancer and connections between FASN and various oncogenic pathways have been described indicating that this metabolic enzyme has oncogenic features (Menendez and Lupu, 2007). Cyclin D1 has also been shown to interact with fatty acid biosynthesis (Buchakjian and Kornbluth, 2010). Interestingly, the fatty acid synthase inhibitor Orlistat was identified in the present study to interfere with NOXA protein turnover in Cyclin D1 overexpressing MCL cells. Hence, Cyclin D1 expression might affect fatty acid synthesis in MCL cells and therefore influence the sensitivity of the cells to FASN inhibitors.

3.5.1 Cell death upon Orlistat treatment is dependent on Cyclin D1 and palmitate depletion

To investigate whether Cyclin D1 overexpression has any impact on fatty acid synthesis and therefore on sensitivity of the MCL cells to Orlistat, Cyclin D1 was down regulated via RNAi prior to treatment. Bortezomib and MLN4924 were used as controls to exclude unspecific effects. In contrast to Bortezomib and MLN4924, knockdown of Cyclin D1 substantially rescued the cells from Orlistat induced cell death (Figure 26) indicating that overexpression of Cyclin D1 renders MCL cells sensitive to FASN inhibitor. However, these effects might still be independent of FASN activity. FASN is catalyzing the final step of the synthesis of palmitate, the most important saturated fatty acid in the cell. To investigate if cell death upon Orlistat treatment is indeed related to depletion of palmitate in MCL cells, the fatty acid was supplemented to the medium after treatment of the cells with the FASN inhibitor. Analysis of cell viability by Annexin V/PI staining revealed that addition of palmitate was able to rescue the cells from induction of cell death demonstrating that apoptosis upon Orlistat treatment is due to its inhibitory effects on FASN and depletion of palmitate (Figure 27, upper panel). Moreover, as observed by Western blot analysis, supplementation of palmitate also partially reduced accumulation of NOXA protein following FASN inhibition (Figure 27, lower panel). These data imply the existence of a crosstalk between Cyclin D1, fatty acid metabolism and UPS-mediated NOXA turnover.

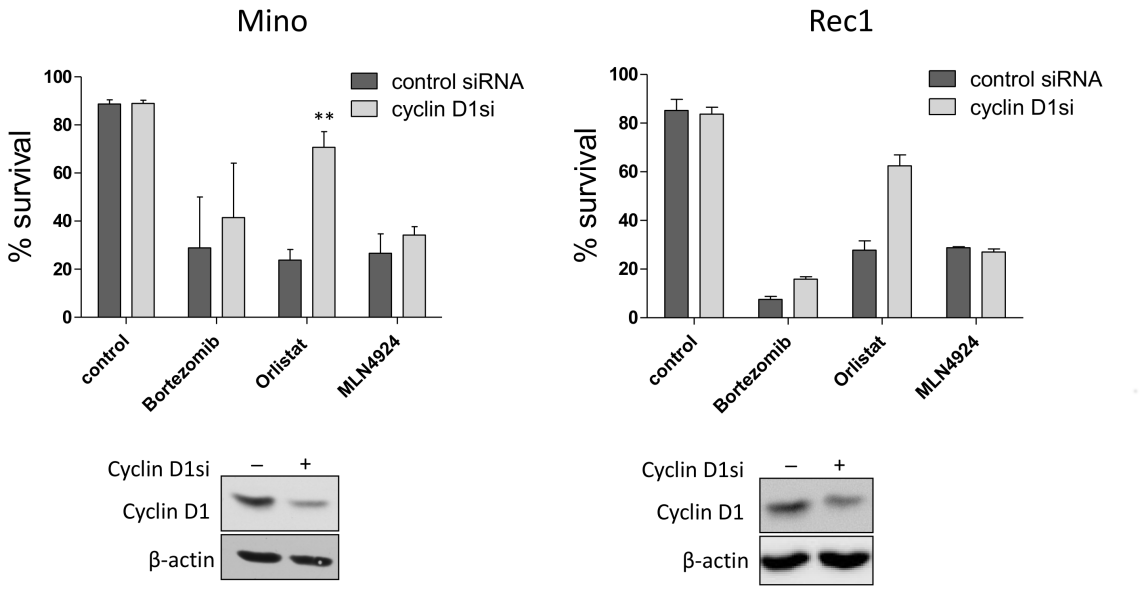


Figure 26: Knockdown of Cyclin D1 rescues MCL cells from Orlistat induced apoptosis.

MCL cell lines Mino and Rec1 were transfected with control siRNA or siRNA targeting *Cyclin D1*. 16 hours upon transfection cells were treated with Bortezomib (10 nM), Orlistat (15 µM), or MLN4924 (0.5 µM) and further cultivated for 24 hours. The cells were then harvested for quantification of cell death by Annexin V/PI staining and flow cytometry (**: $P < 0.01$). Data represent means ±SD from 3 experiments (Mino) or means ±SD from 2 experiments (Rec1). Efficacy of Cyclin D1 silencing was measured by Western blot analysis.

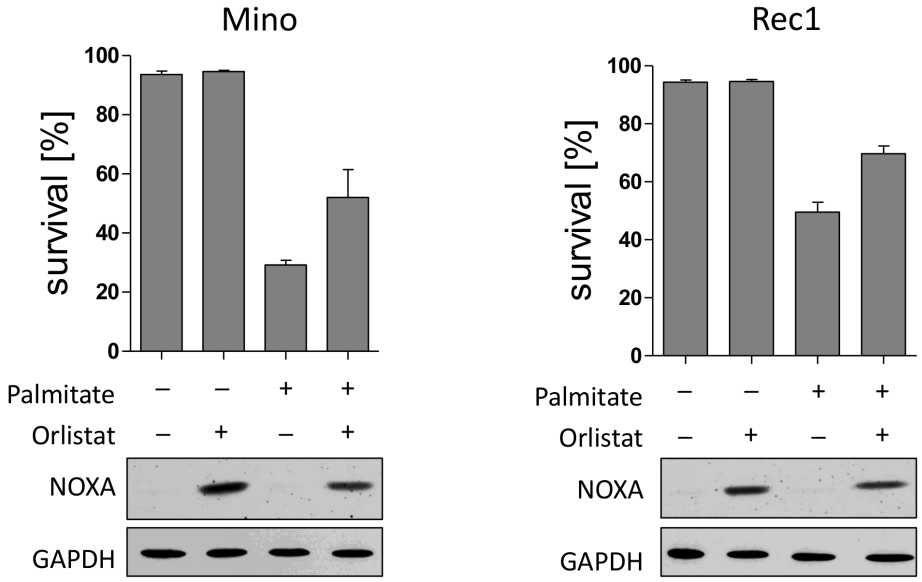


Figure 27: Cell death upon Orlistat treatment is dependent on palmitate depletion in MCL cells.

MCL cell lines Mino and Rec1 were treated with 15 µM Orlistat in presence or absence of 20 µM palmitate. 24 hours upon treatment cells were harvested for Western blot analysis and quantification of cell death by Annexin V/PI staining and flow cytometry. Data represent means ±SD from 2 experiments.

3.5.2 Sensitivity of MCL cells to FASN inhibitors is not limited to Orlistat

To determine whether MCL cells are also sensitive to other FASN inhibitors, the MCL cell lines Mino and Rec1 were treated with the FASN inhibitors Cerulenin and C75 and induction of cell death was then compared with that observed upon treatment with Orlistat. Additionally, PBMCs and fibroblasts from healthy donors were treated to analyze selectivity of these compounds. FACS based cell viability analysis demonstrated that hypersensitivity of MCL cells to FASN inhibitors is not limited to Orlistat. In contrast to the control cells, both MCL cell lines induced severe apoptosis upon treatment with Cerulenin or C75 (Figure 28).

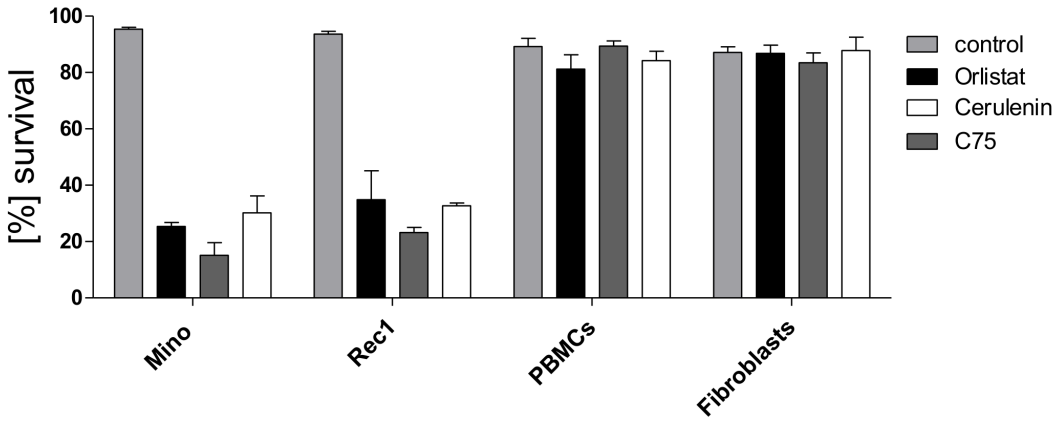


Figure 28: MCL cells are also sensitive to FASN inhibitors Cerulenin and C75.
MCL cell lines Mino and Rec1 as well as PBMCs and fibroblasts from healthy donors were treated with the FASN inhibitors Orlistat (15 µM), Cerulenin (10 µM) and C75 (10 µM) for 24 hours. Cell viability was then analyzed by Annexin V/PI staining and flow cytometry. Data represent means ±SD from 3 experiments.

3.5.3 FASN inhibitors influence the balance between NOXA and Mcl-1

The natural compound Cerulenin and its chemical analogue C75 represent an alternative class of FASN inhibitors which targets a different domain of FASN than Orlistat (Flavin et al., 2010). To investigate whether these inhibitors also induce NOXA protein expression and if FASN inhibitors have additional effects on the expression of other Bcl-2 family members, protein expression of NOXA and its antagonist Mcl-1 as well as a panel of important pro- and anti-apoptotic Bcl-2 family members was analyzed by Western blot. As depicted in Figure 29, both C75 and Cerulenin were also inducing NOXA protein expression in the MCL cell lines Mino and Rec1. Accumulation of NOXA, however, was not as pronounced as observed upon

Orlistat. Interestingly, in contrast to Orlistat, treatment of the cells with C75 and Cerulenin was not accompanied by induction of Mcl-1 expression suggesting that targeting different sites of the multi-domain enzyme FASN may have slightly different effects. Nevertheless, the mechanism of apoptosis induction of both classes of FASN inhibitors seems to be the disturbance of the balance between pro- and apoptotic proteins by altering the expression of NOXA and Mcl-1 since expression of most other Bcl-2 family members was not altered upon FASN inhibition in both cell lines. Interestingly, the pro-apoptotic effector protein Bak seems to be post-translationally modified only upon treatment with Orlistat indicated by a slower migrating band.

