Dissertation

submitted to the

Combined Faculties for the Natural Sciences and for Mathematics

of the Ruperto-Carola University of Heidelberg, Germany

for the degree of

Doctor of Natural Sciences

presented by

Diplom Biochemist Katharina Filarsky

born in Augsburg, Germany

Oral-examination: 15.07.2015

Identification and characterization of genes deregulated by DNA methylation in chronic lymphocytic leukemia

Referees: **PD Dr. Karsten Rippe**

Prof. Dr. Peter Lichter

To my family

Zusammenfassung

Die chronische lymphatische Leukämie (CLL) ist eine der am häufigsten auftretenden Leukämieformen bei Erwachsenen in der westlichen Welt. Die Anreicherung von scheinbar ausgereiften B-Zellen im peripheren Blut, den lymphatischen Organen und dem Knochenmark ist charakteristisch für diese Krankheit. Obwohl die Applikation von neu entwickelten Medikamenten in den letzten Jahren zu einer Verbesserung des Krankheitsverlaufs der Patienten geführt hat, ist die CLL nach wie vor unheilbar. Daher ist die Entwicklung neuer Behandlungsstrategien von großer Bedeutung.

Die CLL ist unter anderem eine Krankheit des Epigenoms, definiert durch genomweite Hypomethylierung und parallel auftretender Hypermethylierung in spezifischen Regionen. Hieraus resultiert die Deregulation von Tumorsuppressoren, nicht-kodierenden RNAs und Onkogenen. Die DNA Methylierung bleibt interessanterweise in der CLL über lange Zeit stabil. Gene die durch diese DNA Modifikation dereguliert werden könnten daher permanent beeinflusst sein, unabhängig vom Status und Verlauf der Krankheit.

Das Ziel dieses Projektes war es, Gene zu identifizieren und zu charakterisieren, die in der CLL durch DNA Methylierung dereguliert sind und somit eine Rolle im Pathomechanismus der Krankheit spielen könnten. Zu diesem Zweck wurden mit Hilfe von Arrays Expressions- und Methylierungnsanalysen von $CD19^{+}$ CLL Zellen und B-Zellen gesunder Spender durchgeführt. Es konnte eine unterschiedliche Expression zwischen den Krebszellen und den normalen B-Zellen von 1866 Genen gezeigt werden. Von diesen wiesen 33 signifikant differentiell methylierte Regionen (DMR) auf, welche eine negative Korrelation zur Genexpression zeigten. Durch Quantifizierung der DNA Methylierung mit Hilfe von Massenspektrometrie konnten 17 dieser Gene validiert werden. Unter diesen befand sich auch der Stammzell- und Transkriptionsfaktor *KLF4.*

Frühere Studien zeigen, dass *KLF4* eine wichtige Rolle in der Differenzierung und Reifung von B-Zellen spielt und in anderen B-Zell Krankheiten als Tumorsuppressor agiert. Des Weiteren ist *KLF4* ein Ziel-Gen von *NOTCH1*, welches in der CLL mutiert und konstitutiv aktiv ist. Tatsächlich, zeigte die Behandlung mit γ-sekretase Inhibitor von sechs verschiedenen lymphoblastoiden und leukämischen Zelllinien, sowie von mononukleären Zellen des peripheren Blutes (PBMCs) von 8 CLL Patienten, nicht nur eine Inhibition der NOTCH1 Aktivität in diesen Zellen, sondern auch eine Re-Expression von *KLF4* sowohl auf RNA als auch auf Protein Ebene. Es kann somit eine transkriptionelle Regulation von *KLF4* durch NOTCH1 angenommen werden. Überdies korrelierte die Expression von *KLF4* in primären CLL- und B-Zellen mit den Mitgliedern der BCL-2 Proteinfamilie *BAK*, *BAX* und *BCL-2,* sowie mit dem Zellzyklus Regulator *CCND1*.

Die transiente Überexpression von *KLF4* in drei Leukämie Zelllinien führte zu einer Deregulation von Genen welche an BCR- und PI3K-Signaltransduktion beteiligt sind. Zusammenfassend deuten diese Ergebnisse darauf hin, dass die Repression von *KLF4* durch DNA Methylierung und NOTCH Signaltransduktion dazu beiträgt die CLL Zellen in einem hoch aktivierten Zustand zu halten, welcher die Expansion und das Überleben der malignen Zellen unterstützt.

Summary

Chronic lymphocytic leukemia (CLL) is one of the most common leukemias in adults in the Western world characterized by the accumulation of mature-appearing B-cells in the peripheral blood, lymphoid tissues and bone marrow. The use of novel inhibitors targeting BCR- and PI3K-signaling in CLL as well as the application of CD20-antibodies in the clinic have led to improved outcome for the patients. Nevertheless, CLL remains an incurable disease and in order to develop new treatment strategies, there is still a strong need to elucidate the pathomechanism of CLL further.

CLL is amongst other aspects a disease of the epigenome, displaying genome-wide hypomethylation while specific regions are hypermethylated, resulting in the deregulation of tumor suppressors, non-coding RNAs and oncogenes. Interestingly, DNA methylation in CLL patients was shown to be relatively stable over time suggesting that genes which are deregulated by this DNA modification are affected permanently, independent of disease stage and course.

In this project, it was therefore aimed to identify and characterize genes deregulated in CLL by DNA methylation, which are likely involved in the pathomechanism of the disease. To this end, expression and methylation array profiling was conducted in $CD19^{+}$ sorted CLL cells and B-cells of healthy donors. Out of the 1,866 genes identified as differentially expressed between cancer cells and their non-malignant counterpart, 33 showed significantly differentially methylated regions (DMR) that displayed negative correlation with gene expression. Technical validation using mass spectrometry based quantification of methylation confirmed the presence of DMRs in 17 of these genes including the transcription and stem cell factor *KLF4*.

KLF4, of which repression and hypermethylation could be confirmed in a larger patient subset, was previously shown to play an important role in B-cell differentiation and maturation and further to act as tumor suppressor in other B-cell malignancies including classical- and non-Hodgkin lymphoma. Moreover, *KLF4* was shown to be a downstream target of NOTCH1 which is recurrently mutated and constitutively active in CLL.

In fact, treatment of six different lymphoblastoid and leukemia cell lines and of primary peripheral blood mononuclear cells (PBMCs) from eight CLL patients with γ-secretase inhibitor resulted in the inhibition of NOTCH1 activity and in the re-expression of *KLF4* on RNA and protein level, suggesting transcriptional regulation of *KLF4* by NOTCH1. Furthermore, expression of *KLF4* was correlated with levels of BCL-2 family members *BAK*, *BAX* and *BCL-2* as well as the cell cycle regulator *CCND1* in primary CLL and B-cells. Although, overexpression of *KLF4* in three leukemia cell lines did not induce apoptosis in these cells, it resulted in the deregulation of a number of genes involved in prominent signaling pathways which are highly relevant for the pathogenesis of CLL, including BCR- and PI3K-signaling.

In summary, these findings suggest the repression of *KLF4* by DNA promoter methylation and NOTCH signaling in CLL. Overexpression of *KLF4* results in the deregulation of genes involved in signaling pathways important for B-cells which indicates that *KLF4* is in part involved in keeping the CLL cells in a highly activated state.

Abbreviations

2-HG	2-hydroxyglutarate
5-CaC	5-carbosylcytosine
5-fC	5-formylcytosine
5-hmC	5-hydroxymethylcytosine
5-mC	5-methylcytosine
ADRB2	beta-2 adrenoreceptor
ALL	acute lymphoblastic leukemia
AML	acute myeolid leukemia
Ann-V	Annexin-V
APOBEC	apolipoprotein B mRNA editing enzyme
ASM	allele-specific methylation
ATM	ataxia telangiectasia mutated gene
ATRX	ATP-dependent helicase ATRX
BAK	BCL-2 antagonist / killer 1
BAX	BCL-2 associated X protein
BC	buffy coat
BCL-2	B-cell lymphoma 2
BCR	B-cell receptor
BL	Burkitt lymphoma
BMSC	bone marrow stroma cells
CCND1	cyclin D1
CGI	CpG island
CGU	CpG unit
ChIP	chromatin immunoprecipitation
cHL	classical Hodgkin lymphoma
CIMP	CpG island methylator phenotype
CLL	chronic lymphocytic leukemia
CML	chronic myelogenous leukemia
CMML	chronic myelomonocytic leukemia
DAXX	death-associated protein 6
DLBCL	diffuse large B-cell lymphoma
DMR	differentially methylated region
DMSO	dimethyl sulfoxide
DNMT	DNA methyltransferase
DSB	double strand break
EBF1	early B-cell factor 1
EMT	epithelial-mesenchymal transition
ER	endoplasmatic reticulum
ER-α	estrogen receptor alpha
ESC	embryonic stem cell
EZH2	enhancer of zeste homolog 2
FL	follicular lymphoma
GBM	glioblastoma multiforme
GSI	y-secretase inhibitor
H	healthy donor
HNSCC	head and neck squamous cell carcinoma
HRP	horse radish peroxidase
ICGC	International cancer genome consortium
IFN-α	interferon alpha
IGFPB4	insulin-like growth factor-binding protein 4
IGHV	heavy chain variable region
iNOS	nitric oxide synthase

iPS	induced pluripotent stem cells
ITAM	immunoreceptor tyrosin-based activation motif
ITGB2	integrin beta 2
JHDM	Jumonji-C domain histone demethylases
KLF4	Krüppel-like factor 4
KMT	histone lysine methyl transferases
LEF-1	Lymphoid enhancer-binding factor 1
LILRA4	leukocyte immunoglobulin-like receptor subfamily A member 4
LINE-1	long interspersed element 1
MA	MassARRAY amplicon
MCIp	methyl-CpG-immunoprecipitation
MCL	mantel cell lymphoma
MCL	mature B-cell like
MCL1	myeloid cell leukemia 1
MDS	myelodysplastic syndromes
miR	micro RNA
MLL	mixed lineage leukemia
MM	multiple myeloma
MPN	myeloproliferative neoplasm
NBC	naive B-cell like
NLC	nurse like cell
NGS	next generation sequencing
NHEJ	non-homologous end joining
NHL	non-Hodgkin lymphoma
NICD	NOTCH1 intracellular domain
NuRD	nucleosome remodeling deacetylase
OS	overall survival
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
pDC	plasmacytoid dendritic cell
pdCR	promoter downstream correlating regions
PFS	progression free survival
PI3K	phosphoinositide-3-kinase
PRC2	polycomb repressive complex 2
RIPA	Radio-Immunoprecipitation Assay buffer
sAML	secondary AML
Sat-α	satellite alpha
STAT1/6	signal transducers and activators of transcription 1/6
TAM	tumor associated macrophages
TBE	Tris-borate-EDTA
TBS	Tris-buffered saline
TCGA	The Cancer Genome Atlas Research Network
TERT	telomerase reverse trancriptase
TET	ten-eleven translocation
TF	transcription factor
TFN-α	tumor necrosis factor alpha
TLR7	toll-like receptor 7
TSS	transcription start site
TP53	tumor protein 53
UTX	ubiquitously transcribed tetratricopeptide repeat, X chromosome
VHL	von Hippel-Lindau
α-KG	alpha-ketoglutarate

Definitions

Gene symbols written as capital letters indicate human protein (e.g. KLF4).
Gene symbols written in italic as capital letters indicate human gene (e.g. *KLF4*).
Gene symbols written in lower case starting with a capital letter indicate mouse protein (e.g. Klf4).
Gene symbols written in italic in lower case starting with a capital letter indicate mouse gene (e.g. *Klf4*).

I. Understanding the development and progression of cancer

1. Numbers of cancer incidence reveal the need for new treatment options

According to Cancer Research UK[1] in the year 2012 about 14.1 million new cases of cancer occurred worldwide. Among those the four most common entities, which account for 42% of all cases, are lung, female breast, bowel and prostate carcinoma (Figure 1).[2,3] In Germany alone, in 2012 about 221,000 deaths were cancer related and in addition, about 0.5 million new cases registered.[4,5] This number was shown to increase over the last decade, which is partly caused by the demographic change as the cancer incidence for over 80-year old is about 200 to 300 fold higher than for kids with age under 15 years.[6] Although, since the 1970s, the overall survival rate of all cancers has increased from about 25% to 50% due to faster diagnosis and advanced treatment options, it is estimated that about four in ten cases of cancer could still be prevented. Environmental factors and lifestyle including smoking, alcohol, UV light and certain infections, have a profound influence on cancer development. For example, tobacco smoking is currently responsible for more than one in five cancer cases among men and nearly one in twelve among women in Germany.[7] Furthermore, primary prevention measures for early cancer diagnosis like screenings of skin, breast and prostate, could further reduce cancer mortality. This is shown by an increased incidence of skin cancer in 2008 after the introduction of preventive screening in Germany.[8]

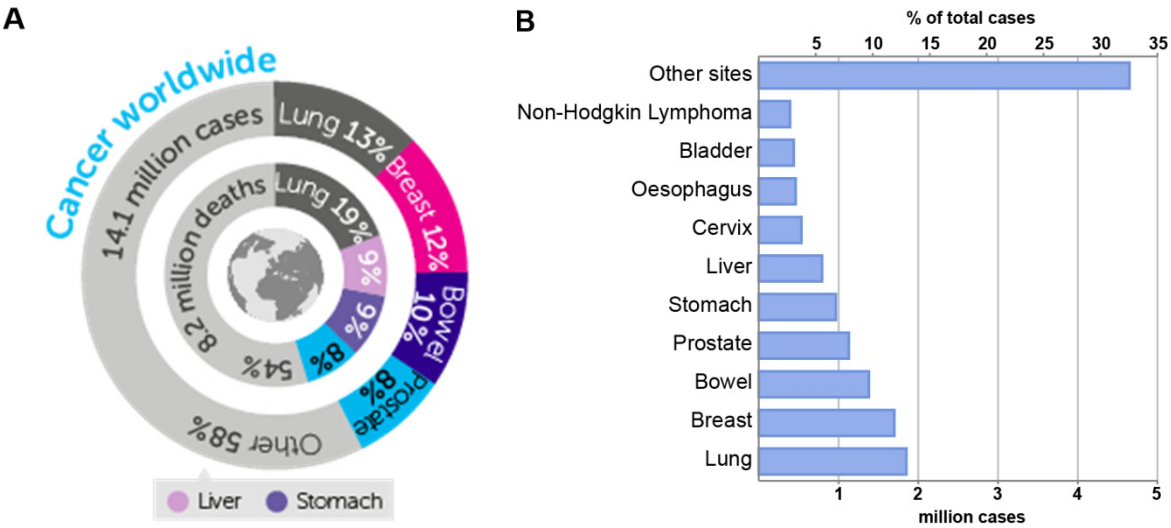

Figure 1. Cancer statistics in the year 2012 worldwide. *(A) Incidence and mortality of cancer worldwide in the year 2012. (B) The 10 most commonly diagnosed cancer 2012 estimates. Total number and percentage of new cases diagnosed per year, worldwide. Modified from www.cancerresearchuk.org*[I]

But still, some types of cancer like lung and pancreas cancer show very little improvement and low survival rates causing about 90% of patients to die within 10 years after diagnosis. This led to about 8.2 million cancer related deaths in the year 2012 worldwide (Figure 1). In addition to the prevention of cancer, there is still a strong need for new and improved diagnostic tools and treatment options which could avert cancer related deaths via early onset and more efficient disease management. To gather these tools it is

[I] Statistics are from the International Agency for Research on Cancer GLOBOCAN database and also the World Health Organization Global Health Observatory and the United Nations World Population Prospects report.

necessary to further expand our understanding of the molecular characteristics underlying the development and progression of cancer.

2. Defining the molecular characteristics of cancer

On cellular level, cancer is a disease of the body's own cells. Triggered by some environmental, microenvironmental or intrinsic stimuli, cells of various types can start growing and cause previous healthy tissue to develop tumors. This oncogenesis is a multistep process which can be seen as a sequence of clonal expansions. These are believed to be based on genomic instability and the inflammatory state of the cells and their surrounding tissue as well as their microenvironment. In line with this process, the neoplastic cells need to acquire functional skills which allow them to survive, proliferate and disseminate.

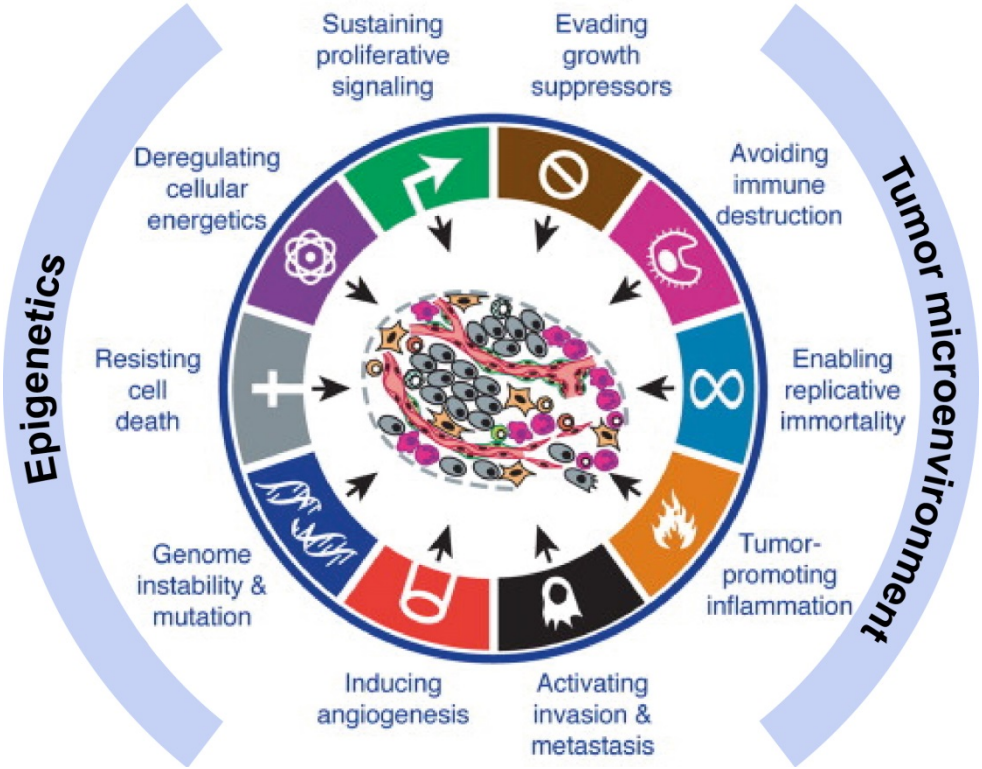

Figure 2. The 8+2 hallmarks of cancer. *Interplay of all hallmarks represents the molecular basis for cancer development and progression. Modified from Hanahan & Weinberg et al.*[9]

In the year 2000 Hanahan and Weinberg first defined such capabilities which they called the 6 hallmarks of cancer. They later amended these hallmarks, resulting in 8 biological aptitudes cells acquire during their multistep development of human tumors. These include (1) sustaining proliferative signaling, (2) evading growth suppressors, (3) resisting cell death, (4) enabling replicative immortality, (5) inducing angiogenesis, (6) activating invasion and metastasis, (7) reprogramming energy metabolism and finally (8) evading immune destruction (Figure 2). Further, the two "enabling characteristics", genomic instability and inflammation, were defined which seem to act as the basis for cancer development.[9,10] However, most hallmarks are tightly connected and moreover influenced by the tumor microenvironment and epigenetic changes within the cancer cells.

2.1. Disruption of genomic stability is supposed to trigger tumor development

In cancer, the integrity of the genomic stability can be disturbed by mutations, which can affect the activity or expression of oncogenes as well as tumor suppressor genes. In addition, chromosomal rearrangements can lead to the formation of fusion genes. Due to the high impact of these genetic aberrations on the development of cancer, networks like the ICGC Cancer Genome Project (www.ICGC.org) and The Cancer Genome Atlas Research Network (TCGA; www.cancergenome.nih.gov) are using large scale next generation sequencing (NGS) to obtain a comprehensive description of genomic, transcriptomic and epigenomic changes in different tumor types.[11,12] Hence, new recurrent mutations have been identified in close to all cancer entities, as well as new chromosomal rearrangements.

One of the most prominent fusion genes that was already discovered in 1960 is the *BCR-ABL* fusion gene which results from a translocation between chromosomes 9 and 22 giving rise to the so called Philadelphia chromosome.[13] It is associated with chronic myelogenous leukemia (CML) with 95% incidence in these patients and also to a lesser extend (25-30%) with acute lymphoblastic leukemia (ALL) and other leukemias.[14] The protein ABL is a tyrosine kinase which in its BCR-ABL fusion form is constitutively active and influences transformation of the cells by activating downstream signaling like the NFκB and PI3K/AKT pathways.[15–17] In addition, *BCR-ABL* was recently shown to cause increased acetylation of p53 which protects the cells from mitochondria-dependent apoptosis in response to DNA damage.[18]

TP53 (tumor protein 53) which was also referred to as the “guardian of the genome” is one of the most recurrently mutated and deregulated tumor suppressors in the course of cancer. The gene gives rise to the protein p53, which can act as tetrameric transcription factor that functions to induce cell cycle arrest or apoptosis after the detection of chromosomal and cell cycle abnormalities as well as hypoxia. Further, it can directly interact with members of the BCL-2 family of apoptotic and anti-apoptotic proteins like PUMA and NOXA and translocate to the mitochondria to induce cell death.[19,20] Loss of p53 function is a strong inducer of cancer and as mentioned before, it was shown to be mutated in various types including mammary carcinoma, medulloblastoma and chronic lymphocytic leukemia (CLL).[21–23] The effect of the mutations is diverse, resulting in most cases in a loss of the wild type function of the protein and in some cases a gain-of-function that helps to contribute to malignant progression and earlier cancer onset.[24–28]

Interestingly, in medulloblastoma and other tumors, *TP53* mutations were recently linked to another phenomenon observed in cancer cells, the shattering of whole chromosomes resulting in catastrophic genome rearrangements.[29] This event, called chromothripsis[II], provokes a high number of gene fusions, disruption of tumor suppressors and amplification of oncogenes.[30] Originally, it was first detected in a case of CLL but since then it has been associated with many other cancer entities and shows an incidence of about 2-3% depending on the type of cancer. It was associated with poor outcome of the patients in multiple myeloma (MM), AML and melanoma.[29,31,32] The mechanisms underlying this catastrophic event are still unclear, but two possible scenarios were suggested: (I) DNA damage caused by ionizing radiation and (II) critical telomere

[II] Chromothripsis composed from the Greek words *chromos* which represents chromosomes and *thripsis* which means “shattering into pieces”

shortening.[30] In the first case, radiation induced double strand breaks (DSBs) are repaired by low-fidelity and error prone repair mechanisms like non-homologous end joining (NHEJ) which could explain the massive rearrangements. Of note, NHEJ was shown to play a greater role in the repair of DSBs when p53 activity is reduced.[33] Another hypothesis is that chromothripsis is the result of critical telomere shortening followed by chromosome end-to-end fusion and subsequent breakage.[34] This is further supported by a higher incidence of chromothripsis in AML patients with advanced age at diagnosis.[29] Unprotected telomeres in p53-deficient cells were found to be shorter than average and prone to form end-to-end fusions which could again point towards a possible connection between chromothripsis and p53 activity. However, so far it is still unclear whether *TP53* mutations predispose cells for chromothripsis or rather enable the cells to survive after the catastrophic chromosome shattering. It also remains to be elucidated if cases of chromothripsis, which are not connected to precedent *TP53* mutations, show other deregulation of the p53 pathway.

Another striking phenomenon which was discovered in recent years is the so-called kaetegis[III]. This term describes areas of localized hypermutations, which were first found in breast cancer cells. Often these mutation clusters co-localize with clusters of genomic rearrangements and in addition, the same type of mutations occur over several megabases.[35,36] By now the APOBEC-family (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like) of cytosine deaminases are thought to be associated with kaetegis. They are a known source of DNA damage, and APOBEC binding motifs seem to be enriched in the affected areas.[37]

The exact mechanisms underlying the events of chromothripsis and kaetegis remain to be clarified, but confirm the assumption that genomic instability is one of the key factors determining the development of cancer and giving rise to the hallmarks.