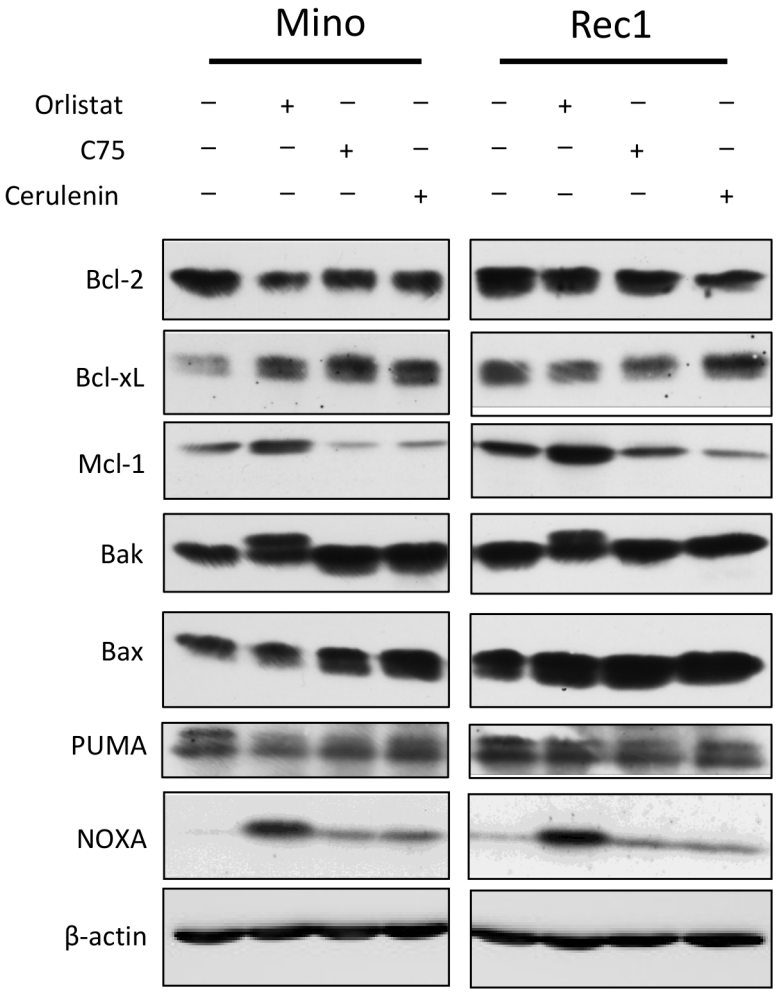


Figure 29: FASN inhibitors disturb the balance between NOXA and Mcl-1.

MCL cell lines Mino and Rec1 were treated with the FASN inhibitors Orlistat (15 μM), Cerulenin (10 μM) and C75 (10 μM). After 16 hours, cells were harvested and protein expression of the Bcl-2 family members Bcl-2, Bcl-x_L, Mcl-1, Bak, Bax, PUMA and NOXA analyzed by Western blot analysis.

To confirm that induction of NOXA protein is also the major mechanism of apoptosis induction in response to the second class of FASN inhibitors, *NOXA* was silenced via RNAi prior to treatment with Cerulenin. Such as observed for Orlistat, induction of cell death following Cerulenin was highly dependent on NOXA induction (Figure 30). Knockdown of *NOXA* almost completely rescued the cells from induction of cell death (Figure 30, upper panel) and significantly reduced NOXA accumulation upon treatment (Figure 30, lower panel) demonstrating the important role the BH3-only protein also for Cerulenin-induced cell death.

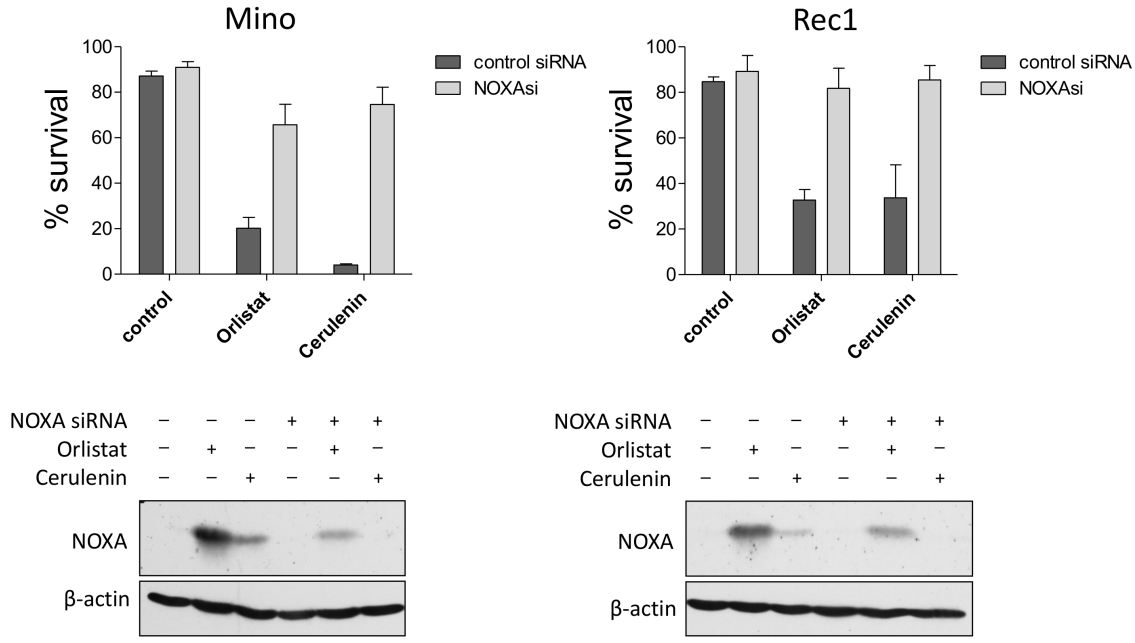


Figure 30: Cell death upon Cerulenin is dependent on NOXA.

MCL cell lines Mino and Rec1 were transfected with control siRNA or siRNA targeting *NOXA*. 24 hours after transfection cells were treated with Orlistat (15 µM) or Cerulenin (10 µM) and further cultivated for 24 hours. Cells were then harvested for Western blot analysis and quantification of cell death by Annexin V/PI staining and flow cytometry. Data represent means ±SD from 2 experiments.

3.5.4 Cyclin D1 renders MCL cells susceptible to FASN inhibitors through direct effects on metabolic activity of the cells

The previous experiments have shown that MCL cells are sensitive to various inhibitors of FASN. Cyclin D1 overexpression seems to render MCL cells susceptible to these inhibitors since down-regulation of Cyclin D1 substantially rescues the cells from Orlistat-induced cell death (Figure 26). This observation was also confirmed for Cerulenin. As quantified by Annexin V/PI staining and FACS analysis, knockdown of Cyclin D1 recues the cells from Cerulenin-induced apoptosis to a similar extent as observed for Orlistat (Figure 31).

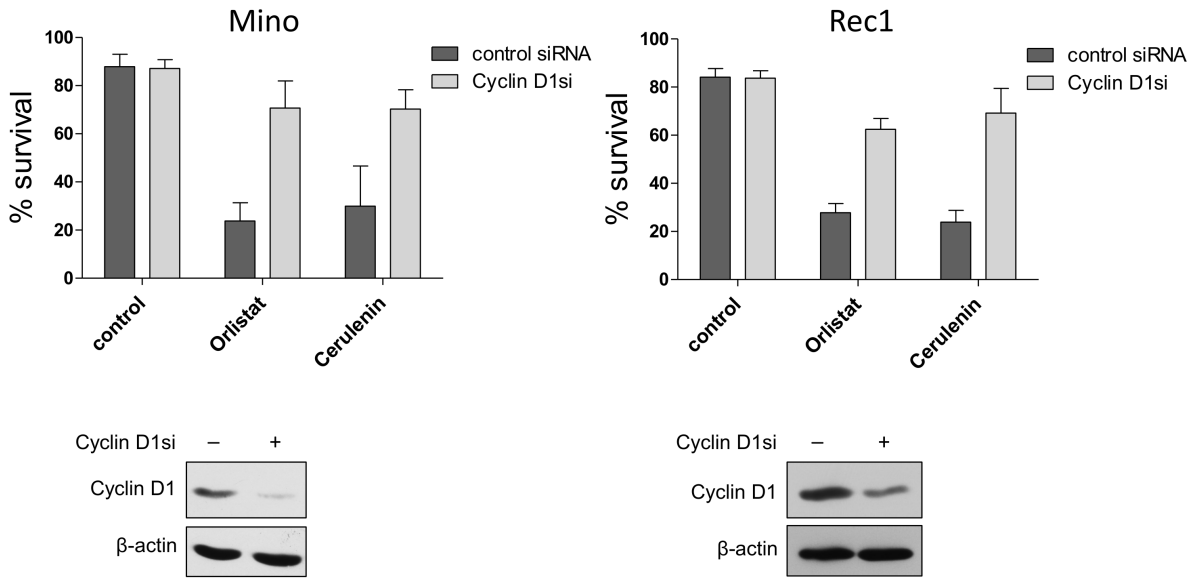


Figure 31: Sensitivity of MCL cells to FASN inhibitors is dependent on Cyclin D1 overexpression.
MCL cell lines Mino and Rec1 were transfected with control siRNA or siRNA targeting *Cyclin D1*. 16 hours upon transfection cells were treated with Orlistat (15 µM) or Cerulenin (10 µM) and further cultivated for 24 hours. The cells were then harvested for quantification of cell death by Annexin V/PI staining and flow cytometry. Data represent means ±SD from 3 experiments (Mino) or means ±SD from 2 experiments (Rec1). Efficacy of Cyclin D1 silencing was measured by Western blot analysis.

These data further support the notion that overexpression of Cyclin D1 is an important determinant of the sensitivity of MCL cells to FASN inhibitors. However, the mechanism of how Cyclin D1 mediates sensitivity remains unclear.

Compared to their normal counterparts, many cancer cells depend on *de novo* synthesis of fatty acids. Consequently, FASN hyper-activation and overexpression of FASN has been observed in many types of tumors (Menendez and Lupu, 2007). To determine whether FASN

is also overexpressed in MCL and if Cyclin D1 expression has any impact on FASN protein levels, Cyclin D1 and FASN expression in MCL cell lines and PBMCs of healthy donors was analyzed by Western blot analysis. Interestingly, compared to PBMCs, all MCL cell lines express very high levels of FASN supporting the previous results that these cells may be dependent on expression of this enzyme (Figure 32A). A correlation of FASN expression to Cyclin D1 levels, however, was lacking in the panel of cell lines analyzed, indicating that Cyclin D1 has no inhibitory effect on FASN expression. To confirm this hypothesis, the impact of RNAi mediated silencing of Cyclin D1 on FASN protein levels was examined in the MCL cell lines Mino and Rec1. As expected, FASN was not enhanced upon down-regulation of Cyclin D1 (Figure 32B). The expression of the metabolic enzyme was even slightly reduced after Cyclin D1 knockdown indicating that Cyclin D1 influences the sensitivity of MCL cells on a different level.

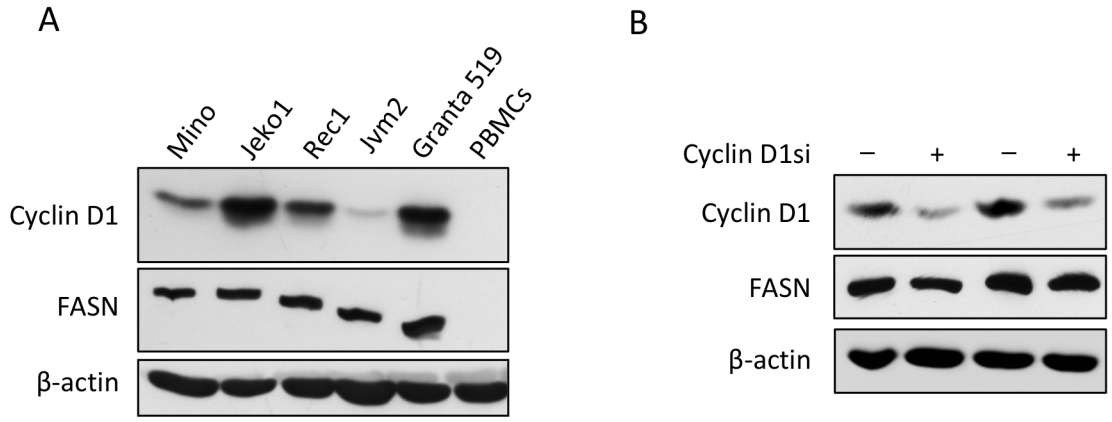
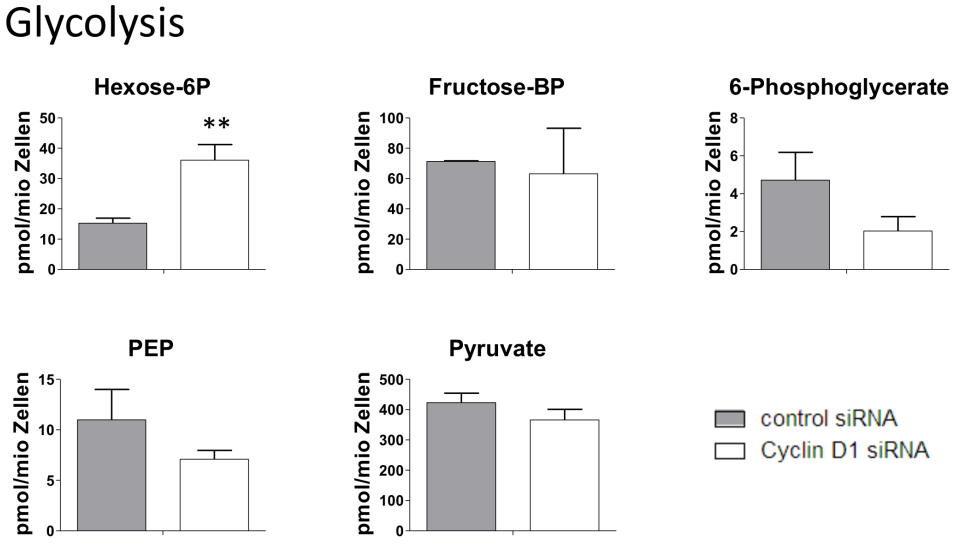


Figure 32: FASN protein levels in MCL are independent of Cyclin D1.
(A) Constitutive protein expression levels of Cyclin D1 and FASN were analyzed in MCL cell lines (Mino, Jeko1, Rec1, Jvm2 and Granta519) and PBMCs from healthy donors by Western blot analysis. (B) MCL cell lines Mino and Rec1 were transfected with control siRNA or siRNA targeting *Cyclin D1* and incubated for 24 hours. Knockdown efficacy and expression of FASN was then analyzed by Western blot analysis.

Fascinatingly, recent studies screening for proteins interacting with Cyclin D1 showed that Cyclin D1 directly binds to several metabolic enzymes including the fatty acid synthase (Jirawatnotai et al., 2011). To investigate whether Cyclin D1 overexpression determines hypersensitivity of MCL cells to FASN inhibitors through direct effects on metabolic activity of the cells, changes in metabolite pools of the central metabolic pathways and cellular concentration of different triacyclglycerides (TAGs) upon down-regulation of Cyclin D1 were quantified by GC-MS and LC-MS-MS. Astonishingly, knockdown of Cyclin D1 significantly

changed concentrations of several intracellular metabolites (Figure 33). Levels of central metabolites such as hexose-6-phosphates (glycolysis), citrate (TCA cycle) as well as seduheptulose-7-phosphate (PPW) were significantly enhanced. In contrast, intracellular concentration of some metabolites, such as malate (TCA cycle), was reduced following silencing of Cyclin D1. Interestingly, concentration of total triacylgycerides (TAGs) was increased in Cyclin D1 depleted cells indicating elevated lipogenesis and subsequent triacylglyceride synthesis in these cells. Especially, TAG48:0, which is consisting of glycerol and three palmitate residues, was found to be significantly higher in cells with reduced Cyclin D1 expression suggesting that palmitate synthesis might be enhanced in the cells and excessive palmitate stored in form of triacylgylcerides. The finding that levels of citrate are increased upon knockdown of Cyclin D1 supports this hypothesis. Citrate is a precursor for fatty acid synthesis and elevated levels activate fatty acid synthesis.

Taken together, Cyclin D1 expression appears to have strong impact on metabolism of MCL cells in general. Especially, fatty acid synthesis and lipogenesis seem to be directly negatively affected by Cyclin D1 overexpression thereby rendering MCL cells susceptible to FASN inhibitors.



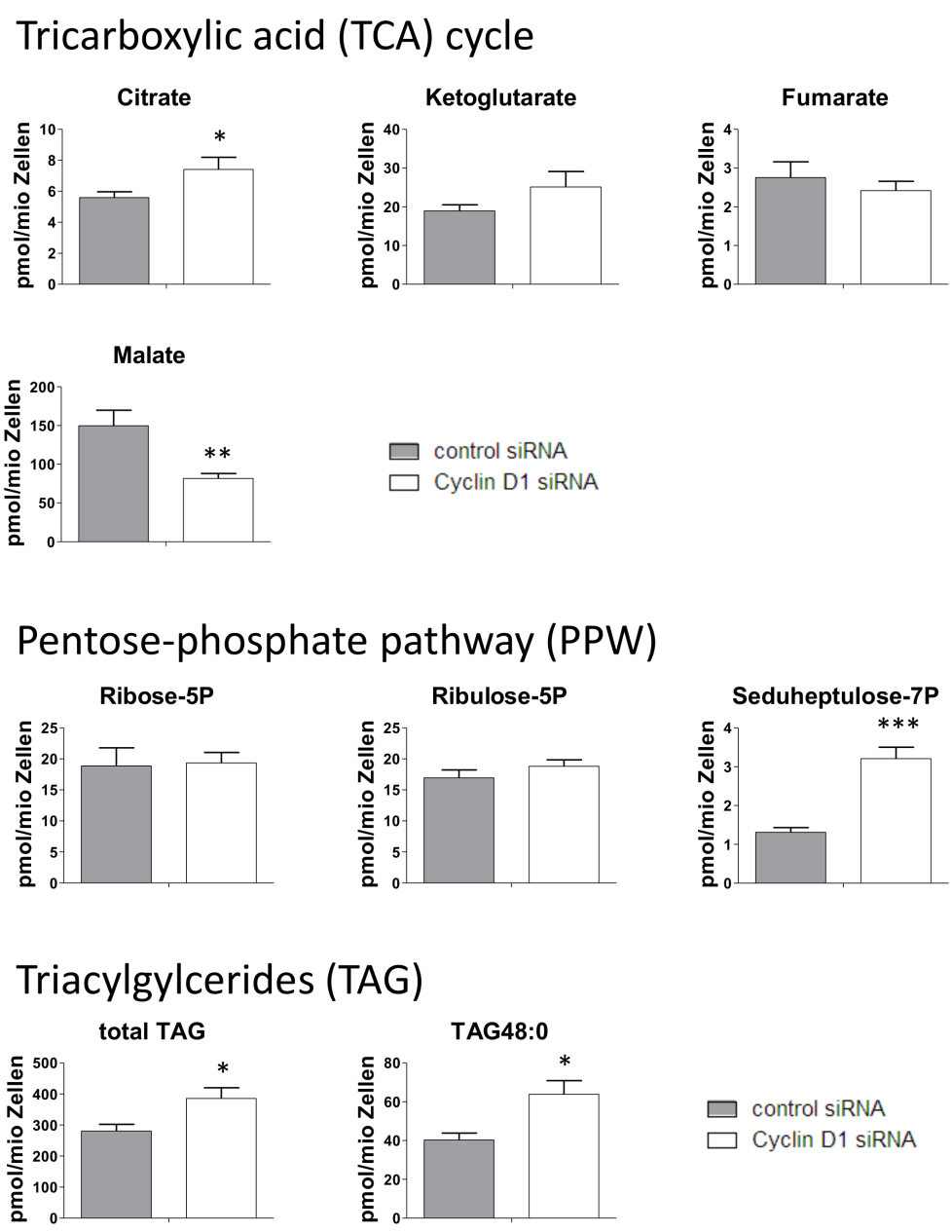


Figure 33: Knockdown of Cyclin D1 changes concentrations of intracellular metabolites in MCL cells.

MCL cell line Rec1 was transfected with control siRNA or siRNA targeting *Cyclin D1* and incubated for 48 hours. Cells were then harvested and lysed. Intracellular metabolites were isolated from cell lysate by methanol/chloroform extraction. Concentrations of intracellular metabolites were measured by GC-MS and LC-MS-MS.

3.6 Screening for potential treatment combinations to enhance efficacy of FASN inhibitors in MCL

So far, this study has shown that MCL cells express high levels of *NOXA* mRNA. However, elevated expression of this pro-apoptotic Bcl-2 family member was not observed on protein level. NOXA protein was found rapidly degraded in MCL cells. Bortezomib, Orlistat and MLN4924 were identified to target UPS-mediated NOXA protein turnover in MCL on different levels. Treatment of the cells with these inhibitors effectively accumulated NOXA protein and induced apoptosis. In contrast to Bortezomib and MLN4924, cell death upon Orlistat treatment was dependent on overexpression of Cyclin D1 indicating that this compound might be selective for MCL. Although all MCL cell lines tested showed enhanced sensitivity to Orlistat when compared to non-malignant cells, the response of the different cell lines to the drug was relatively variable (Figure 25). Combination of FASN inhibitors with other drugs may enhance efficacy of the metabolism inhibitors in cell lines such as Jvm2 or Granta519 that are slightly less sensitive to Orlistat when compared to the hypersensitive cell lines Mino and Rec1. Therefore, drug combination screening based on high-throughput fluorescence microscopy was performed in Jvm2 and Granta519. In this screening low doses of Orlistat or Cerulenin were combined with a panel of commonly used drugs. PBMCs of healthy donors were used as a control to assess selectivity of the drug combinations. Cell viability was measured by staining the cells with Hoechst 33342, propidiumiodide and NucView™ 488 caspase-3 substrate and analyzed using the InCell Analyzer high throughput microscope. As shown in figure 34, in both cell lines most single treatments had only minor effects on cell viability of both cell lines. However, several drug combinations were able to induce apoptosis in the cancer cell lines. Combination of Orlistat or Cerulenin with DNA damaging agents such as Cisplatin or Etoposid, for example, enhanced efficacy of the FASN inhibitors. Interestingly, the p53-activating agent Nutlin-3 was also observed to have combinatorial effects with Orlistat and Cerulenin in the p53 wild type cell lines Jvm2 and Granta519. The most active combination, however, was the treatment of the cell with Orlistat or Cerulenin and the BH3-mimetic ABT737. This approach was found to strongly induce cell death in both cell lines. Remarkably, combination treatments found to be effective in MCL cells were relatively selective for the tumor cells since cell viability of PBMCs from healthy donors was only slightly reduced.

In summary, this screen revealed that combination strategies for treatment might further enhance the efficacy of FASN inhibitors and therefore represent a promising concept for the clinic.