2.2. Activating the proliferative capacity of cancer cells

The sustainment of (1) proliferative signaling and (2) evading growth suppressors are two hallmarks which directly interact. In contrast to normal cells, cancer cells acquire the ability of chronic proliferation by deregulating growth factor mediated, kinase-dependent and -independent intracellular signaling cascades. These signaling pathways, which normally serve to maintain normal tissue architecture and function, are manipulated by the cancer cells. For this purpose the cells can either enhance autocrine proliferative stimulation or trigger other cells to provide the necessary stimuli. This was shown for fibroblasts in mammary carcinoma and tumor associated macrophages (TAMs) in several types of tumors.[38–40] In other cases, the amount of receptors on the cell surface can be increased or constitutive activation of signaling pathways can circumvent the necessity of external stimulation of the cells altogether.[41] One striking example for this is the PI3K/AKT pathway which is hyperactivated in about 30% of invasive breast cancer. Causative for this activation are gain-of-function mutations in members of this pathway including mutations in the catalytic subunit of *PI3KCA*, *AKT1* and amplification of *AKT2*. This is accompanied by a disruption of a negative feedback loop through loss-of-function mutations of the *PTEN* phosphatase, which otherwise counteracts PI3K activity.[42–45]

In addition to increased proliferation, the cancer cells are able to evade growth suppressors amongst other effects by avoiding contact inhibition. For example, the tumor

[III] Kateagis from the greek word for thunder *kataigis*

suppressor MERLIN, which is encoded by the gene *NF2* (neurofibromatosis factor 2), mediates contact-dependent inhibition of proliferation by stabilizing adherent junctions and negative regulation of epidermal growth factor receptor (*EGFR*). Loss or mutations of *NF2* was shown to result in cell proliferation and tumorigenesis.[46–48]

2.3. Replicative immortality enables nonfinite tumor growth

Normal cells go through a limited number of successive cell cycles before they either reach a viable but non-proliferative state, cell senescence or go into cell death. However, in cancer some cells escape the underlying mechanism which enables them to reach (4) replicative immortality. It is believed that telomeres which protect the ends of chromosomes sustain the capability of cells for unlimited proliferation. In fact, the enzyme telomerase which is necessary for maintaining functional telomeres, is almost absent in most non-immortalized cells whereas expressed in most immortalized cells. This would suggest that in cancer cells, either the up-regulation of telomerase or recombination based telomere maintenance would be able to preserve the cells in a persistent replicative state.[49–51] However, as mentioned before it was shown that shortened telomere length increases the risk to develop cancer, probably due to decreased chromosome integrity and stability. It even seems that transient telomere deficiency during the early stages of cancer development facilitates malignant progression.[52–55] It is known that telomerase, especially its subunit TERT (telomerase reverse transcriptase), has additional functions which include the interaction with the NFκB pathway, amplifying WNT signaling, RNA polymerase function, impact on DNA damage repair and cell proliferation as well as mediating resistance to apoptosis.[56–61]

2.4. Evading cell death and senescence

Another requirement for tumor formation is for the cells to circumvent the machineries triggering apoptosis, autophagy and necrosis and hence (3) to resist cell death. In the case of apoptosis upstream and downstream effector components are necessary which can prompt an extrinsic and intrinsic apoptotic program. By now it is known that the apoptotic state of a cell is in part determined by a balance of members of the B-cell lymphoma 2 (BCL-2) protein family, which are in a tight interplay with other proteins like p53, as mentioned before. One or more BCL-2 homology domains (BH) that regulate interactions among the family members characterize these proteins. The anti-apoptotic family members (e.g. BCL-2, BCL-XL, MCL-1) and pro-apoptotic proteins (e.g. BIM, BID, NOXA and PUMA) can directly interact and hence sequester or inhibit each other's effects.[62,63] BCL-2 itself for example, acts via binding the BH3-only proteins BAX and BAK and thus prevents the formation of a homodimeric complex in the mitochondria membrane.[64] This complex otherwise destroys the integrity of the mitochondria and consequently results in the release of Cytochrome c which again activates caspases with proteolytic activity causing the cells to go into apoptosis.[65] Some of the anti-apoptotic proteins like BIM antagonize this mechanism by binding to BAX/BAK, blocking its interaction with BCL-2 and directly activating the pro-apoptotic complex.[66] Another member, Beclin-1, was shown to trigger autophagy as long as it is not bound by BCL-2 or BCL-XL which again can be inhibited by the influence of the other anti-apoptotic family members. In fact, some cancers like T-ALL are dependent on anti-apoptotic proteins like BCL-2 and show a down-regulation of others like Beclin-1.[67–71] While apoptosis and

autophagy are counteracted and circumvented by cancer cells, necrotic cells can recruit inflammatory cells which display tumor promoting effects.[72,73]

2.5. Tumor-associated neovasculature ensures tumor maintenance

As any other type of tissue, tumors are dependent on tumor-associated neovasculature which facilitates the delivery of necessary nutrients and oxygen as well as the disposal of metabolic waste. In general, stimulatory and inhibitory cell-surface receptors on vascular endothelial cells like VEGF-A can trigger ligand induced vessel growth and were shown to be up-regulated under hypoxic conditions and through oncogene signaling.[74,75] On the other side, endogenous angiogenesis inhibitors like TSP-1, Angiostatin, Endostatin and various interleukins counteract this mechanism and their increase was shown to impair tumor growth in different *in vitro* and *in vivo* models.[76–80] Anyway, most important for the process of tumor angiogenesis induction, the angiogenic switch, is the tumor microenvironment with pericytes, the extracellular matrix, endothelial precursor cells and cells which are recruited via inflammatory processes.[81] The latter include different types of bone marrow derived cells being part of the inert immune system like macrophages and neutrophils which can promote angiogenesis *in vivo* and stimulate tumor growth.[82–84] Pericytes act as bystander cells for endothelial cells, attached to the outer surface of normal tissue vasculature and were shown just to be as important for tumor-associated neovasculature.[85] In addition, to inflammatory processes, hypoxic conditions in the tumor cells and its environment can induce factors like *HIF1-α* which in turn drives the release of pro-angiogenic factors like VEGF further supporting the angiogenic switch.[75,81,86] Due to its high importance for maintaining a functional tumor structure, tumor vasculature is a promising target in cancer therapy and in line with this a number of inhibitors like Sorafenib and Sunitinib acting on angiogenic stimulators like VEGF are currently under investigation as cancer therapeutics.[87,88]

2.6. Targeting and preventing tumor metastasis is necessary to fight cancer

One big challenge in the treatment of many types of cancer is not only to kill the tumor itself but also to prevent its progression into other organs and tissues where it can build metastasis. The formation of these secondary metastatic tumors happens in a multistep process, the invasion-metastasis cascade, which is similar to the so called epithelial-mesenchymal transition (EMT). In healthy organisms, EMT is necessary for wound healing and developmental processes in adults and during embryogenesis.[89,90] However, in the case of cancer, tumor cells migrate into the stroma of the tumor surrounding tissue and intravasate into the vascular system in which they circulate to new sites of the body. Upon extravasation, the cancer cells form micrometastatic lesions which adapt to the new microenvironment and evolve into metastatic tumors. It is assumed, that throughout the initial stage of EMT in healthy tissues as well as tumors, the cells are to some degree pushed towards a stem cell-like state. This process is regulated by a tight network of epigenetic modifications, differential splicing, small non-coding RNAs and over all on the level of transcription.[91–93]

Three families of EMT-transcription factors (EMT-TFs), namely *SNAIL*, *ZEB* and *TWIST,* play a central role in this network. One of their main functions is the repression of *CDH1* which codes for the protein E-cadherin which again is essential for EMT progression as it helps to maintain epithelial cell sheets. In line with that, EMT-TFs were shown to interact with epigenetic modifiers and are involved in a genome-wide epigenetic reprogramming

taking place during the initial stage of EMT.[94–97] TWIST for example can interact with the NuRD (nucleosome remodeling deacetylase) chromatin remodeling complex and polycomb proteins, hence recruiting them to the promoter of *CDH1* to induce its transcriptional repression.[98,99] SNAI1, a member of the SNAIL family, can in addition recruit HDAC1 and -2 for the same purpose and moreover the expression of *CDH1* is also regulated via DNA methylation in cancer.[100,101] It is also known that EMT-TFs interact with two families of tumor suppressor micro RNAs (miR), the *miR-200* and the *miR-34* family.[102–105] Members of both classes of miRs can be down-regulated, amongst other aspects by promoter hypermethylation, in cancer and are directly interacting with the EMT-TFs.[92,93,106] For example, SNAI1-dependent EMT is activated in colon, breast and lung carcinoma cells but can be blocked by *miR-34* which binds to the 3'UTR of *SNAI1* itself as well as key *SNAI1* regulatory molecules including ß-catenin. The *miR-200* family is a marker for high expression of *CDH1* in cancer cells *and was* further proven to regulate the E-cadherin repressors *ZEB1* and *ZEB2*. In addition, up-regulation of miR-200 members is sufficient to prevent TGF-ß induced EMT, underlining its strong involvement in the course of this process.[102,103]

Of great interest is also the role of alternative splicing during the transmission of the cancer cells. The epithelial splicing regulator genes *ESRP1* and *ESRP2*, which can be regulated by EMT-TFs, are able to induce ESRP-enhanced or -silenced exon splicing changes of over 200 genes.[107] *FGFR2*, *RON* and *CTNND1* (p120 catenin) are among the most prominent examples.[108,109] *CTNND1* has a short epithelium-specific isoform which promotes cell-cell adhesion by stabilizing E-cadherin at the plasma membrane. Its long isoform is expressed during EMT and supports invasiveness of the cells.[110]

Besides these intracellular mechanisms, necessary for the metastatic capacity of the cancer cells, migration and invasion are also dependent on the support of the tumor microenvironment that has to be permissive and also supports the tumor in other aspects as mentioned before.

2.7. Tumor promoting inflammatory processes and the tumor microenvironment

Tumors are infiltrated by cells of the immune system and it was thought for a long time that the immune system tries to eradicate the tumor cells. Although this might be true to some degree, it is now assumed that the tumor-associated inflammatory processes rather enhance tumorigenesis and progression.[111] Growth factors and survival stimuli secreted by cells of the immune system can stimulate proliferation of cancer cells and support invasion and angiogenesis as already mentioned above. Many of the major inflammatory pathways in cancer converge at level of transcription factors like *STAT3* and *NFκB*. Proliferative and survival stimuli activate growth factor receptor tyrosine kinases on the surface of the cancer cells. A few major downstream cascades including the STAT3 pathway and other members of the STAT family are activated and could be linked to inflammatory processes in cancer.[112] *STAT3* for example was shown to be activated by IL-6 which can be secreted by myeloid cells and leads to an up-regulation of cell cycle regulators, the oncogene *MYC* and anti-apoptotic genes of the *BCL-2*-family.[113] In addition, NFκB signaling functions as tumor promoter in inflammation associated cancer for example by enhancing WNT-ß-catenin signaling which was shown to induce dedifferentiation of intestinal non-stem cells that acquired tumor-initiating capacity.[114] NFκB-dependent inflammatory response on the other hand can mediate increased *IL-6*

expression which leads to the amplification of the NFκB-IL6-STAT3 cascade in cancer cells. However, this is just one example for the complex mechanisms which include inflammatory processes and the cells of the immune system in the promotion of tumor development.

Not only inflammatory processes but also other aspects and cells of the tumor microenvironment are currently under close investigation as they seem to be important not only for tumor development but also for disease maintenance. This will be discussed in greater detail, using the example of CLL, below.

3. Cancer epigenetics

One further characteristic of tumor cells which could be included in the hallmarks of cancer are changes in the cancer epigenome. We know now that not only mutations and chromosomal aberrations but also changes in DNA methylation, histone modifications and the chromatin structure can result in the deregulation of oncogenes, tumor suppressor genes as well as non-coding RNAs. Some examples were already mentioned before but as the epigenome, especially DNA methylation in cancer, is central for the project described here, it will be discussed in greater detail.

3.1. Cancer is characterized by global hypomethylation, DMRs and CIMPs

In the last two decades, the establishment of high-throughput techniques for the genome wide analysis of DNA methylation has led to a greater understanding of the distribution of this DNA modification and its role in the regulation of gene expression. Until recently, DNA methylation was mainly thought to impact on gene expression when present in the promoter regions, where high methylation is associated with repression of the associated genes.[115] A striking example is the silencing of the inactive X chromosome in women which is maintained by highly abundant DNA methylation in the promoter regions of almost all genes localized on this chromosome.[116] However, not only methylation in promoters but also in gene bodies impacts on transcriptional activity, however displaying positive correlation with gene expression and marking enhancer regions.[117] Besides its role in controlling gene expression, tissue specific intragenic DNA methylation was shown to result in alternative splicing. Moreover, a genome wide methylation study in prostate cancer indicates that differential methylation could define alternative transcription start sites (TSS) as well.[118,119] Furthermore, DNA methylation was shown to be important for centromere integrity and telomere length. Changes in the DNA modification on repetitive elements are associated with differentiation and embryonic development.[120–122] Nevertheless, one has to keep in mind that DNA methylation is dynamic and varies between different types of tissues and differentiation stages. Considering its important role in developmental and cell maintaining processes, it is not surprising that changes in DNA methylation patterns were by now associated with many types of diseases including cancer.

For the first time, in 1983 Andrew Feinberg and his group showed a pattern of global hypomethylation in small cell lung cancer and colon adenocarcinoma in comparison to healthy tissue counterpart.[123] Today we know, that global DNA hypomethylation occurs in most types of cancer including hematological disorders like CLL, breast and colorectal cancer but also that most cancers show a divergent epigenetic landscape. The latter is defined by cancer specific differentially methylated regions (DMRs) which especially also

include CpG islands (CGIs).[124–128] About 29,000 of these regions with high CG content (>50%) were found in the human genome and are known to be usually unmethylated throughout all stages of development and in all types of tissue.[129,130] In cancer however, about 5% to 10% of CGIs show strong methylation resulting in a so called CGI methylator phenotype (CIMP). CIMPs, amongst others, were identified in colorectal cancer and glioma where the high frequency of hypermethylation supports the idea of a coordinated event rather than a stochastic phenomenon.[131,132] In line with that, the CIMP of glioma is associated with *IDH1* mutations, which are known to impact on DNA methylation.[133]

Besides changes in direct CGI methylation, regions adjacent to CGIs, so called CGI shores and shelves are currently under investigation as they show tissue specific DNA methylation and were found to be hyper- and hypomethylated in cancer.[134,135] CGI shores and shelves are also part of recently discovered promoter downstream correlating regions (pdCRs). They often extend tens of kilobases or more from TSS and were found in about 8% of expressed genes in medulloblastoma. Interestingly, methylation and expression levels of genes harboring a pdCR could distinguish between the WNT, SHH, group3 and group4 subgroups of medulloblastoma. Besides that, these huge DMRs revealed to be a universal feature present in various types of tissue.[136]

Whether the high degree of aberrant DNA methylation is cause or consequence of cancer development remains to be elucidated, but it is clear by now that the effects are tremendous. On the one hand, hypomethylation was shown to strongly correlate with genomic instability. On the other hand, hypermethylation permits or prevents the binding of transcription factors and methyl binding proteins like MBD1, 2 and 3 as well as MeCP2, which can recruit histone modifiers and other chromatin remodelers resulting in the down-regulation of tumor suppressor genes and miRNAs. Some examples are *TP53* in ALL, *BRCA1* in breast cancer and the DNA repair enzyme *MGMT* in glioma as well as the *miR-200* and *miR-34* families which were already described above as interaction partners and regulators of EMT-TFs and which are hypermethylated in breast and prostate cancer, respectively.[92,93,106,137–141]

3.2. New emerging DNA modifications in mammals: 5-hmC, 5-fC and 5-CaC

For a long time 5-methylcytosine (5-mC) was supposed to be the only DNA modification in mammals. Recently, 5-hydroxymethylcytosine (5-hmC) was discovered as a so called 6th base in mouse embryonic stem cells (ESC) and Purkinje Neurons. By now it is known that 5-hmC is generated through oxidation of 5-mC by the ten-eleven translocation protein family (TET) members TET1 to 3.[142–144] Furthermore, these enzymes catalyze the conversion of 5-hmC to 5-formylcytosine (5-fC) and subsequently to 5-carboxylcytosine (5-CaC) which can then be excised from the DNA by thymine DNA glycosylase in the process of linked deamination base excision repair (Figure 3).[145] Hence, these three DNA modifications were identified as intermediates in an active demethylation pathway.

However, recently several groups investigated the genome wide distribution of 5-hmC using either high throughput sequencing of 5-hmC containing DNA or analysis of the latter on micro arrays.[147–150] These groups all found a similar pattern of 5-hmC distribution in mouse ESCs, ES cell lines, mouse cerebellum and human brain frontal lobe tissue. Most of the 5-hmC enriched regions were within gene bodies, CGIs and gene promoters while the modification was depleted in repetitive and intergenic regions. Moreover, 5-hmC was present in CTCF and pluripotency transcription factor binding sites.[147,149] The majority of

the 5-hmC enriched regions were associated with moderate CpG density. It was also observed that intragenic and proximal enrichment of 5-hmC is associated with more highly expressed genes.[147,148,150] In line with this observation, initial evidence was provided that 5-hmC contributes to both transcriptional activation and repression in a context-dependent manner.[148] These findings indicate that at least 5-hmC and potentially also 5-fC and 5-CaC have specific functions and are not just demethylation intermediates. First screens have identified a number of proteins potentially binding to all three modifications, but this has to be evaluated in detail and the functional role of such interactions are still to be determined.[151–153]

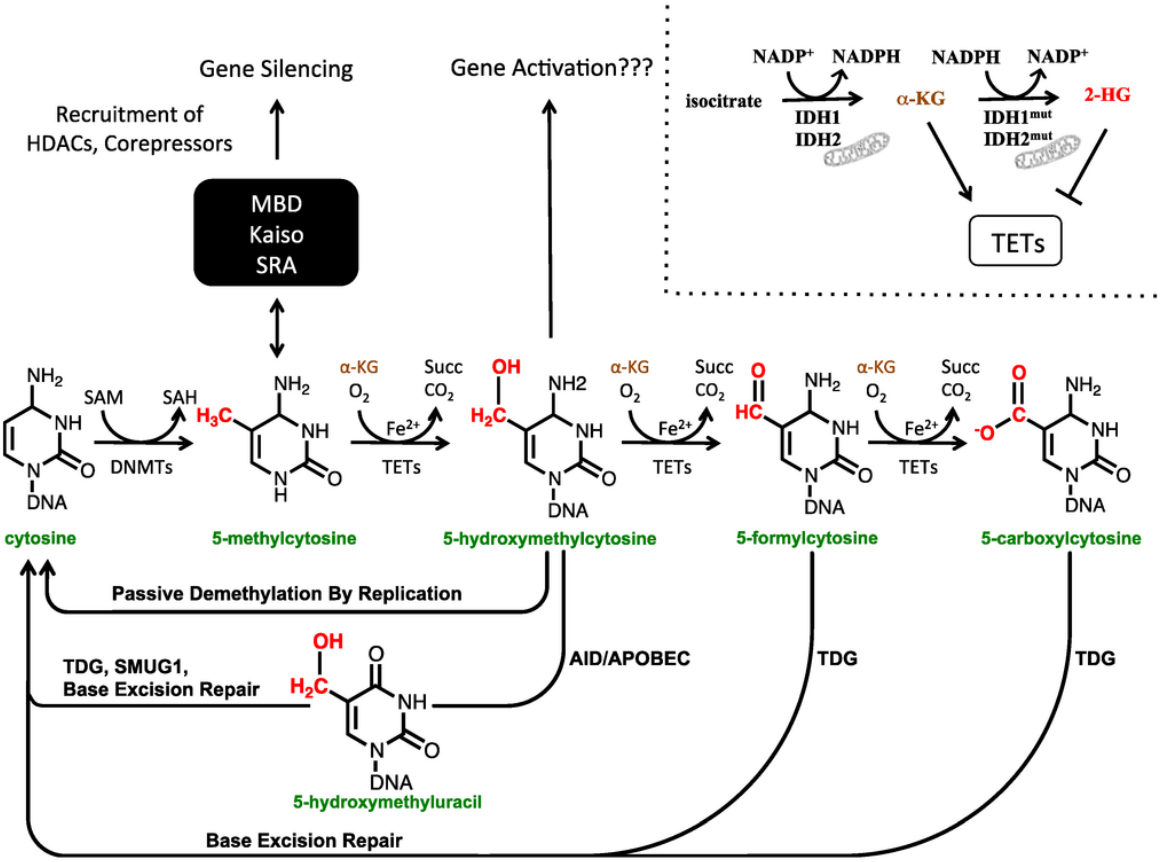

Figure 3. The conversion pathway of emerging DNA modifications. *TET proteins can convert 5-mC to 5-hmC, 5-fC and 5-CaC. Passive demethylation via replication of active demethylation via base excision repair is possible. TET proteins are dependent on α-KG for enzyme activity which is provided by IDH1 and 2. Modified from Mariani et al.*[146]

Besides its specific genome wide distribution, the level of 5-hmC in healthy tissue seems to be an indication for the differentiation state of the cells, with higher 5-hmC levels correlating with differentiation.[154] In cancer cells, the overall levels of 5-hmC are reduced, similar to the global hypomethylation seen for DNA methylation. For example, melanoma cells show significantly low levels of hydroxymethylation genome wide, while increased levels of DNA methylation could be detected in gene bodies and promoter regions, presumably due to lack of oxidation.[155] A similar loss of global 5-hmC could be detected in pancreatic cancer, accompanied by enrichment of this modification in exons and transcription factor binding sites.[156] It is known that loss of DNA methylation can be either result of active demethylation via 5-hmC, deamination or passive loss of methylation during replication. For loss of 5-hmC in cancer cells two reasons are currently under

investigation: inhibition of the TET proteins via *IDH1/2* mutations or inactivating mutations of the *TET* members themselves.

Especially in hematological diseases mutations of *TET1* and *2* as well as *IDH1* where shown to play an important role. In AML, MPN, sAML after MPN, MDS, systematic mastocytosis and CMML[IV] *TET2* shows an overall mutation rate of 19.5% which indicates a biological relevance and a potential impact on the course of these myeloid diseases. A wide range of 237 different somatic mutations were detected for *TET2* ranging from missense over nonsense mutations to frameshifts.[157–166] Although, it was possible to trace *TET2* mutations back to $CD34^+$ progenitor cells and T-cells in some cases, the question if the mutations are in general an early event in cancer development is still open.[167,168] This assumption is however supported by the finding that *Tet2*-deficient mice develop hematopoietic malignancies similar to CMML. Thus it appears that even the lineage determination in the hematopoietic system is controlled in part by deposition of 5-mC and 5-hmC which controls lineage specific gene silencing.[169,170] *TET1* was found to be a fusion partner of the *MLL* (mixed lineage leukemia) protein in cases of acute lymphoblastic leukemia. Translocations, which create fusion genes with *MLL*, are associated with truncation of the protein and often predict a poor prognosis in ALL. However, no somatic mutations of *TET1* have been found in MPN, chronic myelomonocytic leukemia or AML so far.[157]

Remarkably, mutations of *IDH1* and *2*, which were found in several types of cancer, result in a change of the catalytic activity of the enzymes and henceforth, the production of 2-hydroxyglutarate (2-HG) from isocitrate instead of α-ketoglutarate (α-KG) (Figure 3). The TET proteins which normally need α-KG to catalyze the oxidation of 5-mC to 5-hmC and its other derivatives are inhibited by 2-HG, which hence results in a hypo-hydroxymethylation phenotype.[171–175]

3.3. Altered chromatin structure is associated with DNA methylation and genomic mutations in cancer

Besides the effect of DNA modifications, chromatin conformation and activity are influenced by the position and composition of nucleosomes. So-called chromatin remodelers are responsible for altering nucleosome structures by the introduction of histone modifications and variants. These remodelers therewith guide the accessibility of promoter regions, enhancers and other regulatory domains and mediate the binding of transcriptional regulators. More and more evidence suggest that genomic mutations of involved proteins as well as fluctuations in their activity effect changes in chromatin composition and hence, activity in various types of cancer.

The most abundant nucleosomes are composed of an octamer of histones containing two of each histone H2A, H2B, H3 and H4 which are wrapped by about 146 bp of DNA. However, in order to change nucleosome compositions chaperons like e.g. CHZ1, FACT and ASF1, can bind to the nucleosomes and replace the classical canonical histones by variants.[176–178] By now, whole families of histone variants could be identified including the *H2A* family which includes the classical H2A, but also other members like H2A.X, H2A.Z, macroH2A and H2A.B as well as isoforms and splice variants thereof. Of this family the most prominent member, besides H2A itself, is H2A.Z of which the incorporation is

[IV] AML (acute myeloid leukaemia), sAML (secondary AML), MPN (myeloproliferative neoplasms), CMML (chronic myelomonocytic leukaemia), MDS (myelodysplastic syndromes)

replication independent and which can have pleiotropic effects on genome stability, transcriptional regulation, epigenetic memory and heterochromatin boundaries.[179–182] In cancer, high *H2AFZ* expression, the gene coding for H2A.Z, was found to be associated with lymph node metastasis and decreased survival in breast cancer. Apparently, *H2AFZ* overexpression promotes proliferation of breast cancer cells by integrating of the protein into promoter regions of estrogen receptor alpha (ERα*)* target genes and hence regulation of estrogen-mediated signaling.[183,184]

Another example is H3.3 which is a variant of H3 and one of the best characterized histone variants. H3.3 containing nucleosomes are less stable and are associated with telomeres, pericentric regions and active gene promoters. Furthermore, they are incorporated in upstream regulatory regions upon induction of transcription, thus promoting transcriptional elongation. Like for H2AZ, integration of H3.3 is replication independent and depends on chaperones like ATRX and DAXX.[185–188] Both genes were found to be mutated in several GBMs (glioblastoma multiforme) resulting in increased alternative lengthening of telomeres and genomic instability. In addition, mutations of *H3F3A*, one of two genes coding for H3.3, found in high grade gliomas correlate with global reduction of H3K27me3 and aberrant recruitment of the polycomb repressive complex PRC2 as well as inhibition of the histone methyltransferase EZH2 (enhancer of zeste homolog 2).[189–191]

Most chromatin remodelers are recruited by histone modifications, some of which are methylation, acetylation, phosphorylation and ubiquitinylation. Depending on the histone type and the affected amino acid residues, the modifications can have activating, repressing or bivalent effects on gene expression and regulatory elements. They are initiated by so-called “chromatin writers” like acetyl- and methyltransferases and recognized by “readers” which often contain bromodomains or PhD fingers. These domains enable them to recognize and bind histone modifications and subsequently recruit further transcriptional repressors or activators.[192]

Many of these genes were by now shown to be mutated or aberrantly expressed in cancer, resulting in or caused by cancer specific patterns of histone modifications. For example, histone lysine methyl transferases (KMTs) like *EZH2* and *MLL2* mediate the mono-, di- and tri-methylation of histones. *MLL2* is mutated in 90% of follicular lymphoma (FL) and in some cases of medulloblastoma where it is suspected to be involved in the generation of subtype-specific aberrant patterns of H3K4 and H3K27 methylation.[193–195] Overexpression of *EZH2* is associated with poor prognosis in prostate and breast cancer.[196,197] Moreover, *EZH2* was also found to be mutated in diffuse large B-cell lymphoma (DLBCL) and FL which results in increased levels of the repressive histone mark H3K27 tri-methylation (H3K27me3) in these tumors. Besides its histone methyltransferase activity EZH2 is a polycomb group protein and acts as part of the PRC2 complex. Interestingly, PRC2 is mainly recruited and active at bivalent promoters harboring the repressive mark H3K27me3 and the active mark H3K9me3. In cancer, an association between DNA hypermethylation and genes with bivalent histone modification was found. These genes have a low poised transcriptional state and in ESCs they were shown to maintain stemness and self-renewal. In colon and breast cancer the methylation state of these genes can be used to classify and cluster subgroups which points toward controlled mechanisms connecting DNA methylation, bivalent histone marks and gene expression.[198–201]

Another prominent histone modifier is *UTX*, a histone H3 lysine demethylase which was found to be mutated in about 12 different types of cancer including ALL, CML, and glioblastoma. Van Haaften and colleagues could show that upon reintroduction of functional *UTX* in mutated cells, proliferation was slowed down and transcriptional changes could be detected, pointing on the functional impact of de-functional histone modifiers.[202–204]

As mentioned before, it was shown that *IDH1* mutants found in myeloid malignancies and glioblastoma are able to produce 2-HG from α-KG and that these mutations could be associated with a hypermethylation phenotype. Interestingly, some demethylases like for example the Jumonji-C domain histone demethylases (JHDMs) use α-KG as substrate and their activity can, like TET protein activity, be inhibited by 2-HG. In fact, gliomas with *IDH* mutations compared to tumors with wild-type *IDH* show dysregulation of global histone methylation, an effect which could also be confirmed *in vitro*. [171,173,189,205]

Histone acetylation marks open and active chromatin and is mediated by histone lysine acetyltransferase like the *p300-CBP* coactivator family. CBP as well as p300 are known as transcriptional activators not only because of their acetyltransferase activity but also because of interactions with transcription factors like p53 and cMYB. p300 and CBP were shown to be mutated in cancer, effected by chromosomal translocations for example in therapy related myeloproliferative disease, and their expression is associated with poor prognosis in small cell lung cancer. [175,206–208]

The fact that DNA methylation, histone modifications as well as mutations and expression changes in chromatin remodelers could be detected in cancer and associated with disease state, development as well as prognosis, highlights these factors as potential targets in the treatment of cancer. A whole panel of epigenetic treatment options are currently under investigation and also already used in the clinics including HDAC inhibitors like Vorinostat or Romidepsin, BRD inhibitors like JQ1 and demethylating agents like Decitabine and an inhibitor of mutated *IDH1* AG-120.

5-aza-2'-deoxycitidine, commercially available under the name Decitabine (Dacogen), is a cytidine analog which, after incorporation into DNA during replication, inhibits the activity of DNA methyltransferases (DNMT). These proteins maintain DNA methylation during replication (DNMT1) and introduce de novo DNA methylation for example during embryogenesis (DNMT3A and B). As described above, DNA methylation in cancer is disrupted on several levels resulting in the deregulation of tumor suppressor genes and regulatory elements and impact on genomic instability. The use of Decitabine was initially indicated for the treatment of myelodysplastic syndrome but until now it is also used in current treatment trials against AML, CML and ALL where a positive response could be observed.[209] The exact mode of action in the cells however in some cases remains unclear. While in fact, global hypomethylation was observed in ALL patients in a combined trial of Decitabine and Vorinostat in combination with chemotherapy, no changes in DNA methylation and target gene expression could be seen in CLL and non-Hodgkin lymphoma (NHL) upon Decitabine treatment.[210–212] Although Decitabine was only approved for hematological malignancies for quite some time, it is currently also under investigation as treatment option in solid tumors like ovarian and colorectal cancer.[213,214]

Another line of epigenetic treatment are BET family inhibitors. BET family members like BRD4 are chromatin remodelers which have bromodomains that recognize mono-acetylated lysine residues on N-terminal histone tails. Together with the associated co-

activator Mediator, BRD4 builds a complex that is involved in the control of transcriptional elongation by RNA Polymerase II. In multiple myeloma the BRD4-Mediator complex was found to co-occupy thousands of enhancers including a small set of exceptionally large super-enhancers which are associated with feature oncogenes of MM including *MYC*.[215] BRD4 inhibitors lead to the depletion of BRD4 at the *MYC* enhancer and to the down-regulation of *MYC* as well as cMYC target genes resulting in anti-proliferative effects, associated with cell cycle arrest and senescence not only in MM but also in AML and Burkitt lymphoma (BL).[216–218] So far no BET inhibitors are used in the clinic but several are under investigation and being optimized for use in clinical trials.[219]

4. Chronic lymphocytic leukemia

Chronic lymphocytic leukemia (CLL) is diagnosed in patients which show accumulation of small, mature-appearing B-cells in the bone marrow, lymphoid tissues and peripheral blood with at least $5x10^9$ B-cells/l.[220] CLL cells are arrested in early G_0 late G_1 phase of the cell cycle and in addition show an apoptotic defect which leads to enrichment of the malignant cells in the patient. The cells display a specific immunological profile with weak levels of IgM and / or IgD as well as expression of CD23, CD19, CD20 and CD5.[221] Amongst other aspects, CLL is a disease of the genome and the epigenome characterized by genetic aberrations and mutations on the one hand and genome-wide DNA hypomethylation and region specific hypermethylation on the other hand. Furthermore, CLL cells are highly dependent on the interplay with other cells of the immune system and the lymphoid tissues, which represents the leukemic microenvironment.

4.1. Prognostic factors characterizing CLL include surrogate markers and genetic aberrations and are involved in clonal evolution

CLL patients can be subdivided into two groups which are categorized by the mutation status of the heavy chain variable region (*IGHV*) of the B-cell receptor (BCR), which is either classified as mutated (below 98% homology compared to germline) or unmutated. *IGHV* unmutated patients show poor prognosis in comparison to *IGHV* mutated cases, highlighting the status of the BCR as important predictive marker.[222–224] Independent of their mutation status, CLL cells are highly dependent on BCR signaling and interestingly stereotyped and quasi-identical BCRs were found in different CLL patients.[225] Moreover, BCRs in CLL cells are able to induce antigen-independent cell-autonomous signaling which in line with biased *IGHV* usage highlights a signal dependency in the pathomechanism of CLL.[226–228] Although the differences on expression level between *IGHV* mutated and unmutated are relatively low in comparison to CLL vs normal B-cells, it is estimated that these subgroups arise from two different B-cell stages of which the origin is still under debate. However, a recent study of Seifert *et al.* indicates that *IGHV* unmutated CLL develops from mature $CD5^+$ B-cells while *IGHV* mutated CLL could originate form distinct, previously unrecognized $CD5^+CD27^+$ post-germinal center B-cells.[229]

Besides *IGHV* mutation status other predictive markers have been identified in the course of CLL and are used for risk stratification in the clinic, the most important including expression of *ZAP70* and *CD38*. The expression of the zeta-chain associated protein kinase 70 (*ZAP70*) is tightly associated with *IGHV* unmutated CLL cases and hence

another risk factor with even stronger impact than *IGHV* mutation status.[230,231] If present, ZAP70 mediates a more effective BCR signaling in CLL cells, independent of its kinase activity, resulting in increased NFκB signaling after BCR stimulation, enhanced response to survival signals, higher proliferation rates and increased resistance to apoptosis.[232–235] Similar like *ZAP70* the expression of the surface marker *CD38* is associated with *IGHV* mutation status and further linked to aggressive clinical behavior, shorter progression free and overall survival (PFS, OS).[224] However, the abundance of *ZAP70* and *CD38* can vary between disease stages, upon treatment and over time and therefore, are under debate as surrogate markers for disease prediction.

More stable over time, and hence of higher predictive value, are genetic lesions which characterize CLL cells and occur in about 80% of patients. Most frequent are deletions in 13q14, 17p and 11q as well as trisomy 12 (Figure 4).[236] In contrast to the other genetic aberrations, 13q14 is the only one marking favorable course of disease and besides that with an incidence of about 50% the most frequent one. A tumor suppressor gene or mechanism is assumed in this region which would be inactivated by deletion in one allele and mutation in the other one. In fact, it could be shown that the epigenetic up-regulation of the long non-coding RNAs *DLEU1* and *DLEU2* in 13q14 results in the down-regulation of a gene cluster that targets NFκB signaling.[237]

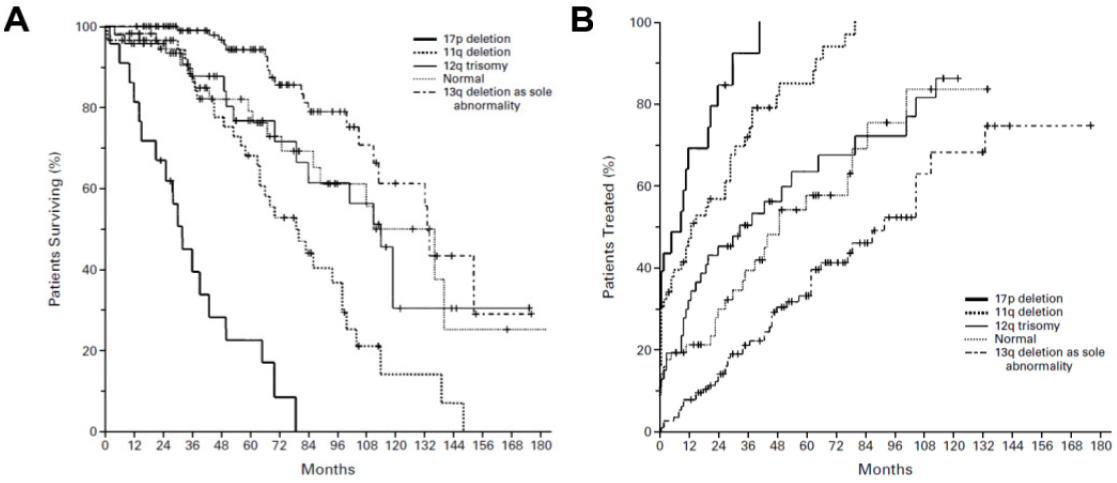

Figure 4. Prognostic impact of chromosomal aberrations in CLL. *(A) "Probability of survival from the date of diagnosis among the patients in the five genetic categories. (B) Probability of disease progression, as indicated by the treatment-free interval in the patients in the five genetic categories." Modified from Döhner et al.*[236]

The deletion of the chromosomal band 11q22-23, which occurs in about 18% of CLL cases, includes the serine threonine protein kinase Ataxia telangiectasia mutated gene (*ATM*) which in addition displays a mutation rate of about 12% in CLL.[238] Both, deletions and mutations are associated with more rapid disease progression resulting in impaired overall and treatment free survival.[239] As an important component of the DNA damage pathway, *ATM* is necessary for the induction of apoptosis upon DNA damage and indeed, mutations in this gene were shown to vitiate *in vitro* DNA damage response.[238]

17p13 is another chromosomal region frequently deleted in CLL (7%) and affects the prominent tumor suppressor gene *TP53*. Most patients with monoallelic 17p deletion show mutations in the remaining *TP53* allele and like the other aberrations this deletion is linked to poor prognosis and in addition poor response to therapy.[240,241]

NGS techniques have allowed to analyze the CLL genome in more detail. Several studies could confirm mutations in *TP53* and *ATM*. In addition, also a panel of previously undiscovered mutations where revealed, of which impact and function have yet to be investigated, including mutations in *MYD88*, *KLHL6*, *FBX7*, *SF3B1*, *KRAS*, *BCOR* and *NOTCH1*.[242–245]

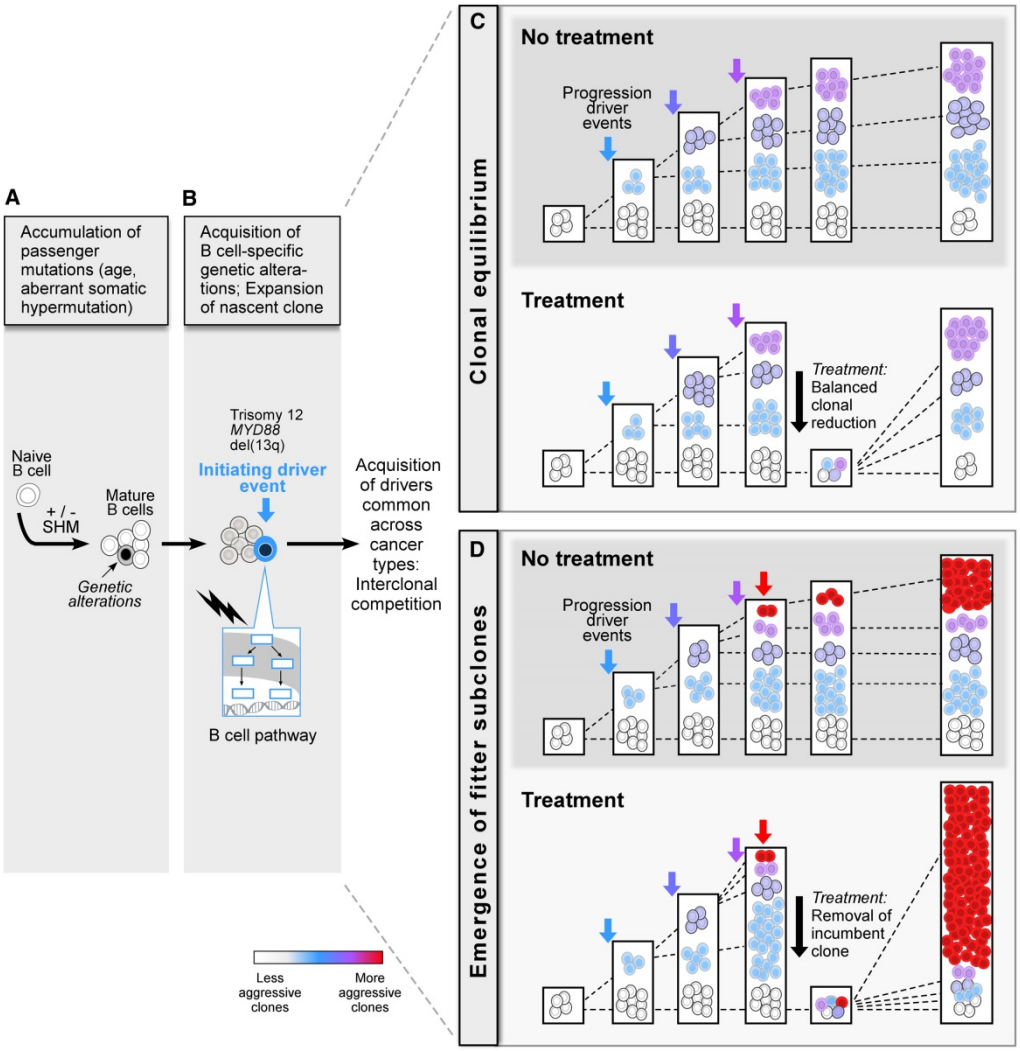

Figure 5. Model for the stepwise transformation of CLL. *"Earlier (A) and later mutations, B-cell specific (B) and ubiquitous cancer events (C and D) drive CLL pathogenesis. Clonal evolution and treatment show complex relationship. Most untreated CLLs and minority of treated CLLs maintain stable clonal equilibrium over years (C). In presence of a subclone containing a strong driver, treatment may disrupt interclonal equilibrium (D)." Modified from Landau et al.*[243]

Besides the discovery of novel mutations, NGS also led to the extensive analysis of another cancer attribute which is clonality and clonal evolution (Figure 5). Whole-exome sequencing studies and copy number analysis showed that CLL displays intratumoral heterogeneity represented by various numbers and types of sub-clones. Genetic aberrations can partly be classified as driver mutations which are predominantly clonal like mutations in *MYD88*, trisomy 12 or 13q deletion, and hence arise early in CLL development. Furthermore, they can be subclonal like *SF3B1* and *TP53* mutations which would reflect late events in CLL pathogenesis and possibly contribute to disease progression. Especially after treatment, clonal evolution could be observed, reflected by the expansion of subclones that harbor driver mutations. It can be assumed that the

outgrowth of subclones after treatment results in the observed resistance and relapse of the CLL patients and is potentially supported by the microenvironment of the CLL cells.[243]

4.2. CLL microenvironment

In *in vitro* culture CLL cells undergo rapid and spontaneous apoptosis which can be prevented by co-culture with bone marrow stromal cells (BMSCs) or other feeder cells that offer survival support.[246] Furthermore, CLL cells in bone marrow and lymph nodes show up-regulation of activation markers and especially the lymph node microenvironment promotes BCR signaling, NFκB activation and tumor proliferation in CLL.[247] These findings support the notion that CLL cells are highly dependent on their microenvironment. As a matter of fact, CLL cells can interact with numerous cell types of the immune system including mesenchymal stromal cells, monocyte-derived nurse like cells (NSCs) as well as T-cells.[248–250]

NLCs and BMSCs protect CLL cells not only from spontaneous but also drug induced apoptosis.[251–253] *Vice versa*, CLL cells can activate BMSCs in the patient which in contrast to healthy-donor derived BMSCs significantly promote normal B-cell proliferation and IgG production.[254,255] NLCs differentiate from $CD14^+$ blood monocytes in co-culture with CLL cells in high-density conditions and can be found in spleen and lymphoid tissues of CLL patients.[251,253] Upon co-culture with NLCs, CLL cells showed characteristic induction of genes in BCR- and NFκB pathways and production of T-cell attracting chemokines.[256] In general, CLL patients display increased number, in particular of $CD8^+$ T-cells in the peripheral blood, which exhibit profound functional defects like chronic activation and exhaustion.[257] In addition, these cells show alterations in genes involved in cytoskeletal formation and similar defects are induced in healthy T-cells after CLL co-culture.[258] Presumably these changes result in defects in the formation of immunological synapse with antigen presenting cells.[259]

However, these are just some examples depicting the microenvironmental factors impacting on CLL pathogenesis. Recent research focuses on the relations of CLL cells with bystander cells and disruption of the supportive interactions is a promising treatment strategy.

4.3. DNA methylation in CLL

CLL is a disease not only defined by genetic but also by epigenomic aberrations. Similar to other cancer types, CLL is known to display global hypomethylation which is strongly associated with repetitive sequences like Alu, LINE-1 and SAT-α.[260] On the other hand, region specific hypermethylation results in the deregulation of a number of CLL prognostic genes and tumor suppressors important for CLL pathogenesis (Figure 6).

These include for example the von Hippel-Lindau gene (*VHL*), which is preferentially methylated and down-regulated in *IGHV* unmutated CLL. The highly prognostic *ZAP70* shows a small DMR in its 5' regulatory region which illustrates large variability in DNA-methylation in CLL cells.[261,262] A single CpG unit seems to be important for expression of this gene and shows strong association with *IGHV* mutation, PFS and OS. Moreover, *DAPK1* is down-regulated through methylation and contributes to heritable predisposition in CLL. The oncogene *BCL-2* displays hypomethylation and overexpression which is a feature of CLL already known for two decades.[263,264] WNT signaling activation in CLL is related to hypermethylation of WNT inhibitor (antagonist) genes and the key transcription

factor of WNT signaling *LEF-1*.[265–267] Besides protein coding genes, aberrant methylation in CLL was shown to be associated with abnormal miRNA expression and in addition, methylation markers can be used to improve risk stratification even independent of *IGHV* and *CD38* expression status.[268,269]

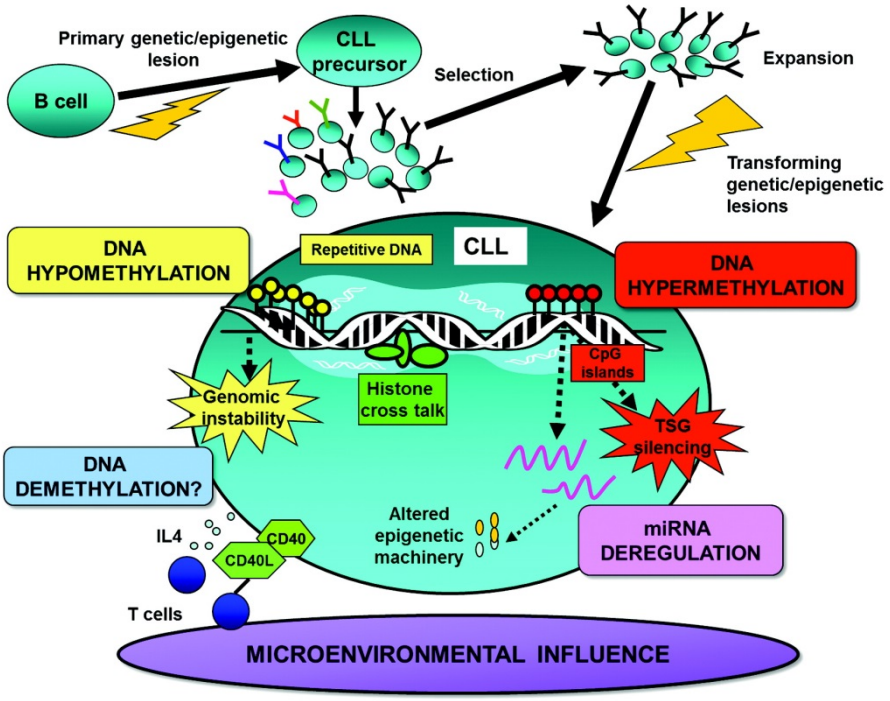

Figure 6. Epigenetic factors shaping the DNA methylome in CLL. *"DNA hypermethylation silences tumor suppressor genes, DNA hypomethylation leading to genomic instability, dysregulation of epigenetic regulators and machinery, interplay between DNA methylation and other epigenetic / microenvironmental factors." Modified from Cahill & Rosenquist*[270]

Recently, NGS techniques and the application of 450K Illumina Bead Chip methylation arrays have allowed to analyze DNA methylation in greater detail. While the genome-wide hypomethylation and region specific hypermethylation could be confirmed in several studies, it was revealed that methylation patterns are more complex than originally thought. Kulis and colleagues analyzed 139 CLL samples with 450K methylation arrays and compared these to different non-malignant B-cell subsets.[271] A widespread gene-body DNA hypomethylation as well as a large proportion of aberrant methylation positioned in gene bodies was observed. Correlation of only 4% of CpGs with expression was detected and interestingly gene body methylation that correlated with gene expression was related to enhancers. Subsequently, three different clinico-biological CLL subgroups could be defined classifying CLL samples as NBC-like (naïve B-cell), intermediate or MCL-like (mature B-cell) CLL. In addition, this re-classification also confirmed again differential methylation between *IGHV* mutated and unmutated CLL where unmutated CLL was closely related to $CD5^+$ NBCs and NBCs and mutated CLLs resembled MBCs.

Another comparison of *IGHV* mutated and unmutated CLL cases by Cahill *et al.* identified 2239 differentially methylated CpG sites, with the majority outside CGIs but many in CGI shores. Numerous CLL relevant genes (e.g. *CLLU1*, *LPL*, *ZAP70*, *NOTCH1*) as well as epigenetic regulators (*HDAC9*, *HDAC4*, *DNMT3B*) and B-cell signaling genes were alternatively methylated between the two subgroups. In addition, this study showed that

global DNA methylation in CLL is stable over time and similar in resting and proliferative compartments.[272]

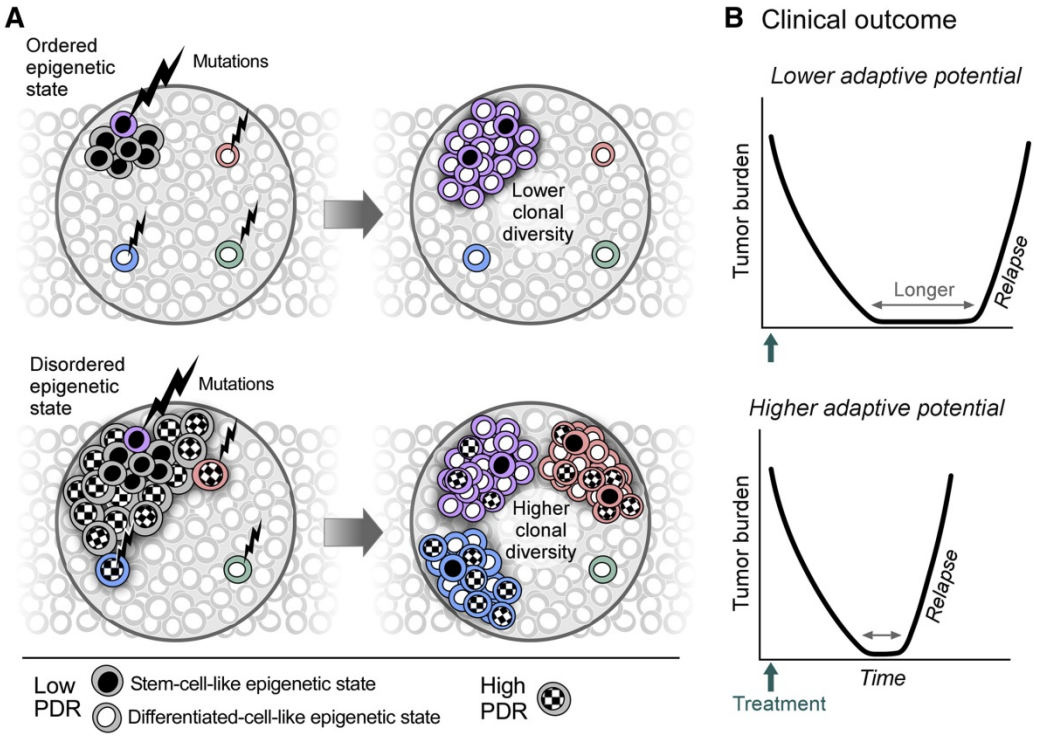

Figure 7. Proposed interaction between methylation disorder and clonal evolution. *"(A) Novel somatic mutation has to coincide with an epigenetic state that will be permissive to the propagation of the new genotype. In a cellular population with ordered epigenetic state, the proportion of cells that are able to actively participate in the evolutionary process is small. In a disordered epigenetic state, a greater proportion of cells can give birth to new subclones, increasing diversity and adaptive capacity of cancer population, resulting in (B) adverse clinical outcome with therapy." Modified from Landau et al.2014*[273]

Of note, NGS identified allele-specific methylation (ASM) within CLL samples which does not reflect imprinting but is a result of methylation loss. Only 0.4% of ASM was recurrent and potentially disease specific. At the same time CLL cells display lower intrasample heterogeneity in comparison to healthy B-cells, but stronger interpatient variability which to some degree is a result of locally disordered methylation in the malignant cells.[273,274] It is assumed that this higher amplitude of epigenetic "noise" allows the cancer cells a greater degree of population diversity, which would go in line with the notion of genetic evolution described above. In fact, these current results indicate that genetic aberrations evolve co-dependently with methylation (Figure 7).[274]

4.4. NOTCH1 signaling is constitutively active in CLL

NOTCH1 is one of 4 NOTCH proteins that have been identified so far and are responsible for NOTCH signaling, a highly conserved canonical pathway also present in mammals. *NOTCH1* codes for a single-pass transmembrane protein which harbors an intra- and an extracellular domain. The latter allows interaction of NOTCH1 with five different ligands (DLL1, DLL3, DLL4, JAGGED1 and JAGGED2) which are consequently able to activate NOTCH1 signaling in a cell-cell contact dependent manner.[275–277] Ligand-engagement results in a series of proteolytic cleavages which leads to release of first the NOTCH1

extracellular domain and finally the NOTCH1 intracellular domain (NICD) by the protein γ-secretase.[278] After translocation to the nucleus, NICD acts together with other proteins like CSL and MAML1 as transcription factor complex which binds to and activates its down-stream targets including *HES1*, *CCND1* and *KLF4* (Figure 8).[279–281]

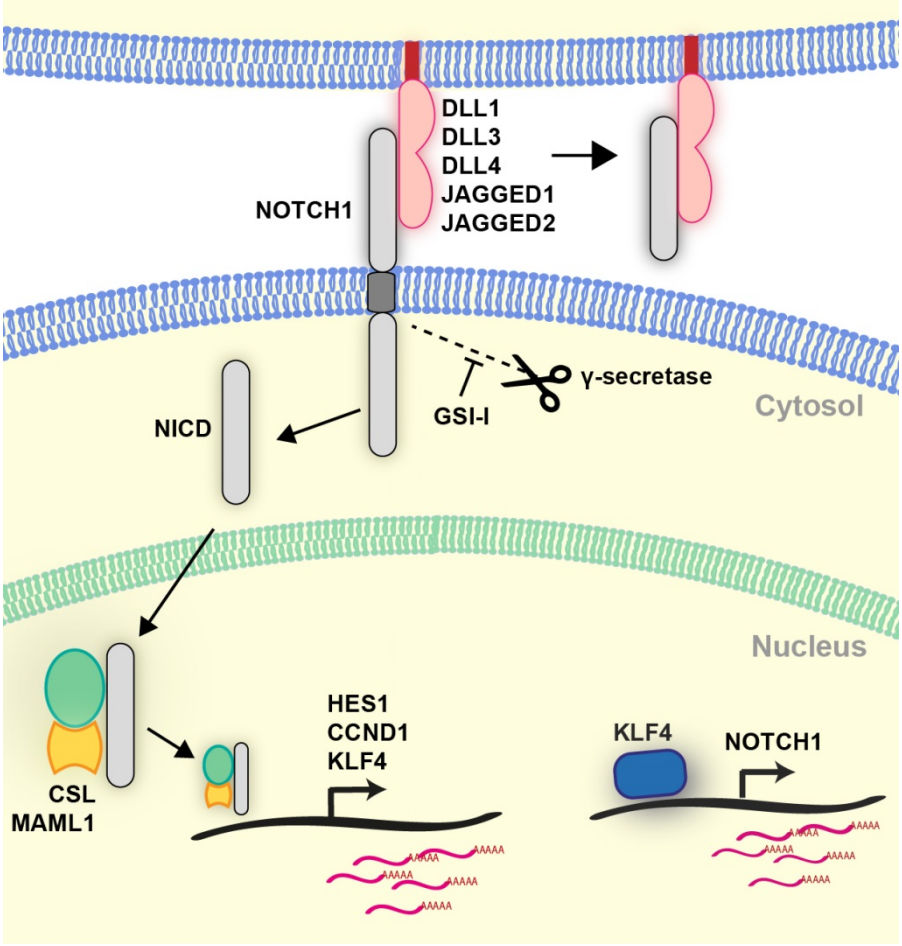

Figure 8. NOTCH1 signaling pathway. *NOTCH1 activation by its ligands DLL1, 3, 4 and JAGGED1 and 2 results in cleavage and translocation of the NOTCH intracellular domain (NICD). As transcription factor complex together with CSL and MAML1 it can activate a number of downstream targets including HES1, CCND1 and KLF4.*

Of note, *KLF4* and *NOTCH1* are able to co-regulate each other resulting in a potential circuitry between NOTCH1 signaling and KLF4 activity. In head and neck cancer (HNSCC), NOTCH1 can increase *KLF4* activity by direct binding of NICD/CSL to the *KLF4* promoter. NOTCH1-mediated repression of *KLF4* expression via up-regulation of HES1 was shown in intestinal epithelium and T-ALL.[282–284] In primary human breast tumors and epithelial cells, KLF4 binds to the *NOTCH1* promoter and induces its transcription while down-modulation of *NOTCH1* was observed in keratinocytes.[285,286] In these cells, knock-down of *KLF4* and in addition another transcription factor (*SP3*) was necessary to increase *NOTCH1* transcription.[287] In summary, these findings suggest that *NOTCH1* and *KLF4* can co-regulate each other, however this regulation is highly influenced by other factors and hence a potential interplay has to be investigated for each case individually.