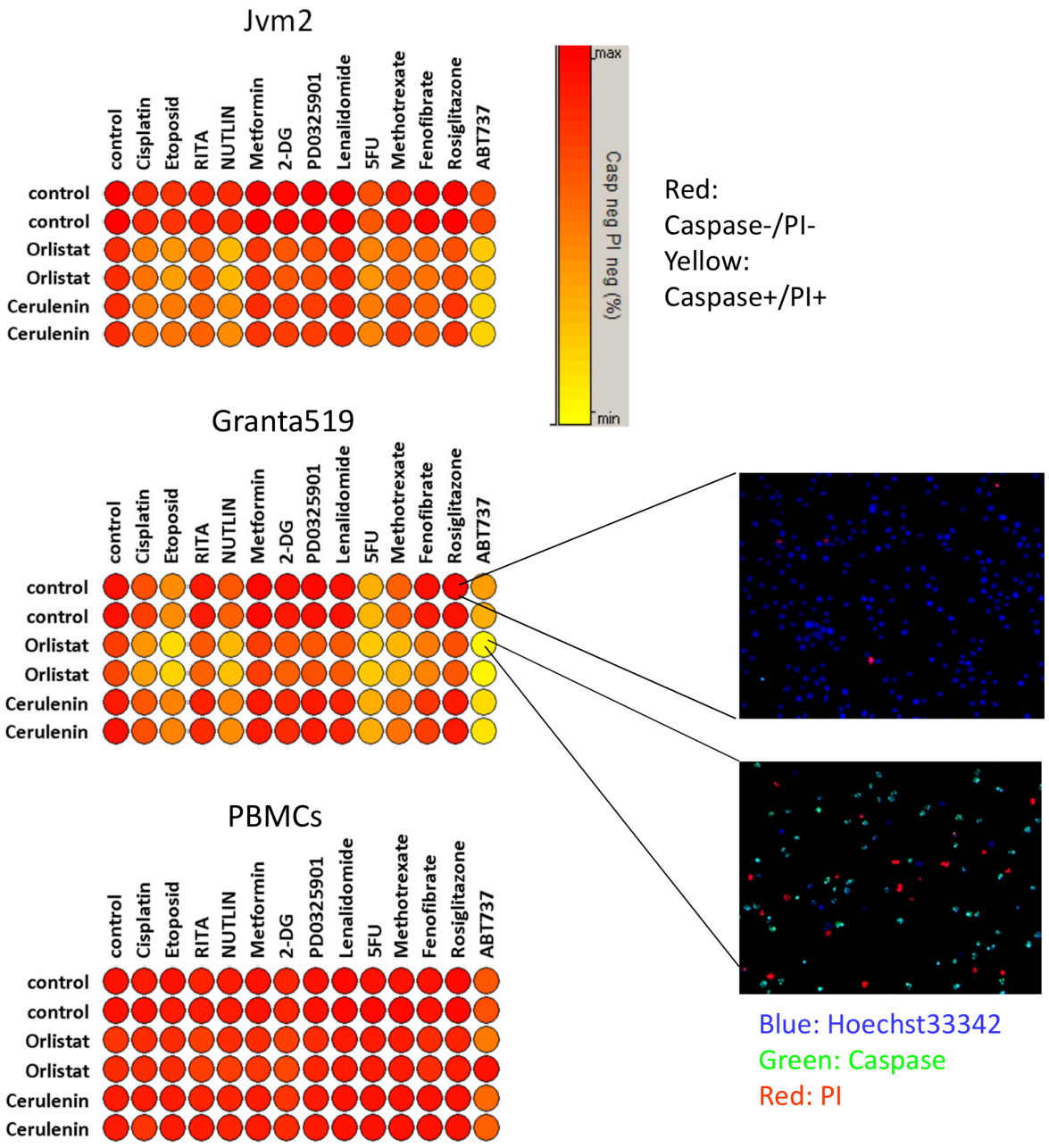


Figure 34: Combination treatment enhances efficacy of FASN inhibitors.

MCL cell lines Jvm2 and Granta519, and PBMCs from healthy donors were seeded in 96well plates and treated with Orlistat (15 µM) or Cerulenin (10 µM) and the panel of indicated drugs. 24 hours upon treatment cells were stained with Hoechst 33342, propidiumiodide (PI) and NucView™ 488 caspase-3 substrate and cell viability analyzed using the IN Cell Analyzer 1000 high throughput microscope.

4 Discussion

MCL is a form of non-Hodgkin lymphoma and characterized by an aggressive clinical course and relative short survival. Although cancer therapy has generally improved during the last decades, MCL remains a difficult to treat neoplasm and only few patients are considered cured. The apoptosis regulating Bcl-2 family members play a major role in the response of tumor cells to anticancer drugs. Especially the BH3-only proteins are important mediators of cell death signals and function as stress-sensors and apoptosis initiators. Not only the stress induced levels of the BH3-only proteins are important for sensitivity of tumor cells to anti-cancer agents but also their constitutive expression levels can be central determinants for drug response. This study provides new insights into the critical role of the BH3-only protein NOXA in MCL and shows why the constitutive expression levels of this pro-apoptotic protein may constitute an Achilles heel of this lymphoma.

The results from the present work demonstrate an obvious discrepancy between *NOXA* mRNA and NOXA protein levels in MCL cells. *NOXA* mRNA expression was found to be very high in MCL cells and astonishingly maintained by chronic BCR signaling as well as Cyclin D1 overexpression, two of the major oncogenic alterations in MCL. In strong contrast, NOXA protein levels were relatively low due to rapid NOXA protein turnover in MCL cells. Targeting the UPS-mediated mediated NOXA protein degradation led to strong increase of the pro-apoptotic protein and subsequent induction of NOXA-dependent apoptosis. In addition to proteasome inhibitors, substances targeting the ubiquitination process were identified to accumulate NOXA and induce cell death in MCL. The high constitutive *NOXA* mRNA levels were a prerequisite for effective killing of these UPS inhibitors indicating that the phenotype of high *NOXA* mRNA/low NOXA protein levels might be a promising target for therapy of this aggressive lymphoma. Astonishingly, fatty acid metabolism was found to be involved in regulation of NOXA protein turnover. Targeting this metabolic pathway accumulated NOXA protein and induced cell death. Furthermore, Cyclin D1 overexpression was identified to render MCL cells susceptible for FASN inhibitors by negatively affecting fatty acid metabolism.

4.1 Discrepant constitutive *NOXA* mRNA and NOXA protein levels in MCL

Deregulation of the Bcl-2 family members leading to defects in programmed cell death contributes to the development of many cancers (Kelly and Strasser, 2011). In MCL, alterations of BIM and Bcl-2 have been detected so far (Beà et al., 2009; Tagawa et al., 2005). This study for the first time demonstrates that regulation of the pro-apoptotic protein NOXA might also be altered in MCL: The malignant B cells are characterized by the phenotype of high *NOXA* mRNA/low NOXA protein levels (Figure 3 and Figure 4). MCL cells seem to adapt to a constant apoptotic signal mediated by high *NOXA* mRNA levels by keeping the pro-apoptotic protein low through rapid NOXA protein degradation (Figure 11).

4.1.1 High constitutive *NOXA* mRNA levels in MCL

Most studies investigating the pro-apoptotic Bcl-2 family member *NOXA* focused on its role in mediating stress-induced apoptosis. Multiple stress and environmental signals have been shown to induce cell death via activation of *NOXA* transcription and protein expression (Ploner et al., 2008). *NOXA* is probably best known as a mediator of p53-induced cell death in response to genotoxic stress (Oda et al., 2000; Shibue et al., 2003). The BH3-only protein can also be induced in a p53 independent manner. For example, *NOXA* is a direct target of the transcription factor HIF1α and is induced under hypoxic conditions (Kim et al., 2004). Furthermore, recent work showed that *NOXA* is transcriptionally activated by ATF3/ATF4 in response to ER stress (Wang et al., 2009) and the transcription factors E2F1 as well as p73 have been implicated in *NOXA* induction upon oncogenic stress (Flinterman et al., 2005; Hershko and Ginsberg, 2004). In contrast, only little is known about the regulation of constitutive *NOXA* levels in normal and tumor cells. In this study the role of constitutive *NOXA* expression was investigated in the hematopoietic neoplasm MCL. Analysis of public available gene expression data from CCLE database and expression profiling of our own collection of cell lines revealed a huge heterogeneity in constitutive *NOXA* mRNA levels in cancer cell lines. However, blood cancer cell lines and especially MCL were found to be among the cell lines with highest amounts of *NOXA* transcript (Figure 1 and 3A). This finding was also confirmed in MCL cells derived from patients (Figure 4A). Astonishingly, *NOXA* transcript was found to be the most abundant mRNA in the whole set of pro-apoptotic Bcl-2 family members in MCL indicating that *NOXA* represents an important player in this malignancy (Figure 2). This phenotype of high *NOXA* mRNA expression might be a typical

feature of stimulated B cells. It has been shown that *NOXA* plays an essential role in B cell development and immune response. The pro-apoptotic gene is transcriptionally up-regulated upon antigen stimulation thereby regulating apoptosis of activated B cells and the formation of high affinity clones (Wensveen et al., 2012). Lymphoid neoplasms are the malignant counterparts of normal lymphocytes that are “frozen” at a certain stage of development. The counterpart of MCL cells appears to be a pre-germinal center mature B cell (Shaffer et al., 2002). Interestingly, malignant B cells maintain BCR expression on their surface and recent studies have shown evidence of constitutive BCR activation in several types of B cell lymphomas including MCL (Shaffer et al., 2012; Young and Staudt, 2013). Indeed, results from this work demonstrate that constitutive BCR signaling is essential for the maintenance of high *NOXA* mRNA levels in MCL (Figure 5). A similar role of *NOXA* was described in T cell development. It was demonstrated that *NOXA* is induced in response to T cell receptor (TCR) activation and involved in selection of high affinity clones (Alves et al., 2006; Wensveen et al., 2010). Protein kinase C (PKC) signaling was implicated in induction of *NOXA* downstream of the TCR in T cells (Alves et al., 2006). In B cells, however, the signaling pathway involved in regulation of *NOXA* expression downstream of the BCR was unknown so far. Just like TCR, stimulation of the BCR triggers the activation of multiple downstream signaling pathways (Young and Staudt, 2013). PKCβ is also involved in BCR signaling and required for BCR-mediated NF-κB activation (Shinohara et al., 2005; Su et al., 2002). Interestingly, PKCβ was also found phosphorylated in MCL (Boyd et al., 2009). However, the data obtained in the present study implicate that PKCβ/NF-κB signaling is not involved in *NOXA* regulation in B cells. Similar observations were made for the MAPK pathway. In contrast, the PI3K/AKT/mTOR pathway was found to be the major mediator of high *NOXA* mRNA levels downstream of BCR in MCL cells (Figure 6). Interestingly, both PI3K and mTOR appear to be involved in *NOXA* regulation (Figure 7). In many respects, these findings are astonishing. First, although *NOXA* has similar functions in B cell and T cell development and BCR and TCR signaling use the same downstream signaling pathways, *NOXA* regulation seems to be different in both types of lymphoid cells. Second, the PI3K/AKT/mTOR pathway is generally considered to be pro-survival and its activation implicated in apoptosis suppression. The PI3K/AKT/mTOR pathway is frequently altered in cancer and mediates survival signals downstream of several receptors (Fresno Vara et al., 2004). For example, AKT has been shown to directly phosphorylate and inhibit the pro-apoptotic proteins BIM and Bad (Engelman, 2009) and activation of PI3K signaling was proposed to increase expression of anti-apoptotic proteins such as Bcl-2 and Bcl-x_L (Tang et