NOTCH1 is supposed to be an oncogenic driver in a number of cancers such as colon and breast cancer and T-ALL.[288,289] In the latter, *NOTCH1* was found to be mutated in about 60% of cases.[290] Two types of mutations were identified resulting either in a

C-terminally truncated NICD or loss of the PEST domain which allows the accumulation of NOTCH1 due to abrogated denaturation of the protein.[291] Activation of *NOTCH1* was shown to induce leukemia in murine models of T-ALL and moreover NOTCH1 signaling is required for hypoxia-related proliferation and chemoresistance.[292,293] Hence, the pathway has emerged as promising treatment target and a number of NOTCH1 inhibitors are currently under investigation.[283,294]

In CLL, *NOTCH1* was recently found to be recurrently mutated in 6-12% of patients. Similar to T-ALL, most mutations are found in the PEST domain and result in the accumulation of a truncated NOTCH1 due to impaired degradation. In these *NOTCH1* mutated cases, NOTCH1 signaling is hence constitutively active and further results in the activation of NFκB signaling.[295] Besides this mutation mediated activation of this signaling pathway, *NOTCH1* as well as its ligands *JAGGED1* and *2* are highly expressed in CLL cells in contrast to normal B-cells.[296] *In vitro* co-culture with BMSCs up-regulates *NOTCH1* and NOTCH1 ligand increases B-CLL survival while blockage of NOTCH1 signaling results in increased apoptosis of the cells.[296,297] These findings support an important role of NOTCH1 signaling activity in the pathomechanism of CLL. Indeed, *NOTCH1* mutations are associated with shorter overall survival and further, *NOTCH1* mutated cases undergo transformation into DLBCL (Richter's transformation) more frequently.[242,244,298–300]

4.5. Role of KLF4 in tumorigenesis and B-cell development

One NOTCH1 target gene is the transcription factor Krüppel-like factor 4 (*KLF4*) which has bivalent functions and can either repress or activate its downstream targets. Initially, *KLF4* was characterized as one of the four Yamanaka factors which are able to induce pluripotent stem cells (iPS) from adult human dermal fibroblasts.[301] Since then, KLF4 was shown to be important not only for differentiation but also for ESC self-renewal, DNA-damage induced apoptosis, cell cycle progression and cell proliferation.[302–305] Furthermore, *KLF4* is important for monocyte differentiation, required for lineage commitment, proliferation and homing of T-cells, acts as mediator of pro-inflammatory signaling in macrophages and seems to have a prominent role in B-cell development and differentiation.[306–309] During B-cell maturation *KLF4* expression increases, and naïve mature B-cells express higher levels of *KLF4* than memory B-cells. Upon activation of B-cells via CD40 and BCR signaling, *KLF4* is down-regulated. *KLF4* overexpression maintains the cells in a resting state.[310] Moreover, *KLF4*-deficient mice show decreased numbers of pre-B-cells in bone marrow and mature B-cells in spleen, suggesting KLF4 to be necessary for regulation of B-cell number and activation-induced B-cell proliferation.[311]

In line with its diverse functions, *KLF4* was shown to have oncogenic as well as tumor suppressive potential. In head and neck as well as breast cancer an oncogenic role of *KLF4* is assumed, while it was shown to act as tumor suppressor in various other types of cancer including urothelial, pancreatic, ovarian and cervical cancer, as well as in several B-cell malignancies.[303,312–315] In AML *CDX2* driven leukemogenesis involves *KLF4* repression and down-regulation of *p21*.[316] In lymphoma cell lines and primary cases of FL, DLBCL, BL and classical Hodgkin-lymphoma (cHL) *KLF4* was shown to be silenced by promoter methylation. Overexpression of *KLF4* induced the accumulation of these cells in G_0 / G_1 phase and growth inhibition as well as apoptosis mediated via *BAK1*.[317] Besides, the expression of KLF4 in B-cell progenitors of transgenic mice blocks transformation by BCR-ABL (Philadelphia chromosome) and depletes leukemic pre-B cells in vitro.[318]

5. Project outline

Chronic lymphocytic leukemia is one of the most common leukemias in the western world and recent work applying NGS has greatly improved our knowledge about this disease. However, CLL remains incurable and patients relapse and in addition have to stay under permanent treatment. It is therefore necessary to further expand our understanding of the pathomechanism of CLL and to identify new treatment targets.

Aim of this project was the identification of genes deregulated by DNA methylation in CLL, as these genes could play a role in CLL development or maintenance. For this purpose, RNA and DNA was isolated from CD19+ CLL cells of 15 untreated patients and B-cells of 9 age-matched healthy donors (Figure 9). While RNA was analyzed on expression arrays, DNA was applied to methyl-CpG-immunoprecipitation (MCIp). The highly methylated DNA fragments isolated with this method were subsequently analyzed on promoter tiling-arrays covering promoter regions -3.8 kb to +1.5 kb around transcription start sites of all RefSeq genes.

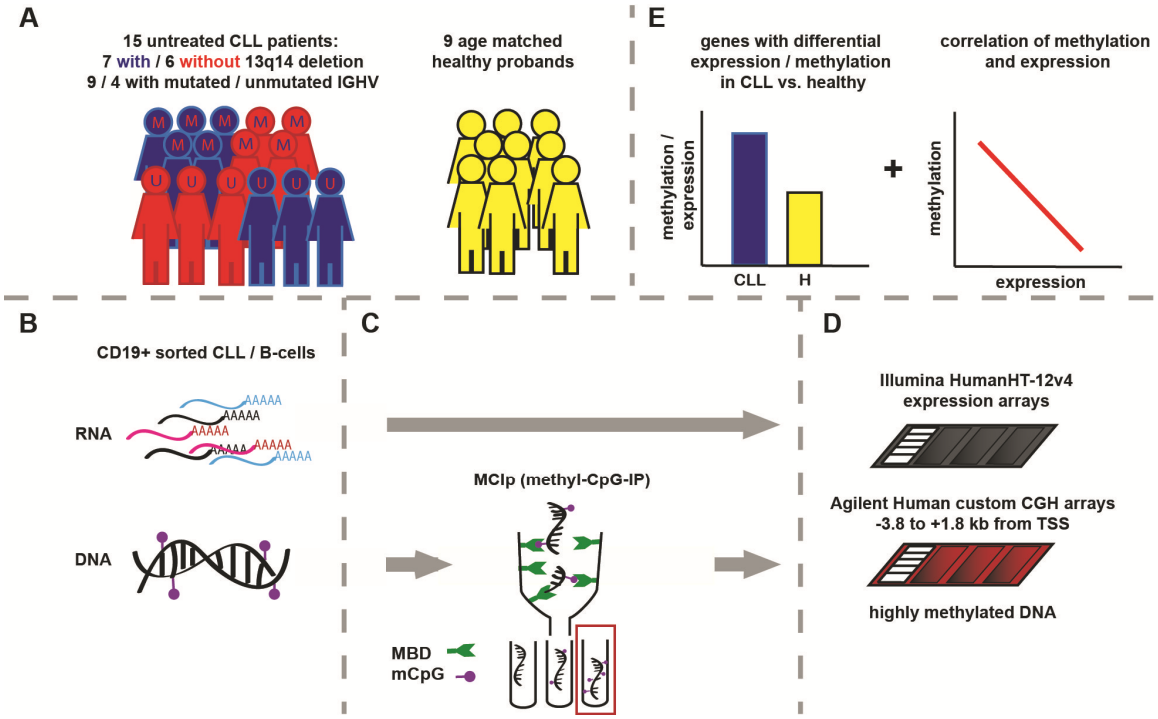

Figure 9. Project outline. *(A) Cohort of 13 CLL patients and 9 age matched healthy donors. (B) Isolation of RNA and DNA from CD19+ CLL/B-cells. (C) Methyl-CpG-immunoprecipitation (MCIp) auf DNA samples. (D) Expression and promoter-array analysis of RNA and DNA. (E) Identification of genes with significantly correlating aberrant expression and DNA methylation.*

Data of MCIp- and expression arrays were analyzed in an integrative approach searching for genes which display differentially methylated regions within their promoters and concurrent correlating, aberrant expression between CLL and B-cells. Genes identified to be potentially deregulated in CLL by DNA methylation were validated in their methylation with mass spectrometry based methylation analysis (MassARRAY) and concerning their expression with quantitative real-time PCR (qRT-PCR). Finally, potential candidate genes were functionally characterized in the course of CLL, to reveal their impact on disease pathogenesis.

II. Material and methods

1. Materials

1.1. Instruments

Instrument	Supplier
ABI PRISM 3100 Genetic Analyzer	Life Technologies, Darmstadt, Germany
ABI PRISM 7900HT Fast RealTime PCR System	Life Technologies, Darmstadt, Germany
Agilent 2100 Bioanalyzer	Agilent, Santa Clara, USA
Agilent Technologies Scanner G2505C US81503234	Agilent, Santa Clara, USA
Amaxa Nucleofector 2b device	Lonza, Basel, Switzerland
BD FACSCanto II	BD Biosciences, San Jose, USA
Bioruptor	Diagenode, Lüttich, Belgium
Eppendorf PCR Cycler (Mastercylcer Gradient)	Eppendorf, Hamburg, Germany
MassARRAY Analyzer 4 System	Sequenom, San Diego, USA
MassARRAY Nanodispenser	Sequenom, San Diego, USA
Matrix Equalizer Electronic Multichannel Pipettes	Thermo Fisher Scientific, Waltham, USA
Mini Protein Gel Transfer System	BioRad, Munich, Germany
Mithras LB 940 Plate Reader	Berthold Technologies, Bad Wildbad, Germany
NanoDrop ND-1000 spectrophotometer	NanoDrop, Wilmington, USA
Photometer Bio Eppendorf	Eppendorf, Hamburg, Germany
ViCell XR cell counter	Beckman Coulter, Krefeld, Germany
XCell SureLock Mini-Cell Electrophoresis System	Life Technologies, Darmstadt, Germany

1.2. Reagents and consumables

Reagent / Consumable	Supplier
1.5 / 2 ml Safe-Lock Tubes	Eppendorf, Hamburg, Germany
15 / 50 ml BD Falcon tubes	BD Biosciences, San Jose, USA
200x S-adenosyl methionine	NEB, Frankfurt am Main, Germany
7AAD	BD Biosciences, San Jose, USA
ABgene Superplate 96-well Plate	Thermo Fisher Scientific, Waltham, USA
Absolute SYBR Green ROX Mix	Abgene, Epsom, UK
Ampicillin	Roche Diagnostics, Mannheim, Germany
Annexin-V binding buffer 10x	BD Biosciences, San Jose, USA
Annexin-V FITC	BD Biosciences, San Jose, USA
Biocoll	Biochrom, Berlin, Germany
Bovine serum albumin	Sigma-Aldrich, Munich, Germany
Cell culture dish (6-well, 12-well, 24-well) TC-plate suspension F	Sarstedt, Nümbrecht, Germany
Cell culture flasks TC-flask suspension (T25, T75, T175)	Sarstedt, Nümbrecht, Germany
CpG Methylated Human Genomic DNA	Thermo Fisher Scientific, Waltham, USA
DMEM medium (4.5 g/L glucose)	Sigma-Aldrich, Munich, Germany
DMSO	Sigma-Aldrich, Munich, Germany
dNTP set PCR grade 100 mM each	Roche Diagnostics, Mannheim, Germany
ECL (plus) WB Detection Reagents	GE Healthcare, Munich, Germany
Ethanol	Sigma-Aldrich, Munich, Germany
Fetal calf serum (FCS)	Biochrom AG, Berlin, Germany

Formamide	Merck Millipore, Darmstadt, Germany
Fuji X-ray Film Super RX, 18x24 cm	Fujifilm, Tokio, Japan
Gamma secretase inhibitor I	Merck Millipore, Darmstadt, Germany
GeneRuler DNA Ladder Mix	Life Technologies, Darmstadt, Germany
Human Cot-1 DNA	Invitrogen, Karlsruhe, Germany
Human type AB serum, heat inactivated	MP Biomedicals, Eschwege, Germany
Immobilon-P PVDF membrane	Merck Millipore, Darmstadt, Germany
Leucosep tubes 50 ml	Greiner Bio-One, Kremsmünster, Austria
MACS CD19 Micro Beads	Miltenyi Biotech, Bergisch Gladbach, Germany
Matrix pipette tips 384 125 µl	Thermo Fisher Scientific, Waltham, USA
MBD2-Fc Protein	Provided by Dieter Weichenhan (DKFZ)
NuPAGE Antioxidant	Life Technologies, Darmstadt, Germany
Nunc F96 MicroWell White Polystyrene plate (white)	Thermo Fisher Scientific, Waltham, USA
NuPAGE Novex 3-8% Tris-Acetate Protein Gels	Life Technologies, Darmstadt, Germany
NuPAGE Novex 4-12% Bis-Tris Protein Gels	Life Technologies, Darmstadt, Germany
NuPAGE sample Reducing Agent (10x)	Life Technologies, Darmstadt, Germany
NuPAGE SDS Running Buffer (20x)	Life Technologies, Darmstadt, Germany
NuPAGE SDS Sample Buffer (4x)	Life Technologies, Darmstadt, Germany
NuPAGE Tris-Acetate SDS Running Buffer (20x)	Life Technologies, Darmstadt, Germany
PageRuler Prestained Protein ladder	Life Technologies, Darmstadt, Germany
Penicillin/Streptomycin (1000U/ml)	Life Technologies, Darmstadt, Germany
Qiashredder	Qiagen, Hilden, Germany
RNase free water	Qiagen, Hilden, Germany
RPMI 1640 media	Sigma-Aldrich, Munich, Germany
SIMAG Protein A/G	Chemicell, Berlin, Germany
Skim Milk Powder	Sigma-Aldrich, Munich, Germany
Spectra Multicolor Broad Range Protein Ladder	Life Technologies, Darmstadt, Germany
Spectro Chips II	Sequenom Inc., San Diego, USA
TMAC (tetramethylammonium chloride solution)	Sigma-Aldrich, Munich, Germany
Tween20	Sigma-Aldrich, Munich, Germany
Universal Human Reference RNA	Agilent, Santa Clara, USA

1.3. Cell lines and bacteria

Characteristics	Supplier
GRANTA-519 cell line	
B-cell lymphoma; established from the peripheral blood from a 58-year old Caucasian woman with high-grade NHL (non-Hodgkin lymphoma) at relapse (leukemic transformation of mantle cell lymphoma, stage IV); previous history of cervical carcinoma	DSMZ, Braunschweig, Germany
JeKo-1 cell line	
B-cell lymphoma; established from peripheral blood of 78-year old female with MCL (Mantle Cell Lymphoma)	ATCC, Manassas, USA
LCL-FM cell line	
B-lymphoblastoid cells; spontaneous developed cell line (WT EBV) from a patient with history of mamma-carcinoma and angiosarcoma	provided by Silke Brüderlein (Ulm)
LCL-MM cell line	
B-lymphoblastoid cells; established from peripheral blood of MCCL (mediastinal clear-cell lymphoma) patient; EBV transformation	provided by Silke Brüderlein (Ulm)

LCL-WEI cell line	
B-lymphoblastoid cells; established from peripheral blood of male patient with melanoma; EBV transformation	DSMZ, Braunschweig, Germany
MEC1 cell line	
Chronic B-cell leukemia; established from peripheral blood of a 61-year old Caucasian man with CLL in prolymphocytoid transformation to B-PLL; serial sister cell line of MEC2	DSMZ, Braunschweig, Germany
MEC2 cell line	
Chronic B-cell leukemia; established from peripheral blood of a 62-year old Caucasian man with CLL in prolymphocytoid transformation to B-PLL prior to therapy; serial sister cell line of MEC1	DSMZ, Braunschweig, Germany
OneShot TOP10 chemically competent *E.coli*	
Genotype: F- *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ *lac*X74 *rec*A1 *ara*D139 Δ(*araleu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Life Technologies, Darmstadt, Germany

1.4. Kits

Kit	Supplier
Absolute QPCR SYBR Green ROX mix	Thermo Fisher Scientific, Waltham, USA
Agilent aCGH Hybridization Kit	Agilent, Santa Clara, USA
AllPrep DNA/RNA Mini Kit	Qiagen, Hilden, Germany
BigDye Terminator v3.1 Cycle Sequencing	Life Technologies, Darmstadt, Germany
BioPrime Total Genomic Labeling System	Invitrogen, Karlsruhe, Germany
CellTiter-Glo Luminescence cell Viability Assay	Promega, Madison, USA
EndoFree Plasmid Maxi Kit	Qiagen, Hilden, Germany
EpiTect 96 Bisulfite Kit (2)	Qiagen, Hilden, Germany
EpiTect Bisulfite Kit (48)	Qiagen, Hilden, Germany
EpiTect Fast Bisulfite Kit (2)	Qiagen, Hilden, Germany
Gateway LR ClonaseII Enzyme mix	Life Technologies, Darmstadt, Germany
Genomic DNA Enzymatic Labeling Kit	Agilent, Santa Clara, USA
MassCLEAVE Kit	Sequenom, San Diego, USA
MinElute PCR Purification Kit	Qiagen, Hilden, Germany
Nucleofector Kit V	Lonza, Basel, Switzerland
Oligo aCGH/ChIP-on-Chip Wash Buffer Set	Agilent, Santa Clara, USA
Plasmid Mini Kit	Qiagen, Hilden, Germany
QIAquick Gel Extraction Kit	Qiagen, Hilden, Germany
QIAquick PCR Purification Kit	Qiagen, Hilden, Germany
QuantiTect Reverse Transcription Kit	Qiagen, Hilden, Germany
REPLI-g Kit	Qiagen, Hilden, Germany
RNA 6000 Nano Kit	Agilent, Santa Clara, USA
RNeasy Micro kit	Qiagen, Hilden, Germany
RNeasy Mini kit	Qiagen, Hilden, Germany

1.5. Buffers and solutions

Buffer	Reagents
10x PBS (Phosphate-buffered saline)	10 mM KH_2PO_4, 155 mM NaCl, 30 mM Na_2HPO_4-$7H_2O$, pH7.4
10x TBE (Tris-borate-EDTA)	89 mM Tris, 89 mM boric acid, 2 mM EDTA
10x TBS (Tris-buffered saline)	50 mM Tris, 150 mM NaCl, pH7.6
10x TME	200 mM Tris/HCl pH8.0, 20 mM $MgCl_2$, 5mM EDTA pH8.0
Buffer A (MCIp, low salt)	1x TME, 0.3 M NaCl, 0.1% NP40
Buffer B (MCIp)	1x TME, 0.4 M NaCl, 0.1% NP40
Buffer C (MCIp)	1x TME, 0.5 M NaCl, 0.1% NP40
Buffer D (MCIp)	1x TME, 0.55 M NaCl, 0.1% NP40
Buffer E (MCIp)	1x TME, 0.6 M NaCl, 0.1% NP40
Buffer F (MCIp, high salt)	1x TME, 1 M NaCl, 0.1% NP40
FACS buffer	1x PBS, 5% FCS, 1% BSA, 0.05% NaAzid
LB-agar	10g Bacto Trypton, 5g yeast extract, 10g NaCl, 15 g Agar, adjust to 1l with ddH_2O
LB-medium	10 g Bacto Trypton, 5 g yeast extract, 10 g NaCl, adjust to 1l with ddH_2O
PBS-T	1x PBS, 0.1% Tween20
RIPA (Radio-Immunoprecipitation Assay buffer)	150 mM NaCl, 1.0% IGEPAL; 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH8.0
TBS-T	1x TBS, 0.1% Tween20
10x Western Blot buffer	1.92 M Glycine, 0.25 M Tris

1.6. Antibodies

Antibody	Source	Supplier
Anti-KLF4 (H-180)	rabbit	Santa Cruz, Dallas, USA
Anti-GAPDH (6C5)	mouse	Merck Millipore, Darmstadt, Germany
Anti-NOTCH1 (mN1A)	mouse	Abcam, Cambridge, UK
Anti-CD20-PE (2H7)	mouse	BD Biosciences, San Jose, USA
Anti-CD19-APC (HIB19)	mouse	BD Biosciences, San Jose, USA
Anti-CD5-FITC (L17F12)	mouse	BD Biosciences, San Jose, USA
anti-mouse IgG HRP-linked	horse	Cell Signaling Technology, Danvers, USA
anti-rabbit IgG HRP-linked	goat	Cell Signaling Technology, Danvers, USA

1.7. Enzymes

Enzyme	Supplier
HotStarTaq DNA Polymerase (1u/µl)	Qiagen, Hilden, Germany
PRECISOR High-Fidelity DNA Polymerase	BioCat, Heidelberg, Germany
T4 DNA Ligase (1 U/µl)	Life Technologies, Darmstadt, Germany
NotI (10,000 U/ml)	Life Technologies, Darmstadt, Germany
HindIII (10,000 U/ml)	Life Technologies, Darmstadt, Germany
CpG Methyltransferase (M.SSsI; 20 U/µl)	NEB, Frankfurt am Main, Germany

1.8. Primers

qRT-PCR		
Gene	**Forward (5' to 3')**	**Reverse (5' to 3')**
BAK	GGTCCTGCTCAACTCTACCC	CCTGAGAGTCCAACTGCAAA
BAX	GCTGGACATTGGACTTCCTC	GTCTTGGATCCAGCCCAAC
BCL-2	AAAAATACAACATCACAGAGGAAGT	GCGGAACACTTGATTCTGGT
CCND1	AACTACCTGGACCGCTTCCT	GCTCCATTTGCAGCAGCTC
HES1	AGAAAGATAGCTCGCGGCATT	TACTTCCCCAGCACACTTGG
HPRT	TGTAGCCCTCTGTGTGCTCAAG	CCTGTTGACTGGTCATTACAATAGCT
KLF4	GCGGCAAAACCTACACAAAG	CGTCCCAGTCACAGTGGTAA
LILRA4	ATTCCAAGACTGCCCCACAC	TGCGGATGAGATTCTCCACT
MCL-1	TAAGGACAAAACGGGACTGG	CCAGCTCCTACTCCAGCAAC
NOTCH1	CTGAAGAACGGGGCTAACAA	CAGGTTGTACTCGTCCAGCA
NOXA	GCTGGAAGTCGAGTGTGCTA	CCTGAGCAGAAGAGTTTGGA
PGK	AAGTGAAGCTCGGAAAGCTTCTAT	TGGGAAAAGATGCTTCTGGG

MassARRAY		
	5'-tag forward primer	AGGAAGAGAG
	5'-tag reverse primer	CAGTAATACGACTCACTATAGGGAGAAGGCT
Gene	**Forward (5' to 3')**	**Reverse (5' to 3')**
ADRB2	GAGTTTGTTGATTAAGAATAAGGTT	CAAAAAATAAAAATACCCATAATAATA
ADRM1	TTGGTTTTTGTATATTTGGTTTTTG	TTTTAAAAAAATATAACCACTTTTCATTTTA
BCKDK	GAGTTTGGGGTTTAGAGTGGTAGA	AATCACCACATCCTTATAATCATCC
C19ORF12	TATGGAAAAATGAGTTAGATATGGTTG	TTCCCAAATAAAAAAACTAAAACAC
C21ORF33	TGATGATATTAGGTGTTTTGTGGTT	CTAACCCTCTTCAAACTTCTCAACT
CBX7	GGAAAAGGTTAAAGTTTAGTAGGAAAAAT	ACTCCCAAAACCCCAATAAAAT
CORO1B	TTTTGTTGGTTTTATTGTTGGTTTT	CACCCCTACTAAACCTTTCTACACA
CXCR3	GGTTTATTTGTGGGAAGTTGTATTG	CCCTCCTAAAAAACTTCAACTCTT
CYB5R3	TTTTTTTGTTTTGTTTTTTTGGTAG	CTCATACCTATAATCCCAATACTCC
EPRS	AGTTTAGGAAGATGAGGTTGTAGTG	AAAAAATATAATCTAAAAAACCCTC
EXOSC3	TTTTAAATTTTTAATTTTAAATGATT	ACTTAACAAAAAAATAACAACAAAC
FKBP3	TTTATTAAAATGGGGTAAGATTTT	ATTACCCAAACTAAAATACAATAAC
GRSF1	TTTGGTTAATATGGTGAAATTGTGTT	AAACTCCAATAATCCTCTCACTTCA
HGS	GTGGGAGGGTTTGGATTAGTATT	AACCCATACAATTCTCCACATAAAA
KLF4	GAGAATAAAGTTTAGGTTTAGGAGAT	ATAACAACAAAAAACCACCACTAAC
LILRA4 $_{MA1}$	GTTAGGAGGGAGGGTTTTTTAGATA	ACCATAAAAAATTCCAAACCCTATT
LILRA4 $_{MA2}$	GGGATGTTTTAATTGATTAAGGTATG	CACCTTAACCTCCCAAAATACTAAA
LILRA4 $_{MA3}$	TTTTTTGTGTGTGTGTGAAAAATAG	ACTCCTAACCTCAAATAATCTACCC
MAFB	GTTGTTTTATTTGTTGAATGAGTTG	TAAACATAAAACCAAAACTACCCAC
MBP	TTTATGGTATTTGTTGTGGTTAGGTATT	CTCTACATACTCAAATAACTTCATC
NOTCH1	GTTTAGGGTTTGGAGATTTTTTTT	ATAATACCCCCATTTCTCTAACAAC
PSMB10	TTGGTTTTAAATTTTTGAGTTTAGGT	TAAAATCCCACACTAAATTTCACTC
PTBP1	GGGTTTTAGATGTTTTTATTTTTTTT	CTTTCTTTAAAACCACCTCTCAAC
PTPLAD1	TTGAGGTAGAAGAAAGGTATGAATT	CAACATCTTTATAACCTCAAACTCC
SCYL1	TTGGTTATTTATTTTTGTTTTTTTT	ATATAATCCAAACCCCCAAACTT
SEPX1	AAGGAGAGGTAAATGTGGAGTTATTAGT	CAACAAAAAAAACCACTTAAAAAAAA
TCF25	ATTTATAGGGGTAGGAGGTGTGATT	AATAAAAATACAAACCAAAAAATCC
TRAPPC2L	GTGGTTTTTAGGAGAAGGTGAGAG	AAACCCAAATACAACTCCCTCTAAT
TSPYL5	GAGATTTTGGTATTGTGAAGGGTT	AAATCCCTCAACAAAAATATTCAAA
UFSP2	AGTTTAGAAGAAAGGTTTAATTTAGAGATA	CCTAAAAAAAACTAATCCTACC
VKORC1	GGTGGAATTAGGTTAGGATTGTTAAT	CCAACCTAACCAACATAATAAAAAC
ZFP90	GAAGATGTAAATGTTTTATTTTTTGA	AAAACTACCCAAAAAAACCTCAAC
ZFYVE21	GTTAAAGTAGTATTAATTGATTGGGTTTAT	AAAACCTCTACCTCATCTTAACCAC

Cloning		
Gene	**Forward (5' to 3')**	**Reverse (5' to 3')**
KLF4	TTTAAGCTTATGAGGCAGCCACCTGGCGAGTCT	TTTGCGGCCGCTTAAAAATGCCTCTTCATGTGTAA

Sequencing		
Target	**Forward (5' to 3')**	**Reverse (5' to 3')**
T7seq	TAATACGACTCACTATAGGG	-
BGHseq	-	TAGAAGGCACAGTCGAGG
CMVseq	GTGTACGGTGGGAGGTCTAT	-

MCIp Control		
Gene	**Forward (5' to 3')**	**Reverse (5' to 3')**
SNRPN	TACATCAGGGTGATTGCAGTTCC	TACCGATCACTTCACGTACCTTCG
MCctF1/R3	GGTTCTCTGCTGCCTTGC	CTGAGAAGGGCGAATACGAA
ZAP70	CAGCTGGACAACCCCTACAT	CGACCAGGAACTTGTGCAG

1.9. Plasmids

Name	Insert	Supplier
pDEST26	-	Genomic and Proteomics core facility, DKFZ
pcDNA3.1/V5-His	-	Life Technologies, Darmstadt, Germany
pcDNA3.1-KLF4	*KLF4*	-
pmaxGFP	*GFP*	Lonza, Basel, Switzerland

2. Methods

2.1. Collection and purification of patient and healthy donor samples

Peripheral blood from CLL patients was collected with informed written consent (Ethics Committee, University of Ulm, approval no.96/08). Buffy coats generated from blood of healthy donors were retrieved from the German Red Cross (DRK) in accordance to the declaration of Helsinki. Peripheral blood mononuclear cells were isolated by density centrifugation in Biocoll (Biochrom) separation solution. Blood was diluted 1:2 with PBS and 30 ml centrifuged 20 min at 2200 rpm without brake in a leukosep tube (Greiner Bio-One) containing 15 ml Biocoll. PBMCs were washed two times with 50 ml PBS and viability determined with a ViCell XR cell counter. PBMCs were either directly applied to treatment or CD19+ magnetic selection or frozen in culture medium (see II.2.2) supplemented with 10% DMSO. $CD19^+$ B-cells and CLL cells were isolated via positive magnetic selection using $CD19^+$ Micro Beads (Miltenyi Biotec) as described by the manufacturer. Purity of PBMCs and $CD19^+$ sorted cells was determined by flow cytometry analysis using anti-CD5-FITC, anti-CD20-PE and anti-CD19-APC antibodies (BD Biosciences). Briefly, after washing two times with PBS, up to 1.0×10^6 cells were stained in 100 µl FACS buffer with 5 µl of the respective antibody for 30 min at 4°C. Cells were centrifuged for 5 min with 1200 rpm and washed two times with 1 ml FACS buffer by centrifugation and finally resuspended in 200 µl FACS buffer for measurement on a BD FACSCanto II (BD Bioscience).

2.2. Cell culture conditions of cell lines and primary CLL cells

Cell lines MEC1, MEC2 and GRANTA-519 were cultured in DMEM (Sigma-Aldrich) with 10% FCS (Biochrom) and 1% Penicillin / Streptomycin (Life Technologies) at 37°C and 5% CO_2. The cell lines LCL-FM, LCL-MM, LCL-WEI and JeKo-1 were cultured in RPMI (Sigma-Aldrich) with 20% FCS and 1% Penicillin / Streptomycin (Life Technologies) at 37°C and 5% CO_2. All cell lines were splitet every 2 to 4 days and cultured at a density of 1.0×10^6 to 2.5×10^6 cells/ml. PBMCs from CLL patients were cultured in DMEM (Sigma-Aldrich) with 10% FCS and 20% human type AB serum (MP Biomedicals) at 37°C and 5% CO_2 with 2.0×10^6 to 5.0×10^6 cells/ml.

2.3. Transfection of leukemia cell lines

For transfection of the cell lines MEC1, MEC2 and JeKo-1 an Amaxa Nucleofector 2b device (Lonza) was used for electroporation of the cells. Cell lines were seeded 24h prior nucleofection with a density of 1.5×10^6 cells/ml in 40 ml in T175 flasks. Shortly before transfection, cells were washed one time with PBS and 1.0×10^7 cells resuspended in 100 µl nucleofector solution and 2 µg plasmid added per reaction. Nucleofector solution V (Lonza) and program X-001 were used for electroporation in the supplied cuvettes. After transfection 500 µl of the respective pre-warmed culture medium were added to the cuvettes and all cells transferred to 24-well culture plates containing 1.5 ml pre-warmed medium. Cells were cultured 3h to 48h at an initial cell density of 2.0×10^6 cells/ml at 37°C and 5% CO_2. During this culture period splitting of the cells was not necessary. Culture medium used for each cell line resembled standard medium as described in II.2.2. For each experiment nucleofection with empty vector, without DNA (mock) as well as untreated cells were included as control. Upon harvest, 50 µl (about 1.0×10^6 cells) were

used for apoptosis analysis using Annexin-V / 7AAD flow cytometry (see II.2.6). Remaining cells were washed two times with PBS and for each time point one pellet for RNA and one pellet for protein analysis was frozen and stored on -80°C or directly applied to sample preparation as described in II.2.5.

2.4. GSI-I treatment of cell lines and PBMCs from CLL patients

Cell lines and PBMCs isolated from CLL patient samples were treated for 24h with 2.5 µM and 5 µM GSI-I, respectively. Untreated cells and cells treated with 0.002% DMSO were the controls. Cell lines were seeded 24h prior treatment at a density of 1.5 x 10^6 cells/ml in 40 ml in T175 flasks under standard culture conditions. Treatment was performed in duplicates in 12-well plates with 2 ml of 1.0 x 10^6 cells/ml at 37°C and 5% CO_2. After 24h, 50 µl of the cell solution were used for CellTiter-Glo® Luminsescent Viability assay as described in II.2.6. Rest of the cells was washed two times with PBS and half of the cells applied to RNA the other half to protein analysis as described below. PBMCs of CLL cells were treated at a cell density of 5.0 x 10^6 cells/ml for 24h at 37°C and 5% CO_2. 2ml of the cells were seeded in 24-well plates for the treatment under culture conditions described in II.2.2. In case of frozen PBMCs from CLL patients, cells were thawed rapidly on 37°C and after washing with 10 ml cell culture medium, cultured for 3h to 4h on 37°C prior to treatment, to allow adjustment of the cells. After treatment 150 µl cells were used for apoptosis measurement with Annexin-V / 7AAD flow cytometry as described below. Rest of the cells was washed with PBS two times and applied to protein and RNA analysis (see II.2.5).

2.5. RNA, DNA and protein isolation

For isolation of DNA, RNA and protein, cells were washed two times with PBS. Pellets were either frozen in liquid nitrogen and stored on -80°C or directly lysed in 350 µl buffer RLT (Qiagen) and homogenized using QIAshredder (Qiagen) for RNA or DNA isolation. DNA and RNA was than isolated according to the manufacturer's instructions using either the AllPrep DNA/RNA Mini or the RNeasy Mini and Micro kits (Qiagen). RNA and DNA concentrations were measured on a NanoDrop ND-1000 Spectrophotometer (NanoDrop). For expression array analysis RNA quality was assessed by running a RNA 6000 Nano Chip (Agilent) on an Agilent 2100 Bioanalyzer (Agilent) as described in the manufacturer's protocol.

For protein isolation, freshly harvested cells or frozen pellets were resuspended in 20 µl to 50 µl 1x RIPA by pipetting and incubated on ice for 20 min. Samples were centrifuged with full speed for 5 min and supernatant was used as protein lysate for western blot. Protein quantification was done with 5 µl of each sample using the Pierce BCA assay (Life Technologies) as described in the manufacturer's manual using 1xRIPA as blank and performing measurement on a Mithras LB 940 Plate Reader (Berthold Technologies).

2.6. Determine cell viability

To determine cell number and cell viability of cell lines in everyday cell culture, a ViCell XR cell counter (Beckman Coulter) was used. For transfection and GSI-I treatment experiments, cell viability was determined either with the CellTiter-Glo® Luminescence cell Viability Assay (Promega) or by Annexin-V / 7AAD flow cytometry. For CellTiter-Glo 50 µl of cell solutions together with 200 µl CellTiter-Glo solution were incubated in white

96-well plates, on room temperature, for 20 min, with 200 rpm on an orbital shaker. Luminescence was measured on a Mithras LB 940 Plate Reader (Berthold Technologies) for 0.1 seconds. For Annexin-V / 7AAD flow cytometry up to 1.0 x 10^6 cells were washed two times in PBS and stained in a total volume of 30 µl containing 24 µl 1x Annexin-V staining buffer, 3 µl Annexin-V-FITC and 3 µl 7AAD (BD Bioscience) for 30 min on 4°C. 150 µl Annexin-V staining buffer was added and cells measured on a BD FACSCanto II device (BD Bioscience). Annexin-V-FITC and 7AAD double negative cells were considered as living cell population.

2.7. Western Blot

For western blot analysis cells were lysed with 1xRIPA as described in II.2.5 and 5 µg to 30 µg of protein was denatured with 2.5 µl NuPAGE SDS sample buffer and 1 µl NuPAGE sample Reducing Agent (Life Technologies) per 10 µl protein lysate in a heat block for 10 min at 70°C. Protein was than separated by electrophoresis with SDS PAGE on NuPAGE Novex 4-12% Bis-Tris or 3-8% Tris-Acetate Protein Gels for 45 min to 60 min with 150 V to 200 V in 1xNuPAGE Running Buffer in the XCell SureLock Mini-Cell Electrophoresis System (Life Technologies). Of Spectra Multicolor Broad Range or PageRuler Prestained Protein ladder (Life Technologies) 10 µl were loaded on each gel for size estimation of detected proteins. After separation, proteins were transferred onto a PVDF membrane (Merck Millipore) in a Mini Protein Gel Transfer System (BioRad) by wet blotting procedure in Western Blot buffer. Stepwise increasing current (200 mA 10 min, 300 mA 10 min, 400 mA 10 min, 500 mA 10 min) was used for the transfer. Subsequently, PVDF membranes were blocked in TBS-T with 5% milk powder (Sigma-Aldrich) for 1h at room temperature. For primary detection antibodies, incubations were performed in 3 ml TBS-T with 5% milk in 50 ml falcon tubes at 4°C over night with constant rolling. HRP-coupled secondary antibodies were incubated for 1h at room temperature. Anti-NOTCH1 and anti-KLF4 antibodies were diluted 1:500, anti-GAPDH 1:25,000 for incubation. Secondary HRP-coupled antibodies were diluted 1:3,000. In between and after incubation steps, membranes were washed three times with TBS-T for 5 min with moderate shaking. Chemiluminescent signal detection was performed with ECL (plus) WB Detection Reagents (GE Healthcare) and X-ray films (FUJIFilms) as described in the manufacturer's protocol. Exposure time was individually adjusted.

2.8. Methyl-CpG-immunoprecipitation (MCIp)

MCIp was performed by Angela Garding and is also described in greater detail in her thesis.[319] This method was originally developed in the group of Michael Rehli to detect global hypermethylation in CpG islands. With this approach methylated DNA is precipitated by binding of a recombinant MBD2-Fc fusion protein containing a methyl-binding domain.[320]

For MCIp 2 µg genomic DNA of T-cells or $CD19^+$ B-cells and CLL cells was pushed 10x through 22G needles and further shared with ultrasonication in a Bioruptor (Diagenode, 2x 30 pulses, 24s, 10% amplitude) to obtain fragment sizes of 400 to 500 bp. The shared genomic DNA was mixed in a molar ratio of 1:1 with spike DNA (630 bp artificial *A.thaliana* sequence) which was later used to test for enrichment.

MBD-Fc fusion protein was coupled to SIMAG protein A magnetic beads (Chemicell). To do so 40 µl beads were washed one times with 200 µl TBS. 30 µg MBD-Fc protein was

added to the beads in a total volume of 200 µl TBS and rotated for 3h on 4°C to allow binding. Beads were washed two times with buffer A (low salt) and DNA was added to the beads in a final volume of 250 µl buffer A. After rotation for 3h at 4°C supernatant was taken off and represents eluate A (low salt fraction), which than contained unmethylated DNA that was not bound by the MBD-Fc fusion protein. Beads were washed with buffers B to F each two times with 125 µl to obtain 250 µl eluate per wash buffer. Eluate F (high salt buffer) should contain highly methylated DNA and was later used for array hybridization and analysis. All fractions were desalted using the MinElute Reaction Cleanup Kit (Qiagen) as described in the manual.

Finally, qRT-PCR was performed to control for successful enrichment of methylated DNA. Primers used were for *ZAP70* which is known to be highly methylated in CLL, *SNRPN* an imprinted gene that shows 50% methylation in humans and the artificial 630 bp fragment spike DNA. Enrichment of highly methylated DNA in the high salt fraction and of unmethylated DNA in the low salt fraction could be confirmed for all samples used for array hybridization.

2.9. Expression arrays of CD19$^+$ B-cells and CLL cells

For expression array analysis 0.5 µg RNA of primary samples was checked for sufficient quality on an Agilent Bioanalyzer using the Agilent RNA 6000 Nano Kit as described in the manual of the kit. Expression array analysis was further performed by the Genomics and Proteomics Core Facility of the DKFZ with HumanHT-12v4 Illumina BeadChip Sentrix arrays according to the manufacturer's instructions.

2.10. Promoter tiling-arrays (MCIp-analysis)

For methylation analysis, MCIp samples were hybridized onto custom designed (eARRAY, Agilent) promoter arrays (2527020 G4125A Human custom CGH microarray 4x180K) covering -3.8 to +1.8 kb from the TSS of human RefSeq genes. For array hybridization MCIp samples from CD19$^+$ B-cells and CLL cells were labeled with Cy5, MCIp samples from healthy T-cell pool with Cy3. Labeling was performed with the BioPrime Total Genomic Labeling System (Invitrogen) as described by the manufacturer. Sample hybridization was performed according to protocol number G4170-90012 for Agilent Microarray Analysis of Methylated DNA Immunoprecipitation version 1.0. Read out of arrays was done with an Agilent Technologies Scanner G2505C US81503234.

2.11. Expression arrays of cell lines after *KLF4* overexpression

To analyze effects of KLF4 on its downstream targets, cell lines MEC1, MEC2 and JeKo-1 were transfected with pcDNA3.1-KLF4 as described in II.2.3. Samples were taken 3h and 10h after nucleofection and RNA applied to expression array analysis as described in II.2.9.

2.12. Sanger Sequencing

Sequencing of PCR products and plasmids was performed with the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies). 100 to 500 ng of DNA was used as template and assembled with 1 µl BigDye, 2 µl sequencing primer (1 µM), 2 µl BigDye 5x sequencing buffer and H_2O to a final volume of 10 µl. Cycle sequencing was

performed in a Eppendorf PCR Cycler (Eppendorf) for 25 cycles as follows: initial denaturation at 96°C for 3 min, denaturation at 96°C for 30 sec, primer annealing at 51°C for 15 sec and extension at 60°C for 4 min. PCR products were purified by ethanol precipitation. 1 µl 3 M sodium acetate and 26 µl ethanol (Sigma-Aldrich) were added and centrifuged at 4°C for 30 min at full speed. Precipitated DNA was washed once with 70% ethanol, air dried at room temperature and resuspended in 10 µl formamide (Merck) and sequenced using an ABI PRISM 3100 Genetic Analyzer (Life Technologies).

2.13. cDNA synthesis

For the production of cDNA the QuantiTect Reverse Transcription Kit (Qiagen) was used. 0.5 to 1 µg of RNA was used for cDNA synthesis per reaction as described in the manufacturer's manual. For qRT-PCR analysis cDNA was diluted 1 to 4 with H_2O and 2 µl per reaction used as template.

2.14. Quantitative real time PCR (qRT-PCR)

qRT-PCR was used to measure gene expression in cell line samples, primary samples from CLL patients and normal B-cells. In addition, it was used for quantification of control genes and spike DNA in MCIp samples. cDNA was diluted 1 to 4, MCIp samples 1 to 10 and 2 µl template were used per reaction together with 6 µl ABsolute SYBR Green ROX Mix (Thermo Scientific), 0.12 µl of each forward and reverse primer (10 µM) and 3.76 µl H_2O. Samples were amplified on an ABI PRISM 7900HT Fast RealTime PCR System (Life Technologies) for 40 cycles as follows: protein activation and initial denaturation for 2 min at 50°C, denaturation for 15 sec at 95°C and primer binding and elongation for 1 min at 60°C. A dissociation curve was measured in the range of 60°C to 95°C. Standard curves were template dilutions of cDNA generated from Universal Human Reference RNA (Agilent Technologies) and allowed to determine PCR efficiency and exact quantification of template. Results were normalized to house keeper genes *HPRT* and *PGK*.

2.15. MassARRAY

MassARRAY allows the quantification of DNA methylation of DNA fragments ranging from 250 to 750 bp using mass spectrometry.[321] For this method DNA is treated with bisulfite which results in the conversion of unmethylated cytosine to uracil, while methylated cytosine is protected from the reaction (Figure 10). The converted DNA is used as template for PCR amplification of the region of interest. In the PCR amplification, a T7 promoter-tagged reverse primer is incorporated into the product which is necessary for *in vitro* transcription in the next step. The forward primer contains a 10 bp long sequence tag which balances PCR primer length. After *in vitro* transcription, products are applied to U-specific cleavage resulting in a mix of smaller fragments which includes also fragments with one or more CpGs which are referred to as CpG units (CGUs). After purification all fragments are applied to MALDI-TOF MS analysis. Each CpG can result in a mass shift of 16 kD of methylated in comparison to unmethylated DNA as the fragment either contains a guanine or an adenine.

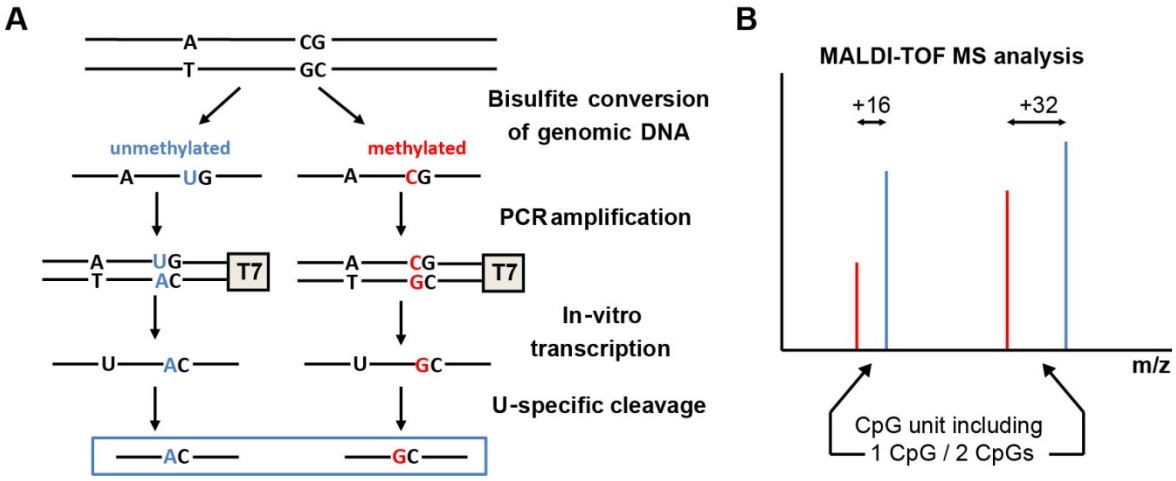

Figure 10. MassARRAY methylation analysis. *(A) Bisulfite conversion, PCR amplification, in vitro transcription and U-specific cleavage results in the preparation of small DNA fragments which vary in their size depending on the original DNA methylation status. (B) Mass spectrometry analysis is used for quantification of unmethylated and methylated CpG units.*

In this project, for MassARRAY analysis 0.5 to 1 µg of genomic DNA was bisulfite converted using either the EpiTectFast Bisulfite or the EpiTect Bisulfite conversion Kit (Qiagen) as described in the manual. DNA was eluted with buffer EB or DNase free water and 1-2 µl used for PCR amplification.

10 µl PCR reactions were assembled with 0.1 µl HotStarTaq DNA Polymerase, 1 µl 10xPCR buffer, 0.2 µl 25 mM $MgCl_2$ (Qiagen), 1 µl 5 mM TMAC (Sigma-Aldrich), 0.8 µl 2.5 µM dNTP mix (Roche) as well as 0.5 µl forward and reverse primer (10 µM). H_2O was added to obtain a final volume of 10 µl. Samples were amplified on an Eppendorf PCR Cycler (Eppendorf) for 40 cycles as follows: initial denaturation and protein activation for 15 min at 95°C, denaturation at 95°C for 60 sec, primer annealing at 60°C for 30 sec, elongation at 72°C for 1 min and final elongation at 72°C for 5 min. 5 µl of the PCR products were analyzed on 1.5% agarose gels.

Subsequently, PCR products were processed with the reagents from the MassCLEAVE kit (Sequenom) as described by the manufacturer. To dephosphorylate any remaining, unincorporated dNTPs, PCR products were treated with shrimp alkaline phosphatase (SAP). A mix of 1.7 µl H_2O and 0.3 µl SAP was added to 5 µl PCR product and incubated for 20 to 40 min at 37°C. Reactions were heat inactivated for 5 min at 85°C and stored on 4°C or -20°C. For *in vitro* transcription and subsequent cleavage a T cleavage transcription RNase A cocktail was assembled which was added to 2 µl of the SAP reaction. Per reaction the cocktail contained 3.21 µl H_2O, 0.89 µl 5x T7 polymerase buffer, 0.22 µl T Cleavage Mix, 0.22 µl DTT (100 mM), 0.4 µl T7 RNA&DNA Polymerase and 0.06 µl RNase A. Samples were incubated for 3h at 37°C and after addition of 20 µl H_2O and 6 mg Clean Resin rotated on room temperature for 15 min. After centrifugation for 5 min at 4000 rpm, samples were spotted onto Spectro Chips II with the MassARRAY Nanodispenser (Sequenom) and analyzed with the MassARRAY Analyzer 4 System (Sequenom).

As controls 0% and 100% methylated DNA were processed together with the patient samples. For the 100% methylated control, CpG Methylated Human Genomic DNA (Thermo Scientific) was purchased or highly methylated DNA was generated *in vitro*. To do so 2.5 µg cell line DNA was treated with 0.5 µl CpG Methyltransferase (M.SSsI, NEB) in a 20 µl reaction including 2 µl 10x NEB buffer 2 and 0.25 µl 200x SAM at 37°C over

night. 2h after start of incubation 0.25 µl 200x SAM were added and 4h after start of incubation 0.25 µl 200x SAM and 0.35 µl M.SssI were added. For the 0% methylated control, 10 ng cell line DNA was applied to whole genome amplification using the REPLI-g Mini Kit (Qiagen) as described in the manual. 0% and 100% controls were purified using the QIAquick PCR purification kit (Qiagen) and 1 µg each applied to the MassARRAY protocol as described above.

2.16. Cloning of pcDNA3.1-KLF4

For cloning of pcDNA3.1-KLF4, *KLF4* cDNA was amplified from whole transcriptome cDNA synthesized from MEC1 RNA as described in II.2.13. For PCR amplification 10 ng cDNA were used in 50 µl reactions containing 10 µl 5x HiFi Buffer, 0.5 µl 25 mM dNTP mix (Roche), 2 µl forward and reverse primer (10 µM), 1 µl PRECISOR High-Fidelity DNA Polymerase (Biocat) and H_2O. Amplification was performed for 30 cycles on a Eppendorf PCR Cycler (Eppendorf) including the following steps: initial denaturation for 2 min at 98°C, denaturation for 30 sec at 98°C, primer annealing at 55°C for 30 sec, elongation for 2 min at 72°C and final extension at 72°C for 7 min. Primers used in this reaction contained tags with NotI and HindIII restriction sites. The PCR product was analyzed on a 1% agarose gel and purified using QIAquick PCR purification kit (Qiagen). Subsequently, 2 µg PCR product as well as 2 µg of the original plasmid (pcDNA3.1/V5-His, Life Technologies) were digested with NotI and HindIII FastDigest restriction enzymes for 1h at 37°C in 20 µl reactions containing 1 µl of each enzyme, 2 µl 10x FastDigest Buffer (Life Technologies) and H_2O. Digestion products were separated on a 1.5% agarose gel and digested PCR fragment and empty vector backbone isolated with the QIAquick Gel extraction Kit (Qiagen). Of the PCR product and plasmid each 5 µl were assembled together with 8 µl H_2O, 1 µl T4 DNA Ligase and 1 µl T4 DNA Ligase buffer (Life Technologies) and incubated for 5 min at room temperature. Of the ligation reaction 5 µl were used for the transformation of chemical competent OneShot Top10 *E.coli* (Life technologies) as described by the manufacturer and plated on LB-agar plates containing 100 µg/ml ampicillin (Roche). After incubation over night at 37°C, colonies were used for inoculation of 5 ml LB-medium containing 100 µg/ml ampicillin (Roche) and again incubated over night at 37°C. Plasmids of these cultures were isolated and subsequent sanger sequencing used to check for plasmids with the right insert.