al., 2000). Constitutive activation of AKT and mTOR was already demonstrated to be present in primary cells from MCL patients (Col et al., 2008). These findings are in line with the observation made in the present work (Figure 8). However, Col *et al.* solely linked constitutive activation of this pathway to proliferation and survival. In contrast, the data in this study suggest that under certain circumstances the PI3K/AKT/mTOR pathway can also mediate a pro-apoptotic signal in form of transcriptional induction of *NOXA*. Data from other work support this hypothesis. Mei *et al.* demonstrated that camptothecin (CPT)-induced apoptosis in HeLa cells depends on PI3K/AKT/mTOR mediated transcriptional induction of *NOXA* (Mei et al., 2007). The cAMP response element binding protein (CREB) was identified as the transcription factor, which mediates CPT-induced *NOXA* induction. The role of CREB in PI3K/AKT/mTOR mediated *NOXA* regulation in MCL cells was not analyzed so far. CREB is involved in the regulation of diverse cellular processes and also plays an important role in immune function (Wen et al., 2010). In addition, CREB is a downstream target of the PI3K/AKT/mTOR pathway (Caravatta et al., 2008; Du and Montminy, 1998) suggesting, that this transcription factor might indeed play a role in the maintenance of high *NOXA* mRNA levels in MCL. Another unexpected phenomenon observed in this study was the possible existence of a positive feedback loop of Cyclin D1 on PI3K/AKT/mTOR pathway. Cyclin D1 overexpression, the hallmark of MCL, seems to contribute to the high *NOXA* transcript levels by exerting a stimulatory effect on the PI3K/AKT/mTOR pathway (Figure 9 and 10). Cyclin D1 is considered a classical downstream target of the PI3K/AKT/mTOR pathway. Upon mitogenic stimulation of cells AKT phosphorylates and inactivates GSK3, a negative regulator of Cyclin D1. The inhibition of GSK3 accumulates Cyclin D1 and promotes cell cycle progression (Chang et al., 2003). The positive feedback loop of Cyclin D1 on PI3K/AKT/mTOR signaling is likely mediated by the Cyclin D1 downstream effector E2F. Data from other groups implicate that RB1/E2F signaling is tightly regulated by several feedback loops (Polager and Ginsberg, 2008). For example, E2F was shown to positively affect AKT activity through transcriptional induction of upstream components such as GAB2 (Chaussepied and Ginsberg, 2004). Taken together, the observed high *NOXA* transcript levels in MCL cells seem to be regulated by constitutive activation of the PI3K/AKT/mTOR pathway mediated by chronic BCR signaling and to a minor degree by Cyclin D1 overexpression. As mentioned before, chronic BCR signaling is also present in other types of lymphomas (Young and Staudt, 2013) and might explain the high levels of *NOXA* transcript in several lymphoid neoplasms. Besides MCL, CLL, for example, is one of the lymphomas found to express elevated *NOXA* mRNA levels such as MCL (Figure 1). Furthermore, the

comparison of the relative gene expression of the pro-apoptotic Bcl-2 family members revealed that *NOXA* mRNA is also the dominant transcript in CLL (Figure 2). Interestingly, such as most cases of MCL, a subtype of CLL is also thought to originate from pre-germinal center mature B cells (Shaffer et al., 2012). The type of BCR signaling in these malignant cells, which represent a very early stage of B cell differentiation, seems to "prime" the cells for death. This might explain why the main anti-apoptotic partner of NOXA, the Bcl-2 family member Mcl-1, is essential for survival of B cells at early stages after activation (Vikstrom et al., 2010). The "priming" of the cells by up-regulation of *NOXA* is thought to play an important role in the control of the selection of high affinity clones and NOXA-induced apoptosis seems to be crucial for the elimination of B cells that express low affinity or auto-reactive antibodies (Wensveen et al., 2012). Malignant B cells such MCL or CLL cells appear to evade this selection process and cope with the constitutive pro-apoptotic signal mediated by enhanced *NOXA* mRNA levels.

As described before, *NOXA* plays similar roles in T cell development (Alves et al., 2006; Wensveen et al., 2010). Adaption to high *NOXA* mRNA levels seems also to be present in T cell lymphomas. Anaplastic large cell lymphoma (ALCL), a T cell neoplasm, was identified to have the same phenotype of *NOXA* mRNA expression as CLL and MCL (Figure 1 and 2). Yet, it can only be speculated what the underlying mechanism of high *NOXA* mRNA levels in ALCL are. Nothing is known about chronic active T cell receptor signaling in ALCL but constitutively active PI3K/AKT/mTOR signaling has also been described in this type of lymphoma (Vega et al., 2006). Furthermore, overexpression of PKC, the protein kinase regulating *NOXA* transcription upon TCR stimulation, was identified as a feature of ALCL (Thompson et al., 2005) suggesting that chronic TCR signaling might be an existing phenomenon in T cell neoplasms.

4.1.2 Low constitutive NOXA protein levels in MCL

Increased *NOXA* expression is generally implicated in induction of cell death or thought to enhance sensitivity to chemotherapy (Ploner et al., 2008). For example, high constitutive *NOXA* transcript and protein levels were recently implicated as critical determinants of the hypersensitivity of embryonal carcinoma cells to the DNA damaging agent Cisplatin (Gutekunst et al., 2013). Astonishingly, the high *NOXA* mRNA levels observed in MCL cells neither impair cell viability nor sensitize MCL cells to chemotherapeutics. MCL patients show only transient response to chemotherapy (Smith, 2011), whereas most patients with

embryonal carcinomas can be cured by chemotherapy (Bosl and Motzer, 1997; Einhorn, 2002). In this study, *NOXA* mRNA as well as protein expression of MCL cells was compared to a panel of cancer cell lines including embryonal carcinoma cells. Astonishingly, although MCL cells express the highest levels of *NOXA* mRNA, NOXA protein expression is relatively low in the B cell malignancy especially when compared to the embryonal carcinoma cell lines (Figure 2). These results indicate that MCL cells adapt to the PI3K/AKT/mTOR mediated constant apoptotic signal by keeping NOXA protein low. This adaptation might provide a survival advantage during selection processes in B cell development and contribute to the pathogenesis of MCL. Such a discrepancy between mRNA and protein expression can be caused by various mechanisms. Protein levels are often post-transcriptionally as well as post-translationally regulated. A recent study showed that in 23 human cancer cell lines only one third of RNA expression levels of more than 1000 genes analyzed correlated with the levels of the corresponding proteins (Gry et al., 2009). One mechanism leading to discrepancies between mRNA and protein expression is decreased protein synthesis. Translation initiation and efficacy is a highly regulated process and often altered in cancer cells (Meric and Hunt, 2002; Silvera et al., 2010). However, a more frequently observed event is a selectively enhanced protein turnover. Targeted degradation of several proteins involved in tumor suppression has been shown enhanced in many cancer cells. Important tumor suppressors affected by deregulation of protein turnover include p27 (Gstaiger et al., 2001) and RB1 (Higashitsuji et al., 2005). In the present work, enhanced NOXA protein degradation was also identified as the underlying mechanism of the observed discrepancy between *NOXA* mRNA and protein levels in MCL. In contrast to other cancer cell lines and PBMCs of healthy donors, NOXA protein was found to be extremely unstable in the MCL cells lines resulting in protein half-life of only 15-30 min (Figure 11). In strong contrast, NOXA protein was very stable in the embryonal carcinoma cell line NTERA-2D1, whose *NOXA* mRNA levels are comparable to those of MCL cell lines. These huge differences suggested that NOXA degradation is a highly regulated process, which might contribute to deregulation of apoptosis and evasion of cell death.

The UPS mainly regulates selective degradation of cellular proteins (Hershko and Ciechanover, 1998). Enhanced UPS-mediated turnover of pro-apoptotic Bcl-2 family members was shown by several studies to contribute to tumorigenesis. For example, increased turnover of the BH3-only protein BIM is frequently found in different types of cancer (Akiyama et al., 2009). In the present work, NOXA was also identified to be a target of extensive polyubiquitination and proteasomal degradation in MCL cells (Figure 12). It was

proposed before, that NOXA protein levels are regulated by the UPS. Baou *et al.* showed that NOXA is ubiquitinated at several lysine residues resulting in its proteasomal degradation. The half-life of NOXA determined by this group was below 2 hours in primary CLL cells (Baou et al., 2010). These findings are in line with the previous discussed hypothesis that CLL cells might have the same phenotype of *NOXA* expression as MCL cells. Although NOXA half-life seems to be slightly longer, these cells also appear to adapt to the high *NOXA* mRNA levels by rapidly degrading NOXA protein. Selective protein degradation therefore seems to represent a strategy of cancer cells to keep the pro-apoptotic BH3-only proteins in check and control their levels. However, the results of this study suggest that there are distinct differences in efficacy of NOXA turnover in cancer cells that seem to have a great impact on the response to chemotherapy. This view is supported by the recent study of Brinkmann *et al.* (2013). They identified UCH-L1 as the specific deubiquitinase (DUB) of NOXA and therefore an important regulator of NOXA turnover. UCH-L1 stabilizes NOXA by removing ubiquitin residues from the pro-apoptotic protein. The DUB was found to correlate with cellular NOXA expression levels and seems to be a critical determinant of the response to genotoxic stress. Interestingly, *UCH-L1* is epigenetically silenced in several chemoresistant tumors (Brinkmann et al., 2013). According to this data, UCH-L1 functions as a tumor suppressor, which contributes to sensitivity of cancer cells to chemotherapeutics. Interestingly, an opposing role of UCH-L1 as a protein with oncogenic features has also been proposed. It was shown that overexpression of the DUB can contribute to oncogenesis by deregulating AKT signaling (Hussain et al., 2010). The exact role of UCH-L1 in cancer seems controversial and its main function may depend on the cellular background. The expression levels of UCH-L1 in MCL were not investigated so far but loss of the DUB might contribute to the enhanced turnover rate of NOXA protein in MCL. In contrast to the DUB, the ubiquitin ligase of NOXA was not identified so far. The SCF E3 ligase subfamily of CRLs was implicated in the ubiquitination process of NOXA (Jia et al., 2010).

Taken together, the levels of the pro-apoptotic Bcl-2 family member NOXA are post-translationally controlled by a complex interplay of various processes. In MCL cells, ubiquitination and subsequent proteasomal degradation appear to be the dominant mechanism leading to a very short half-life of NOXA. This enhanced protein turnover seems to be the adaptive mechanism of the malignant B cells to the high basal levels of *NOXA* mRNA and might also contribute to the poor response of MCL patients to chemotherapy. However, the signal that regulates the high turnover rate of NOXA in MCL cells could not be elucidated so far. Remarkably, NOXA degradation was not affected by inhibition of BCR signaling or

Cyclin D1 knockdown (Figure 13 and Figure 14) suggesting that these central oncogenic alterations do not mediate a dual signal, which results in the observed discrepancy in *NOXA* expression. As described in 1.1.2, alterations in several other oncogenic pathways have been shown in MCL that might mediate the increased turnover rate of the BH3-only protein (Jares et al., 2012). For example, components of the WNT signaling pathway, which has been shown to be constitutive active in MCL (Gelebart et al., 2008), were implicated in regulating the activity of several E3 ubiquitin ligases (Tauriello and Maurice, 2010). Rapid NOXA protein turnover might also be regulated by epigenetic mechanisms since B cell development is controlled by several epigenetic events (Barneda-Zahonero et al., 2012). Interestingly, MCL cells have significant alterations in gene methylation. Multiple genes were found hyper- and hypo-methylated in the malignant B cells (Leshchenko et al., 2010). These events could also influence the expression and activity of the putative NOXA E3-ligase and therefore NOXA protein expression in MCL.