Plasmid separation from *E.coli* was done with Plasmid Mini Kit and EndoFree Plasmid Maxi Kit (Qiagen) as described in the manual. Plasmids were applied to sanger sequencing to control insert identity and sequence.

2.17. Statistical analysis

Expression and MCIp-arrays performed with material from $CD19^+$ B-cells and CLL cells were analyzed by Manuela Zucknick, Natalia Becker und Clemens Phillipen. Array data were quantile normalized and analyzed with the limma package.[322] For expression arrays genes with a $|\log_2 FC| \geq 1$ and adjusted p-value ≤ 0.05 were considered as differentially expressed. For Agilent MCIp-methylation arrays 500 bp windows containing at least two oligonucleotides were tested for differential methylation between CLL and healthy. Differential methylated regions (DMR) with a median $|\log_2 FC \geq 0.5$ (of all CpG containing oligonucleotides within the window) and $p \leq 0.05$ were considered as differentially methylated. Overlapping 500 bp windows were merged to obtain maximal DMRs. Analysis

of correlation between methylation and expression was done by Clemens Phillipen. For all differential expressed genes that had respective hyper- or hypomethylated DMRs, expression values for each available oligonucleotide on the array per gene were tested against each oligonucleotide of the DMRs for significant correlation using Pearson rank statistic. Genes with a correlation coefficient $r \leq -0.47$ and $p \leq 0.021$ were considered to show significant negative correlation. Expression arrays performed of cell lines after *KLF4* overexpression were quantile normalized and analyzed using QIAGEN's Ingenuity Pathway Analysis (IPA, Qiagen).

Furthermore, Students paired t-test and Mann-Whitney rank sum test were used to test for significant difference between two groups as indicated for the single experiments. p-values ≤ 0.05 were considered significant. Correlations were calculated with Pearson or Spearman rank correlation.

3. Patient and healthy donor sample characteristics

3.1. CD19$^+$ CLL cells and B-cells used for array analysis

	Gender	Karyotype	IGHV	Age	MCIp	Expression	CD5$^+$/CD20$^+$ (%)
CLL1	male	normal	unmut	59	yes	yes	99.9
CLL2	male	normal	unmut	64	no	yes	99.8
CLL3	male	normal	mut	66	yes	yes	99.4
CLL4	female	normal	unmut	62	yes	yes	99.8
CLL5	male	normal	mut	61	yes	yes	99.8
CLL6	male	normal	mut	59	yes	yes	99.8
CLL7	female	normal	mut	65	yes	yes	99.7
CLL8	male	13q	mut	67	yes	yes	100.0
CLL9	male	13q	unmut	68	yes	yes	99.9
CLL10	male	13q	unmut	70	yes	yes	99.8
CLL11	male	13q	mut	70	yes	yes	99.6
CLL12	male	13q	unmut	51	no	yes	99.7
CLL13	female	13q	mut	74	yes	yes	99.6
CLL14	female	13q	mut	76	yes	yes	99.5
CLL15	male	13q	mut	50	yes	yes	92.9
H1	NA			NA	yes	yes	94.3
H2	NA			NA	no	yes	97.8
H3	NA			NA	yes	yes	97.8
H4	NA			NA	yes	yes	93.8
H5	NA			NA	no	yes	NA
H6	NA			NA	no	yes	99
H7	NA			NA	yes	yes	96
H8	male			60	yes	yes	96
H9	female			57	yes	yes	95

3.2. CD19$^+$ CLL cells and B-cells for validation of methylation and expression with MassARRAY and qRT-PCR

		CLL (n=56)	B-cells (n=23)
Gender	female	n=18	n=7
	male	n=38	n=15
	NA	-	n=1
Karyotype	normal	n=20	-
	13q14 deletion	n=36	-
Treatment	untreated	n=44	-
	treatment >12 months before sampling point	n=12	-
IGHV status	mutated	n=30	-
	Unmutated	n=25	-
	NA	n=1	-
Median age		62	56

3.3. PBMCs used for GSI-I treatment

Frozen material	n=8	
$CD5^+/CD19^+$ cells	91.6-98.8%	
Gender	male	n=1
	female	n=7
Karyotype	13q deletion	n=7
	13q, 14q deletion	n=1
IGHV status	mutated	n=4
	unmutated	n=4
Age	45-78	
Treatment	untreated	n=8

Fresh material	n=4	
$CD5^+/CD19^+$	47.4-68.8%	
Gender	male	n=2
	female	n=2
Karyotype	13q deletion	n=3
	13q, 11q deletion	n=1
IGHV status	mutated	n=3
	unmutated	n=1
Age	26-76	
Treatment	untreated	n=4

3.4. CLL1 clinical trial

Prognostic factors and risk-adapted therapy in patients with early stage chronic lymphocytic leukemia[323]	
Design	Prospective, open-label, multicenter, risk stratified phase III trial
Objectives	The CLL1-protocol uses a risk stratification strategy to identify a high risk (HR) and low risk (LR) group of early CLL patients. Patients are assigned to the HR arm by using the following definition: Elevated serum thymidine kinase (TK) >7,0 U/l or b2-microglobulin (b2-M) AND non-nodular bone marrow infiltration or short lymphocyte doubling time. Afterwards the HR-patients are randomized to Fludarabine-therapy or "watch & wait
Primary Endpoint	Progression-free survival
Secondary Endpoint(s)	Overall survival, quality of life, incidence of infections
Study Population	B-CLL, Binet-Stage A, No pretreatment, Age ≥ 18 to ≤ 75 years
Treatment	**F Arm**: Fludarabine (25 mg/m2/d, d1-5) q28d; max 6 cycles **Watch & Wait Arm:** watch & wait
Patients recruited	876 patients (198 pts. With high risk of progression)

3.5. CLL2H trial

Subcutaneous Campath-1H (Alemtuzumab; MabCampath®) in Fludarabine-refractory CLL[324]	
Design	Prospective, open-label, multicenter, phase II trial
Objectives	Has the subcutaneous administration of Alemtuzumab similar efficacy compared to intravenous administration, without the first-dose infusion-related side effects? Can subcutaneous Alemtuzumab elicit responses in patients with high-risk genetic abnormalities (unmutated IgV H, del [17p], del [11q]) who have progressed on Fludarabine therapy?
Study Population	B-CLL, Binet-Stage C or A/B with need for treatment, F-refractory max 5 prior chemotherapies Age ≥ 18 years
Treatment	**Dose escalation**: Alemtuzumab s.c. 3, 10, 30mg daily for 1-2 weeks + Pegfilgrastim (Neulasta®) s.c. 6 mg forthrightly **Therapy**: Alemtuzumab s.c. 3x 30mg/week (d1+3+5), max 12 weeks --> SD or PD (optional): Alemtuzumab s.c. (max 12 weeks) + F (25 mg/m2) + C (200 mg/m^2), d1+3, q28d, max 3 cycles
Patients recruited	109 patients

3.6. CLL4 trial

Fludarabine versus Fludarabine plus cyclophosphamide in first line therapy of younger patients (up to 65 years) with advanced chronic lymphocytic leukemia (CLL)[325]	
Design	Prospective, open-label, multicenter, phase III trial
Primary Endpoint(s)	Rate of remission, duration of remission, progression-free survival (PFS), overall survival
Secondary Endpoint(s)	Incidence of side effects, quality of life
Study Population	B-CLL, Binet-Stage C or B/A with need for treatment No pretreatment Age ≥ 18 to ≤ 65 years
Treatment	**Arm F:** Fludarabine (25 mg/m2/d, d1-5); q28d; max 6 cycles **Arm FC:** Fludarabine (25 mg/m2/d, d1-3) +Cyclophosphamide (250 mg/m2/d, d1-3); q28d; max 6 cycles
Patients recruited	375 patients (F-Arm: 189, FC-Arm: 186)

3.7. CLL8 trial

Phase III trial of combined immunochemotherapy with Fludarabine, Cyclophosphamide and Rituximab (FCR) versus chemotherapy with Fludarabine and Cyclophosphamide (FC) alone in patients with previously untreated chronic lymphocytic leukaemia[326]	
Design	Prospective, open-label, multicenter, 2-arm, randomized phase III trial
Primary Endpoint(s)	Progression-free survival (PFS)
Secondary Endpoint(s)	Event-free survival (EFS), overall survival, disease-free survival, duration of remission, time to new CLL or death, rates of molecular, complete and partial remission, response rates and survival times in biological subgroups, rates of treatment-related adverse effects, pharmacoeconomic

	impact, quality of life
Study Population	B-CLL, Binet-Stage C or B with need for treatment No pretreatment Age ≥ 18 years
Treatment	**Arm FCR immunochemotherapy:** Rituximab (Cycle 1: 375 mg/m2, d0;Cycles 2-6: 500 mg/m2, d1) +Fludarabine (25 mg/m2/d, d1-3) +Cyclophosphamide (250 mg/m2/d, d1-3); q28d; max 6 cycles **Arm FC chemotherapy:** Fludarabine (25 mg/m2/d, d1-3) +Cyclophosphamide (250 mg/m2/d, d1-3); q28d; max 6 cycles
Patients recruited	817 patients (FCR: 408, FC: 409)

III. Results

1. Methylation- and expression array analysis reveals genes potentially deregulated by DNA methylation in CLL

CLL is a disease of the epigenome known to show genome-wide hypomethylation and region specific hypermethylation that is able to influence gene expression. To find genes potentially deregulated by DNA methylation in CLL which might impact on disease pathogenesis, MCIp- and expression array analysis was performed.

RNA and DNA of CD19$^+$ CLL cells of 15 patients and B-cells from nine healthy donors were isolated and RNA applied to expression profiling on HumanHT-12v4 Illumina BeadChip Sentrix arrays. DNA was used for methyl-CpG-immunoprecipitation (MCIp) and the high salt fraction, containing highly methylated DNA, hybridized and analyzed on custom designed (eARRAY, Agilent) promoter arrays (2527020 G4125A Human custom CGH microarray 4x180K) covering -3.8 to +1.8 kb from the TSS of all human RefSeq genes. All CLL patients showed low risk disease with seven patients displaying normal karyotype and eight patients harboring 13q14 deletion at time of sampling. Patients had either unmutated (n=6) or mutated (n=9) *IGHV* status and a median age of 65 (see II.3.1). None of the patients had been treated prior to this study. For normalization of the two color arrays, MCIp samples generated from DNA of a pool of healthy T-cells was used as control. Two of the patient samples and three of the healthy donor samples could not be applied to methylation analysis due to loss of material during MCIp preparation and labeling for array hybridization, respectively.

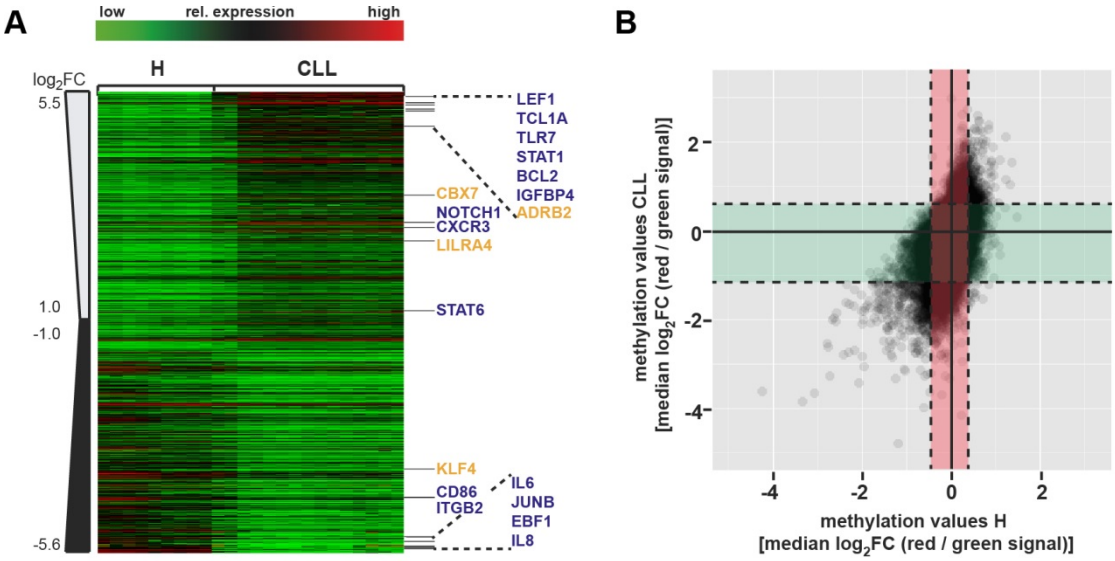

Figure 11. Expression- and MCIp array analysis of CD19$^+$ CLL and B-cells. *(A) Top genes differentially expressed between CLL and healthy donor B-cells ("H"). Genes known to play a role in CLL pathogenesis from previous studies are marked in blue. New potential candidate gens marked in yellow. (B) Median MCIp array methylation values display a broader heterogeneity in CLL samples (y-axis) in comparison to healthy donor samples ("H", x-axis). Lines represent the median, dashed lines represent 1st and 99th percentile. Sample preparation and MCIp arrays were performed by Angela Garding. Data analysis was done by Manuela Zucknick and Natalia Becker.*

Analysis of the gene expression data revealed 1,866 genes to be differentially expressed between CLL cells and normal B-cells with $|\log_2 FC| \geq 1$ and adjusted p-value ≤ 0.05

(Figure 11A). Among these were a number of genes known to be deregulated in CLL or important for CLL pathogenesis from previous studies. These included the oncogene *TCL1* which can, like its interaction partner *JUNB,* induce CLL-like disease in mice.[327,328] In addition, the oncogene *BCL-2* was previously shown to be up-regulated by DNA hypomethylation in CLL and is important for prevention of apoptosis in the cancer cells.[264,329] Similarly up-regulated in this data set were *NOTCH1*, which is known to be deregulated and mutated in CLL, as well as *STAT1* and *STAT6, LEF-1*, *TLR7* and *IGFBP4*.[330–333] *STAT1* is a promising treatment target in CLL as JAK kinase inhibitors and Fludarabine were shown to induce apoptosis in CLL cells via the blockage of STAT1 signaling. *LEF-1* presumably acts as pro-survival factor in CLL and in addition, the high expression of *LEF-1* also present in the preleukemic state of CLL, monoclonal B-cell lymphocytosis, suggests a role for this gene in early CLL leukemogenesis.[334] Genes that are down-regulated in CLL include the NFκB target molecules *IL6* and *IL8*, which are higher expressed upon BCR stimulation in *ZAP70* expressing CLL cells, as well as *EBF1*, *CD86* and *ITGB2*.[229,234,335,336]

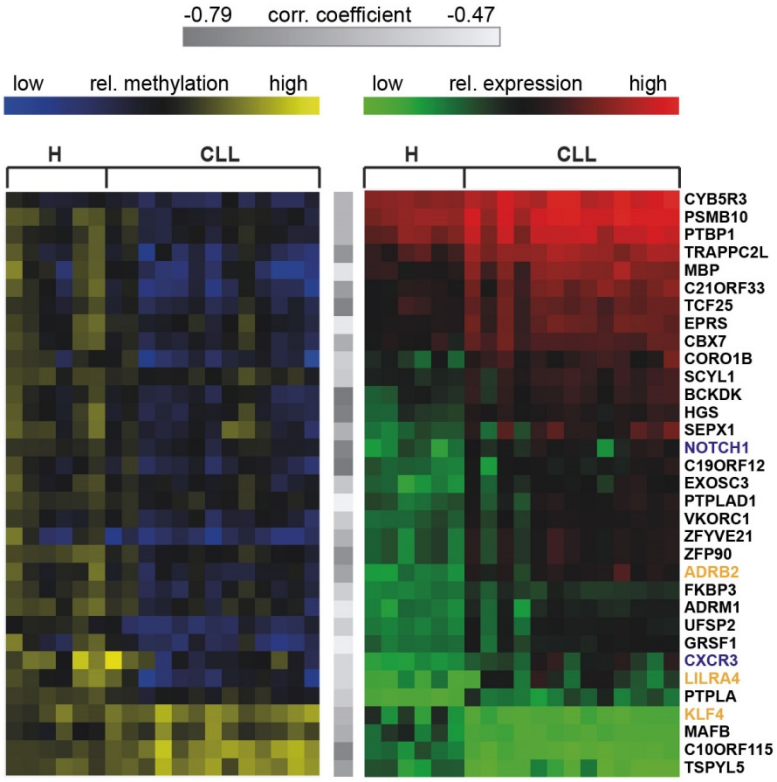

Figure 12. Genes potentially deregulated by DNA methylation in CLL. *Methylation values of MCIp arrays for CLL and healthy donor samples (H) are displayed in blue and yellow on the left, expression values of same samples in green and red on the right. Pearson rank correlation coefficient is indicated in grey. Correlation analysis was done by Clemens Phillippen.*

In addition to quantile normalization, MCIp array data of CLL and normal B-cells were normalized to signals of the T-cell pool control resulting in median $\log_2$FC(red/green signal) values (Figure 12B). Of note, CLL samples displayed a greater heterogeneity in their methylation values than normal B-cells as demonstrated by the range of methylation values within the 1st and 99th percentile of the two groups (1st to 99th percentile of B-cells = -0.46 / 0.37, of CLL = -1.13 / 0.62). To define differentially methylated regions (DMRs) in CLL, all promoter regions represented on the tiling-arrays were screened for 500 bp windows that contained at least two oligonucleotides with differential methylation

between cancer and normal. With this approach, 2,191 genes were identified that contained at least one aberrantly methylated window. 1,472 of these DMRs were hypomethylated in CLL and 719 hypermethylated with a median $|\log_2 FC| \geq 0.5$ and $p \leq 0.05$. Out of all genes displaying aberrant expression and concurrent methylation, 33 were identified to be potentially transcriptionally deregulated by DNA methylation in CLL with at least one oligonucleotide within the DMR showing a significant negative correlation with gene expression (Pearson correlation coefficient of $-0.79 \leq r \leq -0.47$ and $p \leq 0.021$, Figure 12).

2. Aberrant methylation and expression of *KLF4* and *LILRA4* was confirmed in a large patient subset

To further validate aberrant DNA methylation, MassARRAY methylation profiling was performed. Specific regions are amplified from bisulfite converted DNA and applied to enzymatic digest resulting in fragments of different size which contain at least one CpG and are referred to as CpG units (CGU). CGUs are analyzed with mass spectrometry and absolute methylation values for each CGU can be obtained. MassArray analysis of regions covering or lying within the DMRs of the respective 33 genes (Figure 12) was performed in the same samples used for MCIp analysis and could confirm aberrant DNA methylation in at least one CGU of 17 of these genes (Figure 13A).

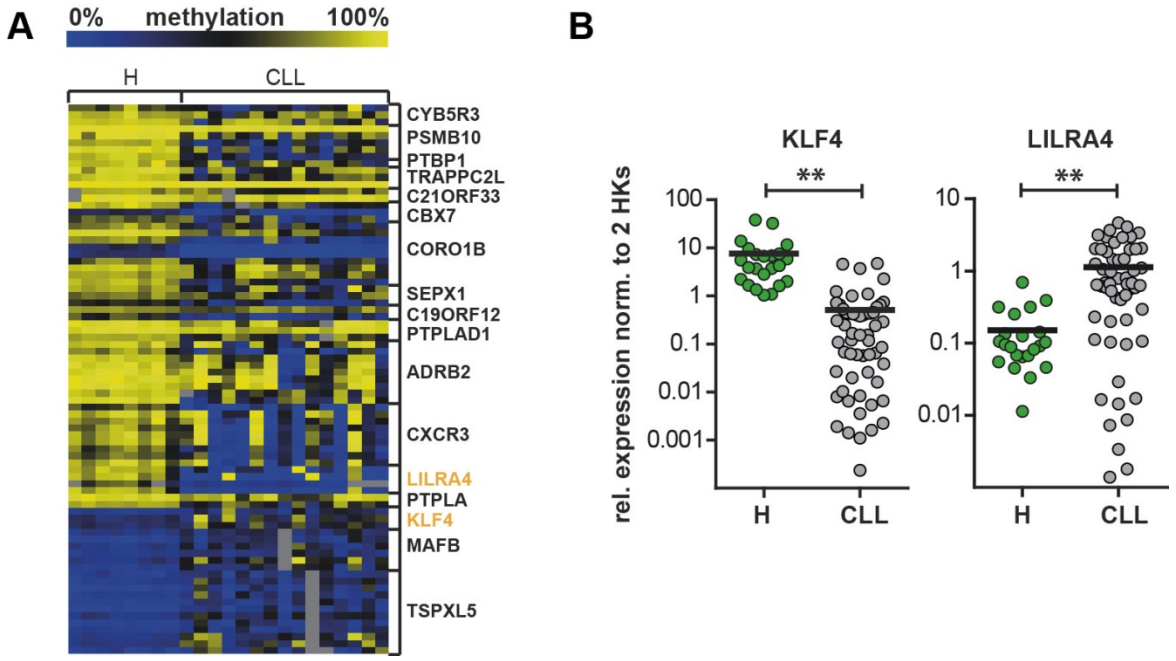

Figure 13. Technical validation of methylation- and expression-array data. *(A) Technical validation of methylation with MassARRAY. 17 out of 33 genes showed at least one significantly differentially methylated CGU. Methylation values from MassARRAY quantification for all significant CGUs ($p \leq 0.05$) between CLL and healthy are shown. MassARRAY analysis was performed together with Angela Garding. (B) Validation of expression for KLF4 and LILRA4 with qRT-PCR in a bigger sample subset; p-values are indicated by asterisk: **≤0.01; Mann-Whitney U test was used to test for significance; lines indicate mean;*

Among these 17 genes were the leukocyte immunoglobulin-like receptor subfamily A member 4 (*LILRA4*, *ILT7*) and the krüppel-like factor 4 (*KLF4*) which are both potentially important for activation, maturation and signaling of B-cells and could hence play a role in CLL pathogenesis.

LILRA4 is a surface receptor so far only shown to be present on plasmacytoid dendritic cells (pDCs), where it is able to activate ITAM (immunoreceptor tyrosin-based activation motif) mediated signaling. The interaction of LILRA4 with its ligand BST2 (CD317) results in the activation of NFAT signaling, phosphorylation of Src family kinases and SYK and the inhibition of the production of IFN-α and TFN-α by pDCs.[337–339] Of note, if present on CLL cells, LILRA4 could interact with the ITAM-containing BCR and hence activate BCR-mediated signaling. Interestingly, a SNP detected in *LILRA4* shows significant genotypic association with progression free survival in CLL and a CLL cell line generated by *in vitro* EBV infection also showed higher expression of *LILRA4* in comparison to lymphoblastoid cell lines generated from normal B-cells.[340,341]

Accordingly, as already described in the introduction (I.4.5), *KLF4* is known to be important for B-cell development and differentiation and was shown to act as tumor suppressor in other cancers and B-cell malignancies. Besides that, the expression of *KLF4* increases during B-cell maturation and is decreased upon B-cell activation suggesting a role of *KLF4* in maintaining the activation status of B-cells.[310,342]

To further validate the aberrant expression of *LILRA4* and *KLF4*, mRNA levels were measured with qRT-PCR in $CD19^+$ cells of a larger sample cohort of 56 CLL patients and 23 age-matched healthy donor samples. Similar to patient samples used for initial array analysis, all patients showed a low risk karyotype with either 13q14 deletion (n=36) or normal chromosomal pattern. The median age of patients was 62, of healthy donors 56. All patients were untreated before samples were obtained for this study (n=44) or had received treatment earlier than 12 months before sampling point. Importantly, expression levels of *KLF4* and *LILRA4* as measured by qRT-PCR confirmed overexpression of *LILRA4* and transcriptional down-regulation of *KLF4* in CLL (Figure 13B).

DNA methylation was quantified in all of the depicted samples using MassARRAY. For *LILRA4* and *KLF4* each, one amplicon (MA1) was analyzed lying within or overlapping the DMRs identified by MCIp array analysis (Figure 14A). Two additional amplicons (MA2 and MA3) within the *LILRA4* promoter region were analyzed, which covered CpGs recently shown to be hypermethylated in CLL by 450K Illumina array analysis from Kulis and colleagues.[271] Five out of ten CGUs analyzed within the *KLF4* amplicon revealed significant hypermethylation in CLL (Figure 14B). The mean methylation values over all CGUs further correlated significantly with gene expression. *KLF4* is therefore likely regulated by DNA methylation (Figure 14C). As mentioned before, *KLF4* was shown to be silenced by hypermethylation in a number of cancers, however in these studies aberrant methylation was detected up-stream of the TSS. Interestingly, no aberrant methylation in this region was detected according to the MCIp array data presented here. Moreover, in a recent DNA methylation screen in a large subset of CLL patients performed by Kulis *et al.* no differential methylation of *KLF4* was detected, presumably as the respective region within the *KLF4* gene body is not covered by the 450K arrays used in this study. For *LILRA4* all CGUs measured in all three MassARRAY amplicons showed hypomethylation of CLL cells in comparison to normal B-cells (Figure 14D). Similar to *KLF4*, correlation of mean methylation values of all CGUs per amplicon with expression, confirmed regulation of the transcriptional activity of *LILRA4* by DNA methylation in CLL. In addition, analogous results obtained for all three MA amplicons indicate a substantial hypomethylation of the whole *LILRA4* promoter region.

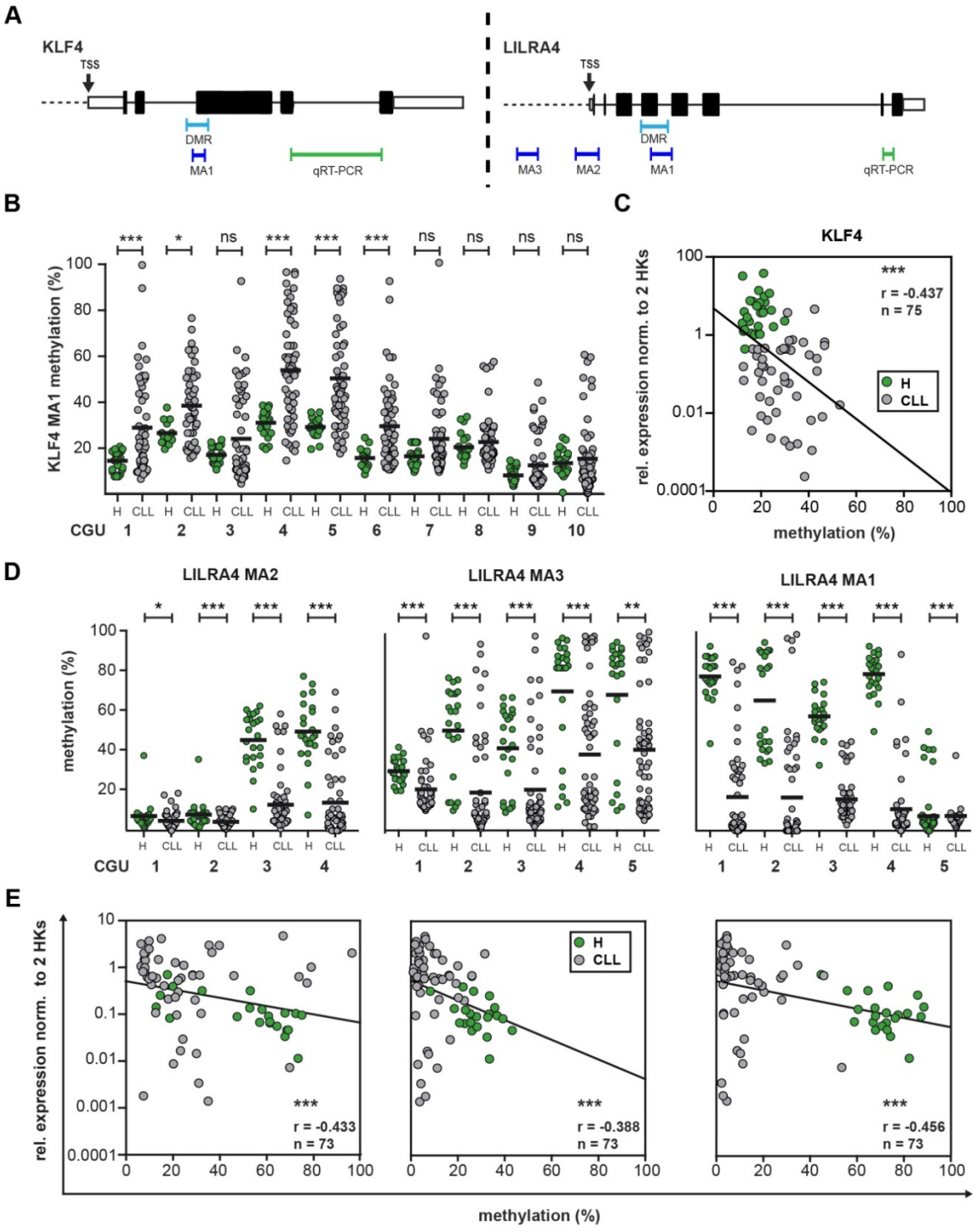

Figure 14. LILRA4 and KLF4 are deregulated by DNA methylation in CLL. *(A) Schematic illustration of KLF4 (left) and LILRA4 (right). Positions of DMRs identified by MCIp array as well as MassARRAY (blue)- and qRT-PCR amplicons (green) are depicted. Black boxes display exons. (B) Results of 10 CGUs measured with MassARRAY in the KLF4 promoter are shown and confirm hypermethylation within this region. (C) Correlation of KLF4 expression and mean promoter methylation was detected. (D) MassARRAY results from three different amplicons within the LILRA4 promoter are shown. MA1 lies within the DMR identified by MCIp array. MA2 and MA3 represent DMRs identified by Kulis et al.*[271] *(E) All three MAs show significant correlation of mean methylation values of all CGUs with LILRA4 expression. p-values are indicated by asterisk: * ≤0.05, **≤0.01, ***≤0.001, ns non-significant; lines represent mean; Mann-Whitney U test and Spearman rank correlation were used to test for significance.*

3. DNA methylation of *KLF4* and *LILRA4* promoter regions show no differences in initial analysis of clinical trial cohorts

DNA methylation in CLL can not only be used to classify CLL cells on a genome wide level and compare them to their non-malignant counter parts, but single DMRs can also help to improve risk stratification like it has been shown for methylation of the *ZAP70* promoter.[261,271] To test if DNA methylation of *LILRA4* and *KLF4* promoter not only impacts on gene expression but also has prognostic impact, MassARRAY was applied to subsets of patient samples from four different clinical trials conducted by the German CLL study group (GCLLSG), namely CLL1 (n=38), CLL2H (n=25), CLL4 (n=34) and CLL8 (n=94).

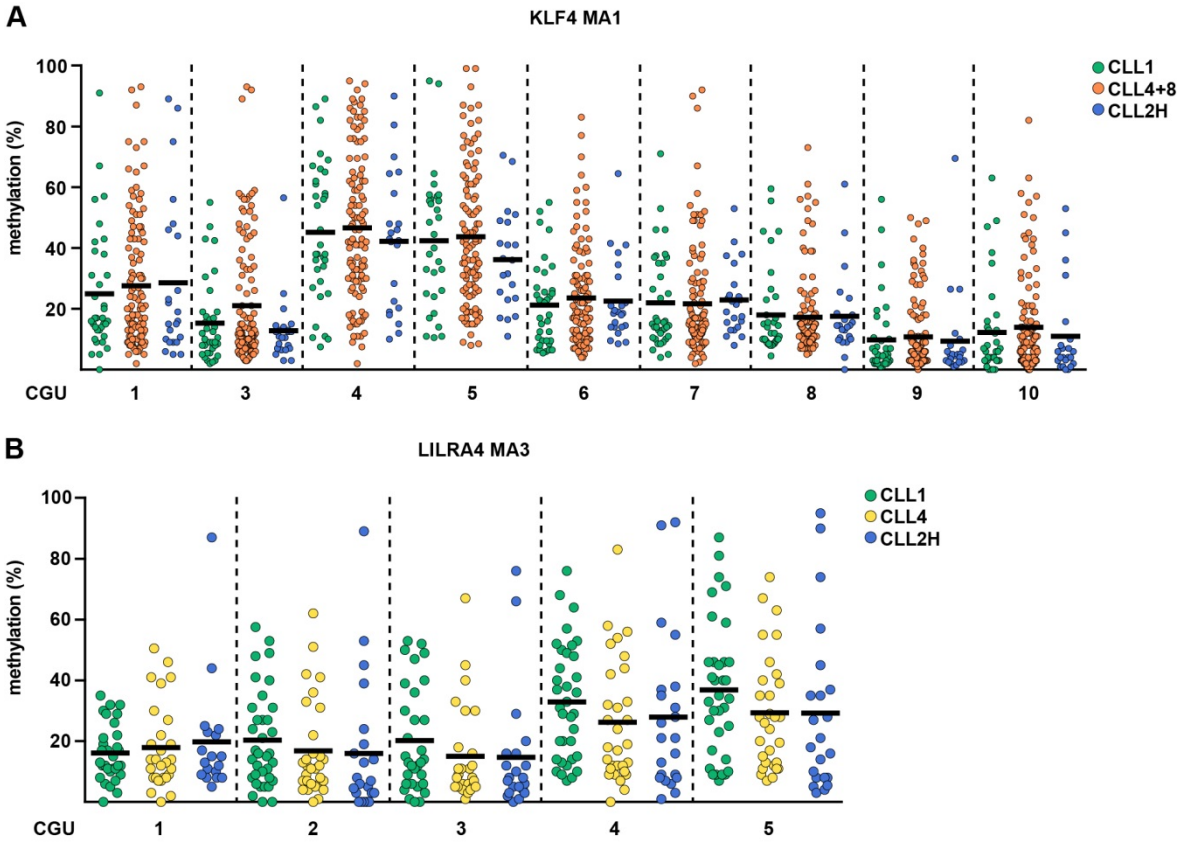

Figure 15. MassARRAY analysis of KLF4 and LILRA4 promoters in clinical trial cohorts. *Methylation in the KLF4 promoter (MA1) (A) and in the LILRA4 promoter (MA3) (B were measured with MassARRAY in samples from four different clinical trials: CLL1, CLL2H, CLL4 and CLL8 (only for KLF4). No differences in DNA methylation could be detected in either of the regions. Mann-Whitney U test was used to test for significance; lines indicate mean values.*

Due to limited amount of material, only *KLF4* amplicon MA1 could be measured in all four trial cohorts in almost all samples available. MA3 within the *LILRA4* promoter region was only analyzed within CLL1, -2H and -4 trial samples (Figure 15). No differences in previously untreated patients with median to strong disease stage (Binet stage B and C; CLL4 and CLL8) and patients with low disease stage (Binet stage A; CLL1) or Fludarabine refractory patients (CLL2H) could be detected in DNA methylation in these regions. However, at time of this work, follow up data of the patients were not available yet, and impact on prognostic factors like progression free or overall survival still has to be determined.

Of note, the methylation patterns for all clinical trials in the two amplicons reflect the pattern which was detected in the patient subset used for validation (Figure 14). For *KLF4* CGUs 4 and 5 showed the highest mean methylation ranging around 40% and CGUs 9 and 10 the lowest mean methylation between 10% and 15% in the patient samples. For *LILRA4* all CGUs showed mean methylation values below 20%, except CGUs 4 and 5 where the mean methylation ranged between 20% to 40% for all trial cohorts.

4. KLF4 does not induce BCL-2 family-mediated apoptosis in leukemia cell lines

Due to its involvement in B-cell development and maturation, the role of *KLF4* in the pathomechanism of CLL was investigated. In NHL and cHL *KLF4* was shown to act as tumor suppressor where promoter hypermethylation is associated with *KLF4* silencing. Here, as well as in CML cells and in advanced epithelial ovarian cancer, it could be shown that KLF4 can induce apoptosis via up-regulation of *BAK1* and down-regulation of the *BCL-2*/*BAX* ratio.[313,317,343] As described in the introduction, *BAX* and *BAK* are pro-apoptotic members of the BCL-2 family. They can form complexes in the mitochondria membrane which leads to the release of Cytochrome c and pro-apoptotic factors in the cells resulting in cell death. This effect is counteracted by BCL-2 which can bind to both proteins and block their complex formation. It is hence possible that the down-regulation of *KLF4* in CLL cells results in the abrogation of *BCL-2* inhibition as well as *BAX* and *BAK* activation. These could be involved in the mechanisms mediating the apoptotic defect of CLL cells (Figure 16).

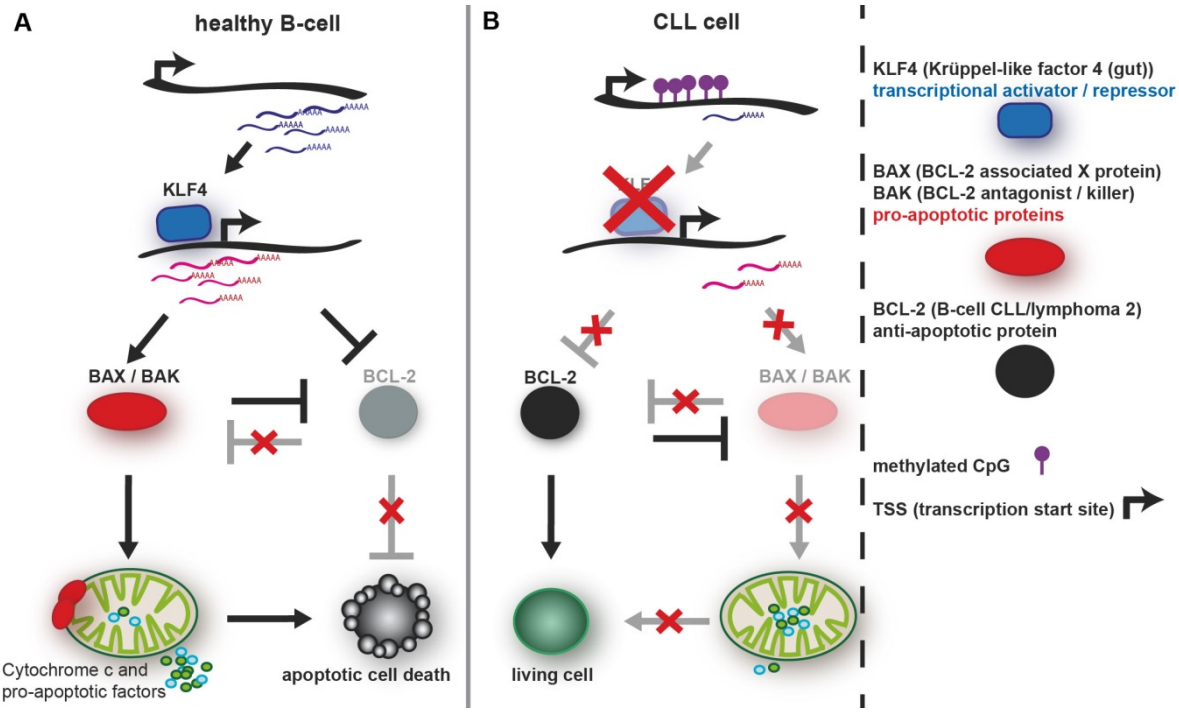

Figure 16. Potential regulation mechanism of BCL-2 family-dependent apoptosis by KLF4. *(A) In healthy B-cells expression of KLF4 could ensure a proper apoptotic mechanism by activation of BAX and BAK and inhibition of BCL-2 which leads to apoptotic cell death. (B) The down-regulation of KLF4 could possibly interrupt the apoptotic mechanisms and contribute to the apoptotic defect of the CLL cells.*

In addition, it was shown for several types of cancer that KLF4 can inhibit cell-cycle progression by repression (amongst others) of the cell cycler regulator cyclin D1 (*CCND1*).[303,315,344]

To investigate the transcriptional regulation of *BAK*, *BAX*, *BCL*-2 and *CCND1* by KLF4 in CLL, the respective mRNA levels were measured in the cohort of CD19^{+} B-cells and CLL cells used for validation of expression and methylation in (III.3). In fact, all four genes showed differential expression between CLL cells and healthy donor samples with *BAK* and *BAX* being down-regulated and *BCL*-2 and *CCND1* up-regulated (Figure 17A). Moreover, the expression of *BAK* and *BAX* showed weak but significant positive correlation with *KLF4* expression, while *CCND1* and *BCL*-2 displayed remote negative correlation. These results suggest that all four genes are KLF4 targets in CLL (Figure 17B).

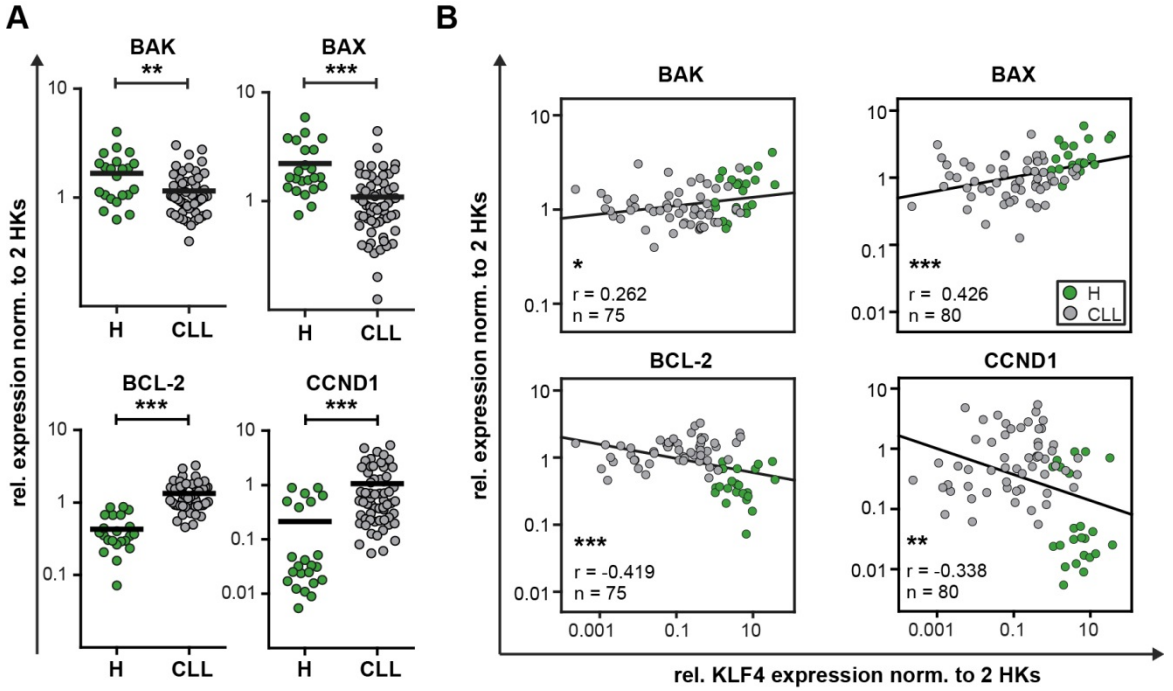

Figure 17. Expression of BAK, BAX, BCL-2 and CCND1 in CD19+ CLL and B-cells. *(A) Expression of BAK, BAX, BCL-2 and CCDN1 mRNA was measured with qRT-PCR and normalized to PGK and HPRT. BAK and BAX show up-regulation, BCL-2 and CCND1 down-regulation in CLL. (B) Expression of BAK and BAX shows positive, of BCL-2 and CCND1 negative correlation to KLF4 expression. Lines indicate mean; Mann-Whitney U test and Spearman rank correlation were used to test for significance; p-values are indicated by asterisk: *≤0.05; **≤0.01; ***≤0.001*

In order to further support this hypothesis, *KLF4* was transiently overexpressed in the CLL cell lines MEC1 and MEC2 as well as the mantle cell lymphoma (MCL) cell line JeKo-1. Cells were transfected by nucleofection with pcDNA3.1-KLF4 and pcDNA3.1/V5-His empty vector control and harvested 24h after transfection. Untreated cells and cells applied to electroporation without DNA (mock) were used as additional controls and three (MEC1 and JeKo-1) to four (MEC2) independent experiments were performed. After 24h, viability was assessed with Annexin-V / 7AAD flow cytometry and RNA applied to qRT-PCR measurements of *BAX*, *BAK*, *BCL*-2 and *CCND1*. The overexpression of KLF4 on protein level was confirmed with western blot (Figure 18B). Of note, no differences in the amount of viable cells could be detected between the empty vector control and *KLF4* overexpression samples (Figure 18A). Furthermore, KLF4 did not impact on the expression of its downstream targets in neither of the cell lines (Figure 18C).

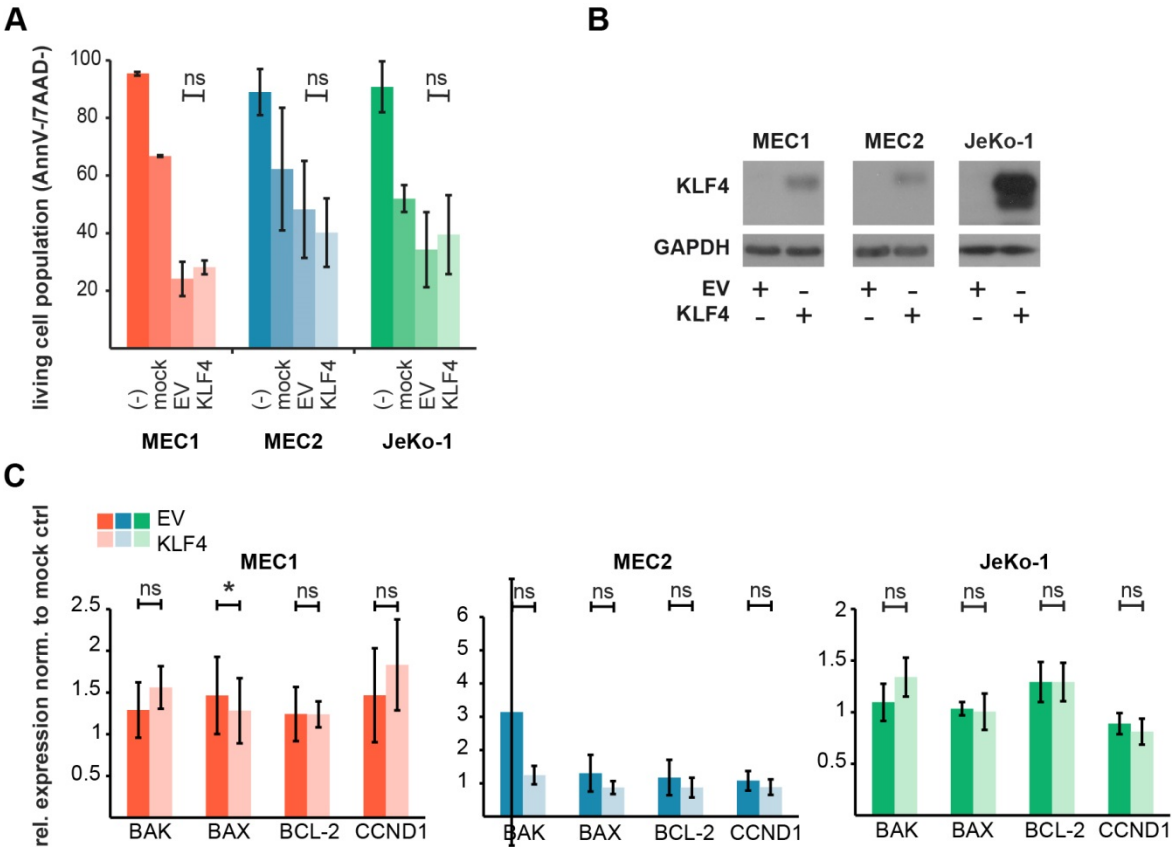

Figure 18. Impact of transient KLF4 overexpression on BAK, BAX, BCL-2 and CCND1 expression levels in leukemia cell lines. *Cell lines MEC1, MEC2 and JeKo-1 were transfected with KLF4 expression plasmid. Results of three (MEC1 and JeKo-1) to four (MEC2) independent experiments are shown. (-) untreated control; "mock" nucleofection w/o DNA; "EV" empty vector; "KLF" KLF4 overexpression; (A) AnnV / 7AAD flow cytometry 24h after transfection. Percent viable cells (Ann-V^{neg} / 7AAD^{neg}) are depicted. No differences in viability could be observed between KLF4 overexpression and empty vector control. (B) Western blot confirms overexpression of KLF4 24h after transfection. (C) No differences in expression of BAK, BAX, BCL-2 and CCND1 were observed 24h after transfection in either of the cell lines. Expression values were normalized to mock control and PGK/HPRT. Error bars represent standard deviation; boxes represent mean; Paired t-test was used to test for significance; p-values are indicated by asterisk: *≤0.05; ns non-significant.*

5. Inhibition of NOTCH1 activity by γ-secretase inhibitor treatment induces the expression of *KLF4* in cell lines and primary CLL cells

As described earlier, the gene coding for the signaling transmembrane receptor NOTCH1 is recurrently mutated in 6-12% of CLL cases.[242] In addition, CLL cells show increased expression and activity of the NOTCH1 pathway in comparison to normal B-lymphocytes. In other cancers, *KLF4* is one of the NOTCH1 downstream targets and is activated by NOTCH1 either by direct binding of the NICD to the promoter or repressed via the activation of *HES1*.[282,284] To assess a potential relationship between NOTCH1 signaling and *KLF4* expression in CLL, *NOTCH1* mRNA levels were measured in the cohort of CD19^{+} CLL and B-cells mentioned before. Similar to results from the expression array data (Figure 12A), up-regulation of *NOTCH1* levels in CLL could be confirmed in our patient subset. However, no correlation between *KLF4* expression and *NOTCH1* was detected (Figure 19A and B).

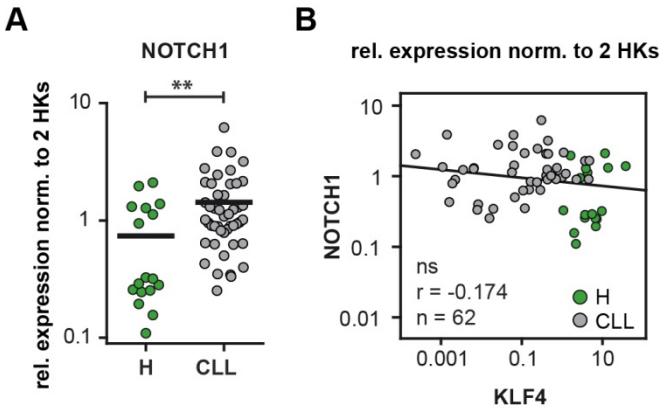

Figure 19. Expression of NOTCH1 in CD19$^+$ CLL- and B-cells. *(A) NOTCH1 expression levels were measured with qRT-PCR and normalized to HPRT and PGK. Overexpression of NOTCH1 in CLL was confirmed. (B) NOTCH1 mRNA levels did not show any correlation with KLF4 expression levels. Mann-Whitney U test and Spearman rank correlation were used to test for significance; p-values are indicated by asterisk: **≤0.01; lines indicate means; ns non-significant;*

Nevertheless, to test whether NOTCH1 activity, independent of gene expression, impacts on the expression of *KLF4* in CLL, peripheral blood mononuclear cells (PBMCs) of CLL patients and six different leukemia and lymphoblastoid cell lines were treated with γ-secretase inhibitor GSI-I. Upon ligand engagement and activation of NOTCH1, its intracellular domain (NICD) is cleaved by the protein γ-secretase and consequently free to translocate into the nucleus where it acts in complex with other proteins like CSL and MAML1 as transcription factor and activates its downstream targets.[280,345,346] Inhibition by GSI-I abrogates the activation of NOTCH1 and hence its target genes.

Frozen PBMCs of eight CLL patients, with a tumor load of at least 92% CD5$^+$CD19$^+$ B-cells, were treated with 2.5 µM and 5 µM GSI-I for 24h. Untreated cells and 0.0002% DMSO were the controls.[347] Similarly, the lymphoblastoid cell lines LCL-FM and LCL-MM which represent normal B-cells and the leukemia cell lines MEC1, MEC2, GRANTA-519 and JeKo-1 were treated with the same concentrations in three independent experiments.

Cell viability was determined with the CellTiter-Glo cell viability assay for cell lines and with Annexin-V / 7AAD flow cytometry for CLL cells, after 24h. A significant, dose-dependent reduction of the living cell population could be detected for all cell lines and PBMCs of CLL patients after 24h (Figure 20A). Effects on LCL-MM were less pronounced, which could be explained by strong cluster formation observed in cell culture. Western blot analysis affirmed inhibition of NOTCH1 activity with 2.5 µM and 5 µM GSI-I, as levels of NICD and full length NOTCH1 (NP) were reduced in cell lines and primary CLL cells (Figure 20B). Despite previous findings from Rosati *et al.* no accumulation of the transmembrane form of NOTCH1 (N-TM) could be detected, but a protein band of unknown identity appeared at about 200 kD.