Although the regulation of UPS-mediated NOXA protein turnover remains elusive, the phenotype of *NOXA* expression in MCL could provide a target for treatment of MCL and other lymphoid neoplasm since stabilization and subsequent accumulation of NOXA could be an efficient strategy to kill or sensitize these cells.

4.2 Rapid UPS-mediated NOXA protein turnover represents an Achilles heel of MCL

During the last years, the UPS has become a prominent target for cancer therapy since deregulation of the UPS has been associated with aberrant expression levels of proteins involved in oncogenesis (Micel et al., 2013). The proteasome inhibitor Bortezomib is the most prominent clinically used agent targeting the UPS. Bortezomib has shown activity in different cancer entities and was approved for treatment of newly diagnosed multiple myeloma and also relapsed/refractory MCL (Chen et al., 2011). The mechanism how Bortezomib induces cell death and acts as an anticancer therapeutic is controversial and appears to depend on the cellular background (Mujtaba and Dou, 2011). Inhibition of NF-κB was proposed as one mechanism leading to apoptosis induction upon Bortezomib treatment in different tumor entities (An et al., 2004; Fahy et al., 2005). Other studies implicated changes in the Bcl-2 family as major mediators of the cytotoxic effects of Bortezomib (Fennell et al., 2008). Up-regulation of the BH3-only proteins BIM and Bik, for example, was observed by several groups (Li et al., 2008; Nikrad et al., 2005; Tan et al., 2005). Interestingly, NOXA induction was identified as the crucial event in apoptosis upon proteasome inhibition in several

lymphoid neoplasms including MCL (Baou et al., 2010; Gomez-Bougie et al., 2007; Pérez-Galán et al., 2006). The screening for agents inducing NOXA protein expression in MCL in the present study confirmed this observation. The proteasome inhibitor strongly induced NOXA protein and led to induction of severe apoptosis (Figure 15 and Figure 16). The previous studies on the role of NOXA in Bortezomib-induced cell death in MCL proposed that *NOXA* is transcriptionally activated in response to induction of endoplasmatic reticulum stress and generation of reactive oxygen species (ROS) upon proteasome inhibition (Pérez-Galán et al., 2006; Weniger et al., 2011). Two mechanisms were proposed to cooperate for a p53 independent induction of *NOXA* transcription: activation of the transcription factors ATF3 and ATF4, and reduced ubiquitination of the histone H2A (Wang et al., 2009). Astonishingly, although NOXA protein was strongly induced in response to Bortezomib treatment (Figure 15), only minor changes in *NOXA* transcript levels were observed in the cell lines used in this study (Figure 21). Furthermore, active PI3K/AKT/mTOR and BCR signaling were required for efficient accumulation of NOXA and induction of cell death (Figure 19 and Figure 20). These data indicate that the high *NOXA* transcript levels are a prerequisite for the sensitivity of MCL cells to proteasome inhibitors and suggest that stabilization of the preexisting pool of NOXA in MCL is the main mechanism of NOXA accumulation. Indeed, Bortezomib was found in the present work to stabilize the short-lived BH3-only protein in the MCL cell lines (Figure 22). These results lead to the conjecture that transcriptional induction of *NOXA* mRNA, as proposed by other groups, may be a secondary event in the response of MCL cells to Bortezomib further increasing the stabilized NOXA protein.

The phenotype of high *NOXA* mRNA/low NOXA protein expression seems to be the critical determinant for the Bortezomib sensitivity of MCL cells. Therefore, high basal expression of *NOXA* mRNA could represent a general predictive marker for sensitivity to proteasome inhibitors. This hypothesis is supported by the finding that other lymphomas with comparable constitutive *NOXA* transcript levels such ALCL and CLL are also sensitive to Bortezomib (Baou et al., 2010; Bonvini et al., 2007). In CLL cells, also stabilization of NOXA rather than transcriptional induction of the pro-apoptotic Bcl-2 family member was suggested as the critical factor for Bortezomib-induced apoptosis (Baou et al., 2010). Intriguingly, the correlation between constitutive *NOXA* mRNA expression and Bortezomib sensitivity seems not to be restricted to lymphoid neoplasms. Thyroid cancer represents the cancer entity with highest basal levels of *NOXA* gene expression according to the data from CCLE (Figure 1). Mitsiades *et al*. recently demonstrated efficacy of Bortezomib in different types of thyroid

carcinoma cell lines (Mitsiades et al., 2006). In addition, first clinical studies showed promising effects of proteasome inhibitor in patients with metastasized thyroid cancer (Putzer et al., 2012) indicating that *NOXA* levels may indeed be a prognostic marker.

The observation that the basal level of *NOXA* mRNA is a critical factor for the response of MCL cells to Bortezomib has also implications for the design of rational combination treatment strategies. According to the results obtained in this study, BCR or PI3K/AKT/mTOR signaling inhibitors such Ibrutinib and Bez235 should not be administered prior to proteasome inhibitors (Figure 19 and Figure 20) since these inhibitors were identified to significantly reduce the high *NOXA* mRNA levels in MCL. However, studies from other groups suggest that starting with Bortezomib or simultaneous administration of the proteasome inhibitor with Ibrutinib could be beneficial (Dasmahapatra et al., 2013) indicating that under certain circumstances the order of drug administration is an important factor for efficacy of combination treatment strategies. It is also conceivable that sequential administration of Bortezomib could prevent drug resistance to Ibrutinib or Bez235 by targeting clones with still active PI3K/AKT/mTOR signaling. To better predict such unexpected combinatory effects of Bortezomib with other drugs, it is important to further explore the pathways regulating constitutive *NOXA* expression levels in MCL and other malignancies.

Although the underlying mechanism is arguable, induction of the pro-apoptotic protein NOXA in response to treatment with the proteasome inhibitor Bortezomib has been shown before (Pérez-Galán et al., 2006; Wang et al., 2009). In the present study, two other substances, MLN4924 and Orlistat, were identified as efficient inducers of NOXA protein and NOXA-dependent apoptosis in MCL cells (Figure 15 and Figure 17). In contrast, Cisplatin and Doxorubicin, two DNA damaging agents and known transcriptional inducers of *NOXA* (Gutekunst et al., 2011; Kurata et al., 2008), were not able to induce *NOXA* mRNA as well as protein in MCL cells and led only to a minor reduction of cell viability (Figure 15, Figure 16 and Figure 21). This might be due to defects in the classical damage response pathways in these cells, a phenomenon frequently observed in MCL (Fernàndez et al., 2005).

Similar to Bortezomib, active PI3K/AKT/mTOR signaling was identified to be a prerequisite for efficient NOXA accumulation and induction of cell death upon treatment with MLN4924 or Orlistat (Figure 19 and Figure 20). Interestingly, in contrast to Bortezomib, both substances were found to stabilize NOXA protein by interfering with the ubiquitination process of NOXA (Figure 22 and Figure 23). MLN4924 is an inhibitor of the NEDD8-activation enzyme (NAE) thereby interfering with the neddylation of many proteins including CRL ubiquitin

ligases (Soucy et al., 2009). Neddylation is required for ligase activity of CRLs and thus involved in regulating protein turnover of CRL substrates. The CRL subfamily of E3 ligases controls the cellular expression levels of several proteins involved in proliferation and survival (Watson et al., 2011). Aberrant activity of certain CRL ligases was implicated in cancer and members of the CRL family such as SCF^{SKP2} have even been described as potential oncogenes (Wei et al., 2012). Targeting CRL activity with MLN4924 has shown promising effects in different types of tumor models (Milhollen et al., 2010; Swords et al., 2010) and is currently investigated in first clinical trials (Wang et al., 2011). Inhibition of the whole CRL ligase subfamily by MLN4924 appears to have multiple effects on the cells and mechanisms of apoptosis induction may vary between different tumor types. NF-κB pathway inhibition, DNA re-replication and DNA damage were proposed as major mediators of MLN4924-induced apoptosis (Blank et al., 2013; Milhollen et al., 2010; Soucy et al., 2009). In MCL cells, stabilization of NOXA seems to be one of the key events for initiation of cell death. This finding is another evidence that CRL family members are involved in ubiquitination of NOXA (Jia et al., 2010). Surprisingly, in contrast to Bortezomib or Orlistat, parallel induction of *NOXA* gene expression was observed after treatment of the MCL cells with the NAE inhibitor (Figure 21). Since classical DNA damaging agents were not able to transcriptionally activate *NOXA* it seems unlikely that classical DNA damage response pathways are responsible for this observation. Accumulation of transcription factors involved in regulation of *NOXA* gene expression might account for enhanced *NOXA* transcription upon CRL inhibition. Turnover of several known transcription factors of *NOXA* including ATF4, HIF1a or MYC (Kim et al., 2004; Nikiforov et al., 2007; Wang et al., 2009) is regulated by members of the E3 ligase family (Jia and Sun, 2011). This data would suggest that the additional transcriptional response of *NOXA* should amplify NOXA protein accumulation in cells treated with MLN4924. Astonishingly, NOXA induction upon CRL inhibition was not as strong as observed in response to Bortezomib or Orlistat treatment although efficacy of NOXA-dependent cell death induction of the three substances was comparable (Figure 15 and Figure 17). The main binding partner of NOXA is the Bcl-2 family member Mcl-1. The anti-apoptotic protein selectively binds and sequesters NOXA with high affinity thereby preventing NOXA from apoptosis induction (Certo et al., 2006; Chen et al., 2005). Cellular levels of Mcl-1 can influence the susceptibility of cells to NOXA-induced apoptosis (Ploner et al., 2008). It could be demonstrated in the present work that in contrast to Bortezomib or Orlistat, which induce Mcl-1 in parallel to NOXA, MLN4924 treatment of the lymphoma cells rather reduce the levels of the anti-apoptotic protein (Figure 18). This observation

indicates that less NOXA protein accumulation is sufficient to efficiently induce cell death upon treatment with MLN4924. At a glance, this finding seems counterintuitive. As previously described, turnover of Mcl-1 is also mediated by CRL ligases (Inuzuka et al., 2011). Consequently, inhibition of NAE by MLN4924 should lead to the stabilization and accumulation of Mcl-1. The missing accumulation of the anti-apoptotic protein might represent a consequence of DNA damage, which is observed upon MLN4924 treatment but not implicated in the response of cancer cells to Bortezomib or Orlistat (Soucy et al., 2009). DNA damage was shown to reduce Mcl-1 levels (Zhong et al., 2005) and could counteract MLN4924-induced Mcl-1 accumulation.

Orlistat is the second substance found in the present study to induce the BH3-only protein NOXA in MCL cells (Figure 15). Orlistat is an inhibitor of the fatty acid synthase (FASN). The metabolic enzyme is catalyzing the synthesis of the fatty acid palmitate and is a key player in *de novo* lipogenesis, an important metabolic pathway that has been linked to the pathogenesis of several types of tumors (Kuhajda, 2006; Menendez and Lupu, 2007). Gelebart *et al*. recently showed overexpression of FASN and efficacy of Orlistat in MCL cell lines and primary MCL cells (Gelebart et al., 2012). However, the underlying mechanisms of the cytotoxic effects of Orlistat in MCL remained unclear. Induction of cell cycle arrest and apoptosis via the extrinsic pathway as well as intrinsic pathway has been shown in response to Orlistat treatment in different tumor models (Kant et al., 2012; Knowles et al., 2004, 2008). In the present work, NOXA was determined as the key mediator of Orlistat-induced cell death in MCL cells (Figure 17). Intriguingly, Orlistat-mediated FASN inhibition stabilized NOXA by impairing ubiquitination of the pro-apoptotic protein (Figure 22 and Figure 23). An existing crosstalk between fatty acid metabolism and the UPS has been proposed by several reports (Little et al., 2008). FASN protein stability, for example, is regulated by the de-ubiquitinating enzyme USP2A (Graner et al., 2004). On the other hand, FASN appears to regulate expression of multiple enzymes involved in protein ubiquitination including E2 ubiquitin conjugating enzymes and E3 ubiquitin ligases (Knowles and Smith, 2007b). Interestingly, FASN inhibition by Orlistat was demonstrated to reduce expression of Skp2, a component of an CRL E3 ligase (Knowles et al., 2004). Therefore, Orlistat might indirectly target the ubiquitin ligase of NOXA by mediating its down-regulation through inhibition of fatty acid metabolism.

These findings indicate that both MLN4924 as well as Orlistat might represent alternative therapy options to classical proteasome inhibitors. This is of high clinical relevance since Bortezomib resistance is a frequently observed phenomenon in cancer. Mechanisms leading

to the resistance to the proteasome inhibitor include enhanced expression of proteasome components, mutations in the chemotrypsin-like subunit or alterations in the cellular stress response (Lü and Wang, 2013). Plasmacytic differentiation and increased levels of the pro-survival chaperone BIP/Grp78 have been linked to Bortezomib resistance in MCL cells (Pérez-Galán et al., 2011b; Roué et al., 2011). The data obtained in the present study suggest that enhanced proteasomal activity/capacity might represent another mechanism of drug resistance in MCL cells. Compared to the Bortezomib sensitive parental cell line, Bortezomib-mediated NOXA accumulation was found to be significantly lower in the resistant clone used in this work indicating that the used concentrations are not sufficient to inhibit proteasomal degradation of NOXA (Figure 24). Of importance, both MLN4924 and Orlistat were still able to accumulate the BH3-only protein and induce severe apoptosis in the Bortezomib resistant clone due to their ability to target UPS-mediated NOXA degradation in MCL cells independently of the proteasome (Figure 24). Consequently, both substances might serve as alternatives in patients showing resistance to Bortezomib. MLN4924 and Orlistat might even offer a greater therapeutic window than Bortezomib since both substances seem to be more selective for MCL cells than Bortezomib (Figure 25). This observation underlines the clinical relevance of the findings made in this study.

4.3 FASN inhibitors selectively kill Cyclin D1 overexpressing MCL cells

The t(11;14)(q13;q32) translocation leading to the constitutive overexpression of Cyclin D1 is thought to be the primary event in oncogenesis of MCL and a driver of tumor cell proliferation (Pérez-Galán et al., 2011a). The importance of Cyclin D1 for cell cycle progression and viability of MCL cells was recently shown by Weinstein *et al*. (2012) who demonstrated that complete knockdown of Cyclin D1 induces cell cycle arrest and apoptosis in MCL cells and once again highlighted the potential of Cyclin D1 as a target for therapy (Weinstein et al., 2012). However, targeting the oncogenic protein is difficult since Cyclin D1 lacks intrinsic enzymatic activity (Musgrove et al., 2011). Inhibition of Cyclin D1 downstream targets such as cell cycle regulating kinases CDK4 and 6 appear to represent alterative targets to circumvent this problem. First studies using a CDK4/6 inhibitor showed promising responses in a subset of MCL patients (Leonard et al., 2012). In addition to the function in regulation of cell cycle progression, Cyclin D1 has several CDK independent functions (Fu et al., 2004; Musgrove et al., 2011). Consequently, Cyclin D1 overexpression might provide other therapeutic targets through its impact on diverse cellular processes.

Aberrant expression of Cyclin D1 was identified in the present study to render MCL cells susceptible to FASN inhibitors. In contrast to Bortezomib or MLN4924, partial knockdown of Cyclin D1 substantially rescued MCL cells from Orlistat-induced cell death (Figure 26). As mentioned in the previous chapter, FASN catalyzes the synthesis of the fatty acid palmitate (Kuhajda, 2006). Apoptosis and NOXA induction upon treatment of MCL cells with Orlistat was found to be indeed dependent on palmitate depletion. Although cellular uptake of free fatty acids is limited, supplementation of palmitate was able to partially rescue the cell from induction of cell death and reduced expression of NOXA (Figure 27). Consequently, further experiments showed that sensitivity of MCL cells to FASN inhibitors is not limited to Orlistat (Figure 28). However, both additional FASN inhibitors, Cerulenin and C75, had slightly different effects on the expression of the Bcl-2 family members NOXA and Mcl-1. Induction of NOXA was less pronounced in presence of Cerulenin or C75 when compared to Orlistat. Interestingly, induction of Mcl-1 was also lacking upon treatment with these inhibitors. Furthermore, similar to MLN4924, both FASN inhibitors rather reduced Mcl-1 expression (Figure 29). Nevertheless, as observed for Orlistat both inhibitors appear to induce cell death by accumulation of NOXA since silencing of *NOXA* almost completely rescued MCL cells from induction of apoptosis (Figure 30). Protein expression levels of other important members of the Bcl-2 family were not significantly altered by any of the FASN inhibitors indicating that indeed disturbance of the balance between NOXA and its anti-apoptotic partner Mcl-1 is the major apoptotic event. The effector protein Bak appeared to be post-translationally modified by Orlistat treatment but not after Cerulenin or C75 (Figure 29). The observed slower migrating band of Bak might represent the phosphorylated form of the pro-apoptotic protein (Tran et al., 2013). However, the function of Bak phosphorylation in apoptosis is controversial (Fox et al., 2010; Tran et al., 2013). In contrast to Orlistat, which targets the thioesterase domain of FASN, Cerulenin and C75 are irreversible inhibitors of the ketoacyl synthase domain (Flavin et al., 2010). Whether these differences in targeting the mutlidomain enzyme FASN are responsible for the distinct effects of the FASN inhibitors on NOXA and Mcl-1 expression as well as post-translational modification of Bak is unclear and can only be speculated. These observations could be the result of off-target effects, just as well. Depletion of Mcl-1 by treatment of the cells with Cerulenin or C75 was demonstrated before in other tumor models (Ho et al., 2007).

Although several differences were observed between both types of FASN inhibitors, this study demonstrates that their cytotoxic effects in MCL consistently depend on Cyclin D1 overexpression (Figure 31). Several reports implicated Cyclin D1 in regulation of cellular

metabolism. Cyclin D1 was found to exert inhibiting effects on aerobic glycolysis, mitochondrial activity as well as lipogenesis in mammary tumors (Sakamaki et al., 2006). Similar findings in B cells confirmed the negative effect of Cyclin D1 on metabolism. The study by Tchakarska *et al*. demonstrated that Cyclin D1 localizes to the outer mitochondrial membrane thereby reducing mitochondrial activity and energy production (Tchakarska et al., 2011). Further evidence for an existing connection between the cell cycle regulatory protein and lipogenesis was given by studies in primary hepatocytes showing that Cyclin D1 expression substantially impairs hepatic lipogenesis in response to glucose stimulation (Hanse et al., 2012). These findings seem to be counterintuitive since lipogenesis and especially aerobic glycolysis, two of the major metabolic pathways in cancer cells, are thought to promote tumor cell growth (DeBerardinis et al., 2008; Menendez and Lupu, 2007). However, in normal cells these interactions might support a proper completion of the S-phase during cell cycle. Reduction of Cyclin D1 as it is observed at the beginning of S-phase might provide extra energy and metabolic intermediates for replication and cell growth (Buchakjian and Kornbluth, 2010). In some malignant cells including MCL, Cyclin D1 expression is deregulated and the cell cycle regulatory protein is also present in S-phase. This aberrant expression of the Cyclin D1 is generally thought to promote cell growth and proliferation (Kim and Diehl, 2009). However, according to the findings mentioned above this might also maintain the inhibitory effects of Cyclin D1 on metabolism in parallel. Since cancer cells rely on metabolic pathway such as glycolysis and lipogenesis Cyclin D1 overexpression might render cells more dependent on the residual cellular metabolic activity. This could explain the sensitivity of Cyclin D1 overexpression MCL cells to FASN inhibitors. The previous studies on the effect of Cyclin D1 on lipogenesis and other pathways proposed that Cyclin D1 inhibits the transcription of several metabolic enzymes including FASN (Hanse et al., 2012; Sakamaki et al., 2006). In contrast to the data demonstrated by Sakamaki *et al*. (2006) in mammary tumors, RNAi mediated silencing did not induce FASN protein expression in MCL cells (Figure 32). Recent findings indicated that Cyclin D1 might not only regulate the expression levels of metabolic enzymes but also directly influence their activity by binding them. A screen for protein binding partners of Cyclin D1 revealed that FASN is a direct binding partner of the cell cycle regulatory protein (Jirawatnotai et al., 2011). Analysis of changes in the metabolome upon RNAi mediated down-regulation of Cyclin D1 that were performed in this study confirmed its strong influence on cellular metabolism also in MCL cells (Figure 33). Several metabolites were significantly changed. For example, concentration of intracellular hexose-6-phosphates is increased upon Cyclin D1 depletion suggesting

enhanced activity of hexokinase II. These results are in line with previous findings (Sakamaki et al., 2006). Several other changes in intracellular metabolites, such as enhanced TAG48:0 and citrate concentrations also point to enhanced fatty acid synthesis and lipogenesis in MCL cells with reduced Cyclin D1 expression indicating that direct inhibitory effects of Cyclin D1 overexpression on cellular metabolism render the cells susceptible to FASN inhibitors. The fact that down-regulation of Cyclin D1 is accompanied by a slight reduction in proliferation might decrease the cellular demand on metabolic intermediates and therefore reduce their dependency on metabolic activity may contribute to the resistance to FASN inhibitors.

Finally, a screen for potential combination therapies revealed that efficacy of FASN inhibitors in slightly less sensitive MCL cells can be further enhanced by combination with other anti-cancer drugs (Figure 34). Most effective combinations include Orlistat or Cerulenin with the DNA damaging agents Cisplatin and Etoposide, the p53-activating agent Nutlin-3 and the BH3-mimetic ABT737. The synergistic effect of FASN inhibitors with Nutlin-3 is most likely limited to tumor cells expressing wild type p53 such as the MCL cell lines Jvm2 and Granta519, which were used in the combination screen performed in the present work. However, the combination with DNA damaging agents or BH3-mimetics might represent a more general approach to kill MCL cells. BH3-mimetics sequester anti-apoptotic proteins and thereby disturb the balance between pro- and anti-apoptotic proteins (van Delft et al., 2006). In cells with high NOXA levels, such as upon treatment with FASN inhibitors, treatment with ABT737 might be sufficient to reach the apoptotic threshold and induce apoptosis. DNA damage might have similar effects by reducing cellular Mcl-1 levels (Zhong et al., 2005). This is in line with the view that constitutive expression levels of BH3-only proteins can determine the response to chemotherapeutics (Davids and Letai, 2012). Stabilization and accumulation of NOXA even by low dose treatment of the cells with FASN inhibitors seems to enhance the cellular “priming” for cell death induction by a second apoptotic stimulus such as DNA damage. Treatment of the cells with NOXA-accumulating agents might therefore generate a more apoptosis-prone phenotype similar to that observed in embryonal carcinoma cells (Gutekunst et al., 2013). Of note, the most effective combination treatments identified in this study are tumor selective since PBMCs of healthy donors were unaffected. The synergistic effects of FASN inhibitors with other substances likely depend on the enhanced NOXA protein levels indicating that NOXA might be an important player for tumor selective induction of cell death. Indeed, it has been shown before that NOXA, in contrast to other BH3-only proteins such as PUMA, selectively induces apoptosis in human cancer cells (Suzuki et al., 2009). Consequently, induction of NOXA protein by FASN inhibitors, for

example, may represent a promising tool to sensitize not only MCL cells but also other tumor entities for conventional drugs used in chemotherapy. An important role of NOXA as mediator of drug combinations therapy was already proposed by previous studies (Ehrhardt et al., 2012). This view is supported by recent work showing synergistic effects of Bortezomib with drugs such as Cisplatin or ABT737 in different tumor model systems (Al-Eisawi et al., 2011; Premkumar et al., 2012, 2013; Reuland et al., 2012).

In summary, Cyclin D1 renders MCL cells susceptible to FASN inhibitors by exerting an inhibitory effect on cellular metabolism. Targeting FASN in MCL cells leads to accumulation of NOXA and cell death, which can be further enhanced by combination with other anti-cancer drugs.

4.4 Conclusion and outlook

The present study provides important insights in the regulation of the pro-apoptotic BH3-only protein NOXA and its critical role in life/death decision in MCL. The results from this study demonstrate that MCL cells express unexpectedly high levels of *NOXA* transcript and adapt to this by rapid turnover of the NOXA protein. The resulting phenotype of high *NOXA* mRNA/low NOXA protein constitutes an Achilles heel of MCL cells. Targeting the rapid UPS-mediated NOXA protein turnover on different levels accumulates the pro-apoptotic protein and efficiently induces cell death in MCL cells. Of importance, the high constitutive mRNA levels of *NOXA* predominantly mediated by constitutive activation of the PI3K/AKT/mTOR pathway are required for efficacy of agents targeting NOXA turnover in MCL cells. Unexpectedly, fatty acid metabolism was identified to be involved in the NOXA ubiquitination process. Especially MCL cells appear to rely on their metabolic activity since Cyclin D1 overexpression, the hallmark of MCL, exerts inhibitory effects on the central metabolic pathways thereby rendering the cells more sensitive to agents targeting cancer cell metabolism such as the FASN inhibitors that were identified in this study to stabilize NOXA protein levels in MCL cells.

In conclusion, the present work is the first study demonstrating aberrant regulation and expression of the pro-apoptotic BH3-only protein NOXA in MCL. The continuous rapid NOXA protein turnover in MCL may provide a strategy of the cells to evade selection processes in early B cell development possibly contributing to pathogenesis of the aggressive B cell lymphoma. However, in contrast to frequently observed gene deletions of the pro-apoptotic Bcl-2 family member *BIM* in MCL cells, the reduced expression of NOXA is a

regulated process and therefore constitutes a potential druggable target. Indeed, the discrepant levels between *NOXA* mRNA and protein were found in this study to represent a promising target for treatment of MCL cells. The combination of high *NOXA* mRNA and a short-lived NOXA protein seems to "prime" MCL cells for cell death by agents interfering with ubiquitination or proteasomal degradation of NOXA protein such as MLN4924 and Orlistat, or Bortezomib. The term "priming" was introduced by Letai *et al*. and describes the proximity of a cancer cell to the apoptotic threshold (Davids and Letai, 2012). They linked the cellular "priming" to the basal protein expression and balance of the Bcl-2 family members and established a method called "BH3 profiling" to assess the priming status of a cancer cell and predict the response to chemotherapeutics. In this study, constitutive *NOXA* mRNA levels were identified as a potential biomarker for sensitivity of cancer cells to NOXA accumulating compounds such as Bortezomib. Consequently, constitutive high mRNA levels of certain pro-apoptotic genes such as *NOXA* may also represent a type of endogenous "priming" for cell death mediated by anti-cancer drugs such as inhibitors of the UPS, which are able to accumulate unstable pro-apoptotic proteins and convert the high transcript levels into high protein levels. In MCL cells, central oncogenic pathways such BCR and PI3K/AKT/mTOR signaling were identified to be involved in maintaining the high *NOXA* mRNA levels and are therefore a prerequisite for the "primed" status of the cells. Thus, administration of drugs targeting these pathways in combination with UPS inhibitors should be done with caution since antagonist effects may occur.

The obtained data in this study suggest that targeting the UPS upstream of the proteasome represents a more selective way to kill cancer cells "primed" by a discrepant phenotype of *NOXA* expression. Unfortunately, the E3 ubiquitin ligase of NOXA, which would represent the most selective target to inhibit NOXA turnover, is unknown so far. Consequently, further experiments are needed to identify the ubiquitin ligase of NOXA and gain deeper insights into the regulation of the ubiquitination and subsequent degradation of the BH3-only protein. This may lead to the identification of novel targets for more efficient and selective therapy of MCL and other neoplasms. The results of the present study partially contribute to the understanding of the complex interplay of multiple cellular pathways involved in this process and may provide hints for novel treatment options. The identification of the neddylation inhibitor MLN4924 to accumulate NOXA in MCL suggests that a certain member of the CRL family of E3 ligases targets NOXA for proteasomal degradation. Furthermore, fatty acid metabolism was identified to be one of the processes involved in regulation of NOXA ubiquitination. Especially the latter may represent a very selective target for treatment of MCL cells. It could

be demonstrated that inhibitory effects of Cyclin D1 overexpression on cellular metabolism render the cells more dependent on the residual metabolic activity and thus susceptible to inhibitors of fatty acid metabolism such as Orlistat. Astonishingly, two essential processes of MCL, namely NOXA turnover and fatty acid metabolism, appear to be cross-linked in this B cell lymphoma pointing to the great potential of targeting this signaling axis. Although the present results are based on *in vitro* studies, MLN4924 and fatty acid metabolism inhibitors such as Orlistat represent interesting clinical alternatives to the currently used proteasome inhibitor Bortezomib. Both, the superior selectivity as well as their ability to kill Bortezomib resistant clones may contribute to a better clinical outcome of MCL patients.

Enhanced NOXA expression has been shown by several groups to promote sensitivity to chemotherapeutics (Brinkmann et al., 2013; Gutekunst et al., 2013). Specific targeting of NOXA turnover by substances such as Orlistat or MLN4924 might not only provide a way to kill MCL or CLL cells but also enhance NOXA expression and therefore lower the apoptotic threshold in other cancer cells resistant to chemotherapy. Synergistic effects of FASN inhibitors with DNA damaging agents or BH3-mimetics as observed in the drug combination screen performed in the present work support this hypothesis.

In summary, this study not only sheds light in the underlying mechanism of the enhanced sensitivity of MCL cells to proteasomal inhibitors but also identified a new druggable Achilles heel of MCL cells. These results might offer novel therapeutic options for improvement of MCL treatment, which are urgently needed for this aggressive B cell malignancy. The findings may also provide new treatment strategies for other neoplasms and help to increase the response of chemoresistant tumors.

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7 Publications

This work was published in part in the following publications:

Original articles

Dengler MA, Weilbacher A, Gutekunst M, Staiger AM, Vöhringer MC, Horn H, Ott G, Aulitzky WE, Van der Kuip H: Discrepant *NOXA* (*PMAIP1*) transcript and NOXA protein levels: a potential Achilles' heel in mantle cell lymphoma. *Cell Death Dis* 2014

Meeting Abstracts

Dengler MA, Weilbacher A, Gutekunst M, Staiger AM, Horn H, Ott G, van der Kuip H, Aulitzky WE: High NOXA (PMAIP1) transcript levels combined with a short-lived NOXA protein primes mantle cell lymphoma (MCL) cells for death by inhibition of the ubiquitin proteasome system. In: Proceedings of the 104th Annual Meeting of the American Association for Cancer Research; 2013 Apr 6-10; Washington, DC. Philadelphia (PA): AACR; 2013. Abstract nr 1717

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Other publications

Original articles

Gutekunst M, Mueller T, Weilbacher A, **Dengler MA**, Bedke J, Kruck S, Oren M, Aulitzky WE, Van der Kuip H: Cisplatin hypersensitivity of testicular germ cell tumors is determined by high constitutive *NOXA* levels mediated by Oct4. *Cancer Res* 2013.

Yilmaz A, **Dengler MA**, Van der Kuip H, Yildiz H, Rösch S, Klumpp S, Klingel K, Kandolf R, Helluy X, Hiller K-H, Jakob PM, Sechtem U: Imaging of myocardial infarction using ultrasmall superparamagnetic iron oxide nanoparticles: a human study using a multi-parametric cardiovascular magnetic resonance imaging approach. *Eur Heart J* 2013, 34:462–475.

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Meeting abstracts

Gutekunst M, Mueller T, Weilbacher A, **Dengler MA,** Oren M, Aulitzky WE, van der Kuip H: Testicular germ cell tumors are hypersensitive to p53 activation based on their Oct-4/*NOXA*-mediated cellular context rather than on differential p53 activity. In: Proceedings of the 104th Annual Meeting of the American Association for Cancer Research; 2013 Apr 6-10; Washington, DC. Philadelphia (PA): AACR; 2013. Abstract nr 1721

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Dengler MA, Staiger A, Gutekunst M, Hofmann U, van der Kuip H, Aulitzky WE: Glucocorticoids inhibit cell death induced by oncogenic stress after imatinib withdrawal in TKI resistant p190Bcr/Abl overexpressing cells. Blood (ASH Annual Meeting Abstracts) 2010 116: Abstract 3150.

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8 Curriculum vitae

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Name:	Michael Dengler
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Education and training

03/10 - 10/13 **PhD thesis at the Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology, Stuttgart**

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Prof. Dr. Walter E. Aulitzky, Robert Bosch Hospital, Stuttgart

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"Regulation of the BH3-only protein NOXA in mantle cell lymphoma: relevance for novel treatment concepts"

10/03 - 10/09 **Degree in Biology at the University of Stuttgart**

Qualification: Diplom

Focus subjects: Immunology / Marine Biology

Diploma thesis at the Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology, Stuttgart

Prof. Dr. Walter E. Aulitzky, Robert Bosch Hospital, Stuttgart

"Charakterisierung Imatinib-resistenter, Bcr/Abl-überexprimierender Zellen in An- und Anwesenheit von Imatinib und deren Ansprechen auf Agenzien verschiedener Substanz-Bibliotheken"

09/07 - 02/08 **Study research project at University of New South Wales, Sydney, Australia**

Prof. Dr. Ian Dawes, School of Biotechnology and Biomolecular Sciences

"*Intron-mediated regulation of the spliceosomal LSM genes in Saccharomyces cerevisiae"*

09/93 - 06/02 **Abitur at Gymnasium Ochsenhausen, Ochsenhausen**

Stuttgart, January 14, 2014