In order to confirm the efficiency of NOTCH1 inhibition by GSI-I treatment, the expression of *NOXA*, *HES1* and *CNND1* was measured with qRT-PCR in all cell lines and in addition of *MCL1* in all CLL samples treated. *HES1* and *CCND1* are direct downstream targets of NOTCH1 signaling and were shown to be repressed upon NOTCH1 inhibition.[281,296,348,349] Furthermore, *MCL*-1 and *NOXA*, two more members of the BCL-2 protein family, were previously shown to be up-regulated by GSI-I treatment and are necessary for GSI induced apoptosis in CLL cells and other cell types.[347,350]

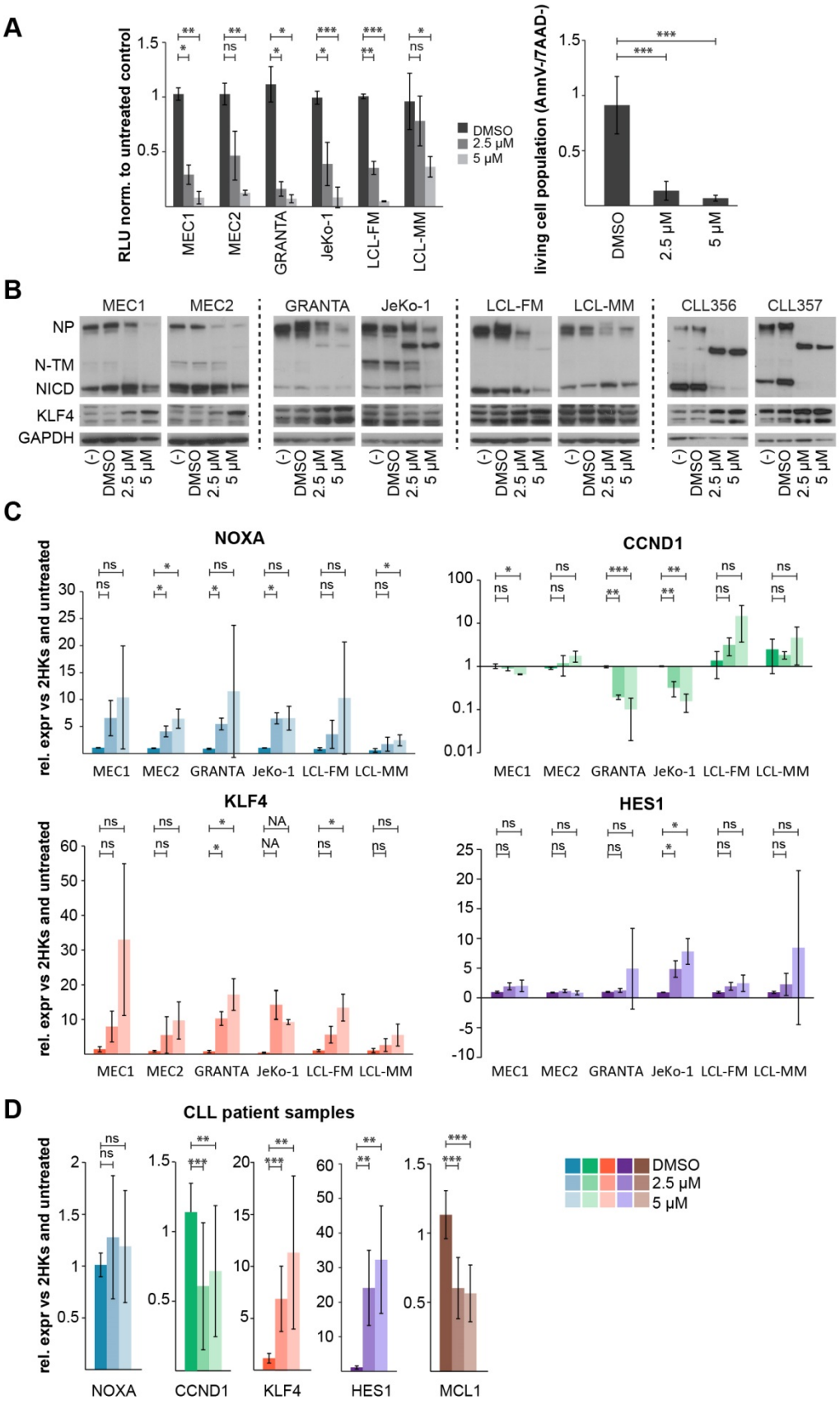

Figure 20. NOTCH1 inhibition with GSI-I in cell lines and frozen PBMCs from CLL patients. *Cell lines MEC1, MEC2, JeKo-1, LCL-FM and LCL-MM (three independent experiments each) and PBMCs form eight CLL patients were treated with 2.5 µM and 5 µM GSI-I for 24h. Untreated cells and cells treated with 0.0002% DMSO were controls. (A) CellTiter-Glo viability assay and Ann-V / 7AAD flow cytometry were used to assess viability of cell lines and CLL cells. RFU and Ann-V^{neg}/7AAD^{neg} cells normalized to untreated control are shown. (B) Western blot for NOTCH1 and KLF4 confirmed inhibition of NOTCH1 activation and up-regulation*

*of KLF4 expression. Examples for one experiment per cell line and two CLL patients are shown. GAPDH was used as loading control. NP full length NOTCH1, N-TM NOTCH1 transmembrane form. (C)+(D) qRT-PCR measurement for the expression of NOXA, CCND1, KLF4, HES1 and MCL1 in cell lines (C) and PBMCs of CLL patients (D). Paired t-test was used to test for significance; Error bars represent standard deviation; p-values are indicated by asterisk: *≤0.5, **≤0.01, ***≤0.001, ns non-significant; NA only 2 out of 3 experiments could be analyzed; boxes represent mean.*

In fact, *NOXA* displayed significant up-regulation in all cell lines and to lesser degree in CLL patient samples (Figure 20C and D). *HES1* also showed up-regulation on mRNA level while *MCL1* was down-regulated in CLL cells upon GSI-I treatment. *CCND1* levels were down-regulated in a subset of cell lines including MEC1, GRANTA-519 and JeKo-1 and CLL cells as expected, but increased in LCL-FM, LCL-MM and to lesser degree in MEC2. In summary, GSI-I treatment led to mixed effects on reported effector genes which might be due to the analysis of mRNA levels instead of protein levels as done in previous studies. Furthermore, GSI-I treatment was shown to result in inhibition of the proteasome and an increase of endoplasmatic reticulum (ER) stress in CLL cells.[347]

However, a strong induction of *KLF4* expression on mRNA and on protein level could be detected in cell lines and CLL samples (Figure 20B to D). This indicates that *KLF4* is in fact a downstream target of NOTCH1 signaling in CLL and that NOTCH1 inhibition results in the transcriptional activation of *KLF4* expression.

The same experimental setup was applied to freshly isolated PBMCs of three CLL patients (47% to 70% $CD5^{+}CD19^{+}$ B-cells) which were less sensitive to GSI-I treatment as confirmed by Annexin-V / 7AAD staining. Western blot and qRT-PCR for downstream targets (Figure 21) also displayed diverse and less pronounced effects on target genes but also a slight increase of *KLF4* on mRNA level.

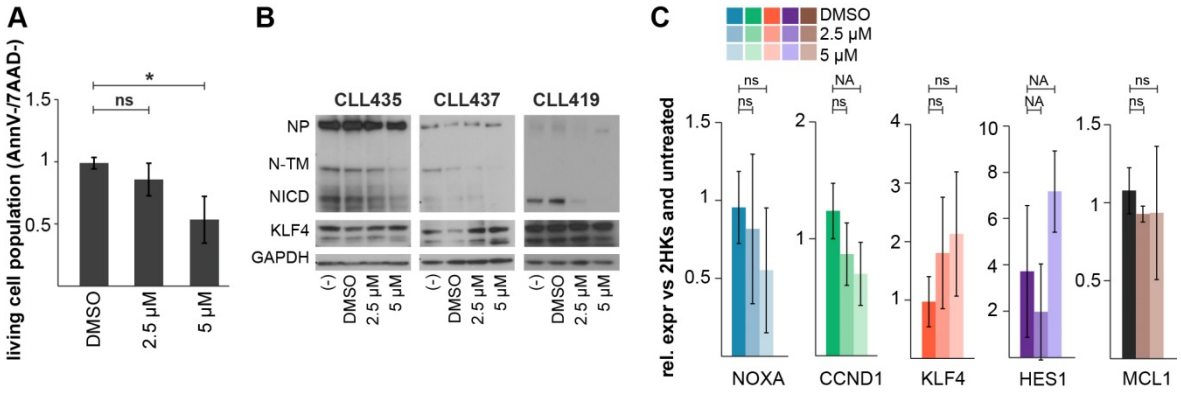

Figure 21. GSI-I treatment of freshly isolated PBMCs from CLL patients. *PBMCs from three CLL patients were treated with 2.5 µM and 5 µM GSI-I for 24h. Untreated cells and cells treated with 0.0002% DMSO were the controls. (A) Ann-V / 7AAD flow cytometry show a drop of viability after 24h. Percent living cells normalized to untreated control are shown. (B) Western blot for NOTCH1 and KLF4 shows weak NOTCH1 inhibition and minor up-regulation of KLF4. (C) mRNA levels for NOXA, CCND1, KLF4, HES1 and MCL1 were determined by qRT-PCR. Paired t-test was used to test for significance; Error bars represent standard deviation; p-values are indicated by asterisk: *≤0.5, ns non-significant; NA only 2 out of 3 experiments could be analyzed; boxes represent mean.*

6. Expression profiling after *KLF4* overexpression reveals iNOS- and BCR signaling genes as downstream targets

The previous experiments showed that overexpression of *KLF4* does not result in the deregulation of its downstream targets *BAX*, *BAK*, *BCL-2* and *CCND1* in leukemia cell lines. Therefore, expression profiling after overexpression of *KLF4* in MEC1, MEC2 and

JeKo-1 was conducted to identify direct targets of the transcription factor in leukemia. Cells were transfected with pcDNA3.1-KLF4 and pcDNA3.1/V5-His as empty vector control by nucleofection. RNA was isolated from cells harvested 3h and 10h after transfection and applied to expression profiling on HumanHT-12v4 Illumina BeadChip Sentrix arrays. Overexpression of KLF4 on protein level could be verified with western blot, already 3h after transfection (Figure 22A). However, scatter plots of quantile normalized expression values (log_2(signal)) demonstrate only minor changes in the transcriptional profile after 3h in the cell lines (Figure 22B). In contrast, 10h after transfection, expression profiles of MEC1 and MEC2 displayed striking up-regulation of a set of genes, while in JeKo-1, only minor effects could be observed.

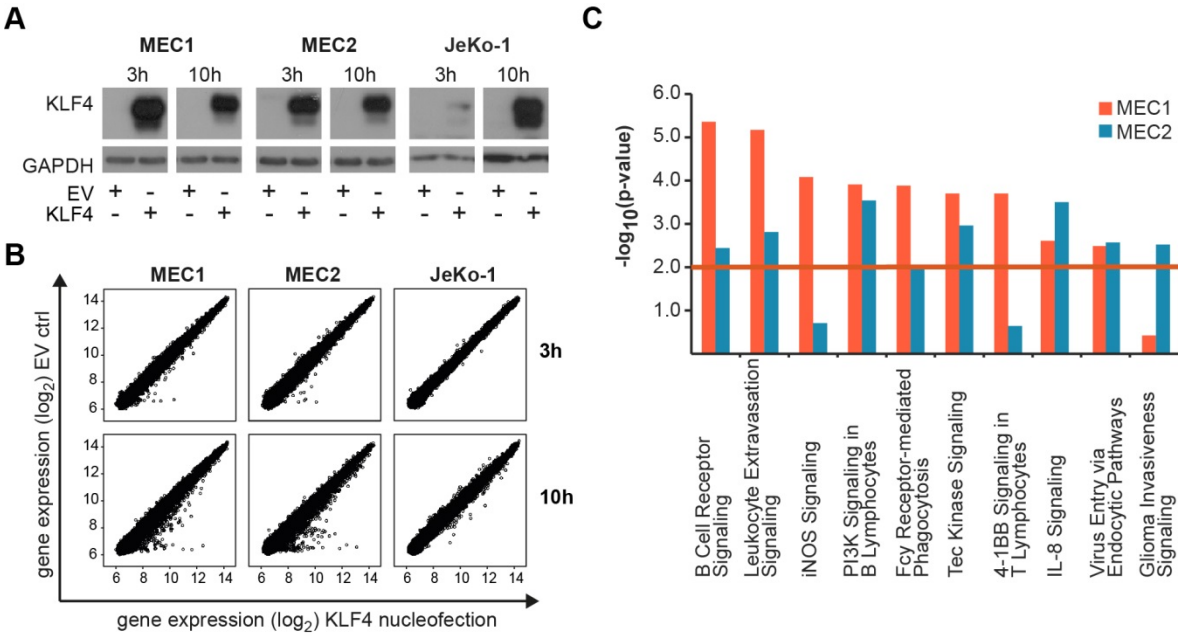

Figure 22. Expression profiling in leukemia cell lines after KFL4 overexpression. *MEC1, MEC2 and JeKo-1 were transfected with pcDNA3.1-KLF4 ("KLF4") and pcDNA3.1/V5-His as empty vector control ("EV"). Samples taken 3h and 10h after transfection were applied to expression profiling. (A) Western blot for KLF4 confirms overexpression already after 3h. (B) Scatterplots for expression values (log_2(signal)) for all cell lines 3h and 10h after transfection. (C) Ingenuity pathway analysis reveals top canonical pathways deregulated in MEC1 and/or MEC2 10h after transfection. Top 7 pathways for both cell lines are shown. Red line marks p-value of 0.01.*

Ingenuity pathway analysis was performed with genes showing a deregulation of $|log_2FC| \geq 0.38$ 10h after transfection for MEC1 (n=1,218), MEC2 (n=1,245) and JeKo-1 (n=705), in order to identify the impact of *KLF4* expression on cellular mechanisms. In case of MEC1 and MEC2 cellular functions potentially affected by the deregulated genes include cell death and survival as well as proliferation, cell to cell signaling and interaction but also hematological system development and hematopoiesis (Supplemental tables 1 to 3, V.1). Furthermore, KLF4 targeted genes that are involved in a number of canonical pathways important for B-cell development, homing and maintenance including BCR-signaling, leukocyte extravasation, iNOS- (nitric oxide synthase) and PI3K-signaling (Figure 22C).

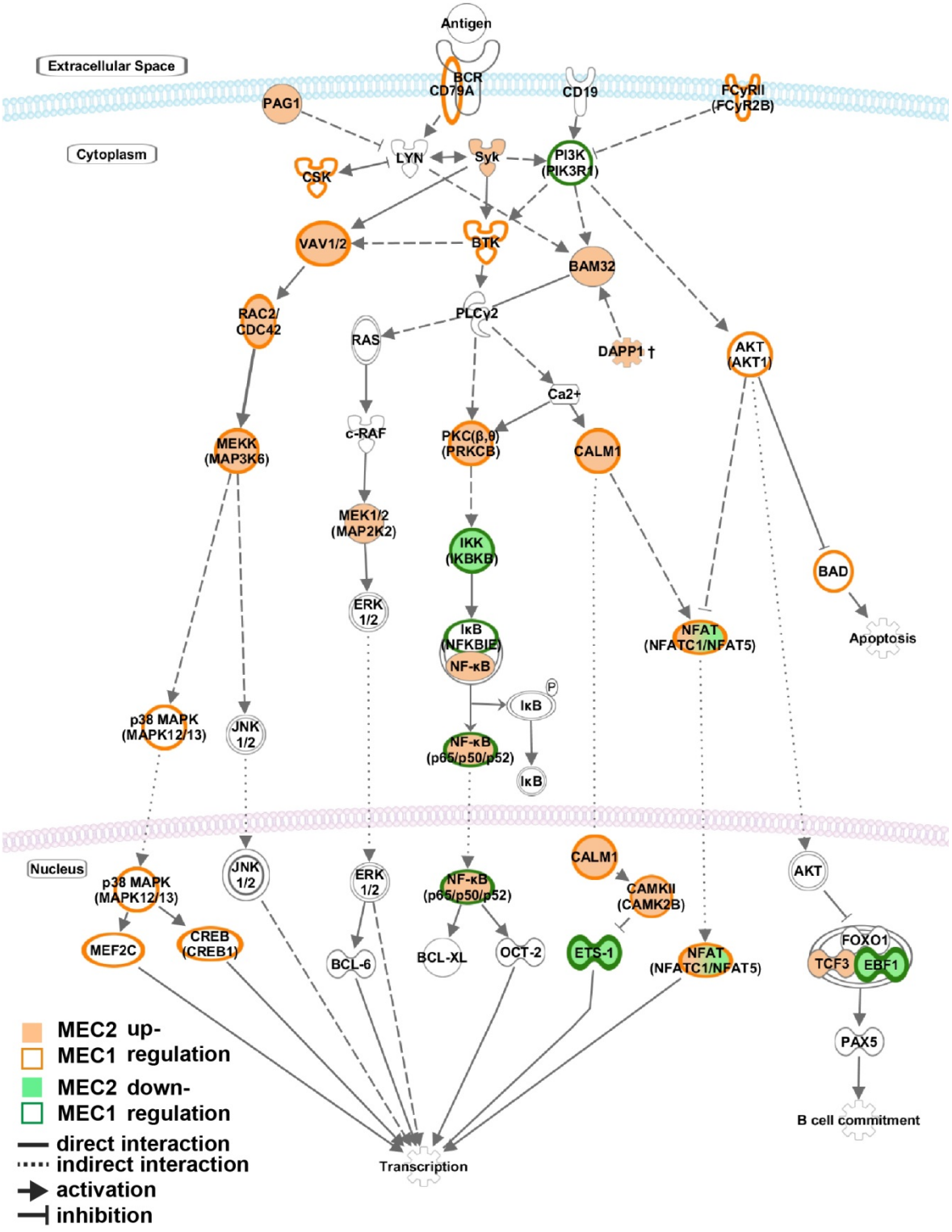

Figure 23. Genes of the BCR signaling pathway are deregulated upon KLF4 overexpression. *MEC1 and MEC2 cells were transfected with pcDNA3.1-KLF4 and EV control. Expression profiling 10h after transfection revealed 32 out of 167 genes of BCR signaling to be up- or down-regulated in one or both of the cell lines. Major elements of BCR signaling are displayed. Genes up- or down-regulated are marked in orange and green, respectively with borders of symbols representing MEC1 and filling MEC2.*

In CLL, apoptosis resistance is associated with enhanced *iNOS* expression and NO release and it was suggested that TLR-7 signaling stimulates NFκB dependent activation of the NO pathway.[351] Moreover, KLF4 was shown to interact with p65 to induce iNOS in

macrophages.[352] In this study, KLF4 deregulated several components of NFκB signaling including p50, p52 and p60 subunits of NFκB complex as well as IKK kinase family (IKBKB) and NFKBIE which are involved in the activation of NFκB signaling important for B-cell development and function.[353–355] These findings indicate that KLF4 might to some degree be involved in the apoptosis resistance mechanism of CLL cells via activation of iNOS signaling. Furthermore, a significant proportion of genes involved in BCR signaling became deregulated after KLF4 overexpression in MEC1 and MEC2, suggesting impact of the epigenetic inactivation of *KLF4* on the BCR signaling pathway that is central to the pathomechanism of CLL.

Ingenuity pathway analysis of genes deregulated in JeKo-1 did not reflect a strong overlap in the expression profiles with MEC1 and MEC2. Top canonical pathways which showed significant deregulation are mitochondrial dysfunction and oxidative phosphorylation. Presumably, this can be assigned to the general weak impact of KLF4 on the transcriptional profile of the cells which might be due to the lower expression of the protein after 3h and 10h.

Of note, upon *KLF4* overexpression *NOTCH1* was upregulated in MEC1 and MEC2 (log_2FC=1.08 and 1.25) and to some degree in JeKo-1 (log_2FC=0.31) indicating the transcriptional regulation of *NOTCH1* by KLF4.

IV. Discussion

1. Genome wide promoter methylation analysis and expression profiling reveals low correlation between DNA methylation and gene expression in CLL

In CLL, global DNA methylation was shown to be relatively stable over time and comparable in resting and proliferative compartments.[270] Therefore, DNA methylation has proven to be a stable clinical marker in oncology, to some degree more reliable than protein or mRNA levels.[356] In CLL as well, DNA methylation was shown to be predictive for biological and prognostic subgroups. Even further, the methylation status of single promoter regions can impact on the expression of its downstream genes and on CLL survival, as has been shown for *BCL-2* and *ZAP70*.[261,264] However, in previous DNA methylation studies no thorough integration of gene expression data has been conducted to elucidate the impact of DNA methylation on the transcriptional profile of CLL cells. Moreover, recent work on the characterization of the methylome of CLL cells was performed with Illumina 450K methylation arrays which quantify DNA methylation at the majority of elements thought to be of functional relevance, however with reduced coverage.[271]

Therefore, in this work, expression- and methylation-array profiling on custom designed promoter tiling-arrays, which densely cover the promoters of all RefSeq genes, was conducted in $CD19^+$ B-cells of CLL patients and healthy donors. This integrative approach aimed at the identification of genes which are transcriptionally deregulated in CLL due to stable, aberrant DNA methylation in their promoter regions. Consequently, genes should be revealed with prognostic and functional impact on the malignant cells, which so far went unnoticed in CLL. The small patient cohort (n=15) used in this study represented low risk disease, displaying no deletions in 17p or 11q nor trisomy 12. Hence, the influence on gene expression of these prominent genetic aberrations could be ruled out. Furthermore, all patients were untreated at time of sampling, therefore representing original disease status that most likely did not evolve yet or undergo clonal evolution due to treatment. Of note, in this present study, CLL patient samples were compared to $CD19^+$ B-cells of healthy donors, which were considered as the healthy counterpart of CLL until recently. However, the cell of origin of this B-cell leukemia is still under debate, and previous expression profiling suggests $CD5^+$ B-cells as source of disease development. Based on DNA methylation profiling, others subdivide the CLL cells into three groups, which they assign to naïve B-cell like, intermediate and memory B-cell like states.[229,271] Hence, a clear definition of the cell of CLL origin is still missing. However, many prominent markers and candidate genes were found by comparison to normal $CD19^+$ B-cells and since then proven to be of functional relevance in CLL, e.g. *BCL-2* and *ZAP70,* underlining the pertinence of this project.[261,264]

Among the 1,866 genes which appeared to be up- or down-regulated in the CLL cells, were a number of genes previously shown to have aberrant expression in CLL and / or to be important for leukemogenesis. These were the oncogenes *BCL-2* and *TCL1*, as well as a number of transcriptional regulators like *LEF-1*, *JUNB*, *EBF1*, *STAT1* and *STAT6*. Further signaling molecules and surface receptors including *CD86*, *TLR7*, *IGFBP4*, *ITGB2*, *IL6* and *IL8* were identified in this screen as aberrantly expressed. These results support the validity of this expression data set.

DNA methylation values measured with MCIp array in the same patients displayed a greater heterogeneity in CLL cells, showing a broader distribution of methylation values in comparison to normal B-cells. This finding goes in line with results from other groups who found in general a lower intratumor heterogeneity in DNA methylation of CLL cells in comparison to normal B-cells but nevertheless strong locally disordered methylation in the malignant cells. This results in intersample methylome variation not present in the healthy counterpart.[273,274] However, a more detailed analysis of the methylation data generated in this thesis, according to genome wide distribution of methylation or representation in CpG islands, CGI shores and shelves as well as enhancers was not done as the custom tiling-arrays used here display promoter regions only. As mentioned in the introduction a thorough and detailed analysis of DNA methylation patterns in CLL was done by other groups already. [271,273,274,357]

To reveal differentially methylated regions in the CLL cells the present dataset was screened for 500 bp windows which showed aberrant methylation in at least two oligonucleotides. Out of the 1,472 DMRs defined with this approach, only about one third showed hypermethylation while two thirds showed hypomethylation. This confirmed that reduced methylation in comparison to normal cells in general is more abundant in cancer than increased DNA methylation. Combining the expression and methylation data, finally 33 genes could be identified which showed significant negative correlation between the expression and the methylation status of at least one oligonucleotide of the respective DMR. Considering the high amount of about 19,000 protein coding genes in the human genome this number appears rather small.[358] However, it is known from other studies that in general the genome wide correlation of DNA methylation with expression in CLL is relatively small and not only restricted to the promoter sites.[271] Hence, many regulatory elements which could show aberrant methylation that impact on gene expression are not covered on the promoter arrays used in this project or might display positive rather than negative correlation to gene expression.

2. *LILRA4* and *KLF4* are deregulated in CLL by DNA methylation

To confirm the differential methylation detected within the promoter regions of the 33 genes depicted above, technical validation was performed with methylation specific mass spectrometry (MassARRAY) in the same samples. 17 out of these genes demonstrated aberrant methylation also with this method (Figure 13A). MassARRAY is a highly quantitative method which is based on mass spectrometry measurements of methylated and unmethylated DNA fragments. MCIp in contrast relies on the physiological binding of an MBD-Fc fusion protein to methylated DNA. While only about half of the DMRs could be validated with MassARRAY, the combinatory results of both methods assure the presence of aberrant methylation in these promoter regions.

Of these genes, *LILRA4* and *KLF4* were selected for further validation, as both could play roles in the maturation and activation of CLL cells. The overexpression of *LILRA4* and the down-regulation of *KLF4* in the leukemia cells could be confirmed in a large patient subset, as well as promoter-wide hypomethylation of *LILRA4* and hypermethylation of a region within the gene-body of *KLF4*. Thus, it appears that in fact both genes could be transcriptionally deregulated in CLL by DNA methylation. Moreover, to test the prognostic impact of abnormal methylation in both genes, MassARRAY was performed with DNA samples from four different clinical trials conducted by the German CLL study group (GCCSG), namely CLL1, CLL2H, CLL4 and CLL8. No differences could be detected

within the two MA amplicons of *KLF4* and *LILRA4* which were investigated, reflecting homogenous methylation status of both genes within patients of different clinical stages. However, the effect on more elaborate prognostic factors like progression free or overall survival and treatment response still remains to be elucidated as soon as the respective clinical information is available.

In contrast to gene-body hypermethylation of *KLF4* detected in CLL, in other cancer entities including cervical cancer and B-cell malignancies like NHL and cHL *KLF4* is repressed by promoter hypermethylation upstream of the TSS.[314,317] This raises the question how the hypermethylation of *KLF4* within a CGI but downstream of the TSS and within the gene-body impacts on the transcriptional activity of its promoter. In CLL, gene body methylation outside CGIs has been described to be positively correlated with gene expression. In contrast, gene body CpGs whose methylation showed negative correlation with expression were related to enhancers.[271] In other tissues and cancers, intragenic DNA methylation was shown to result in alternative splicing or the definition of alternative TSS.[118,119] Furthermore, besides DNA methylation, other epigenetic factors not investigated in this work like histone modifications or the binding of chromatin remodelers, could influence the transcriptional regulation of *KLF4* as well. Taken together, the exact mechanism underlying the deregulation of *KLF4* in CLL is not clear. Also, if the aberrant methylation of *KLF4* detected here solely results in the down-regulation of its expression or also in the transcription of deviant splice variants thereof remains to be elucidated.

Together with *OCT4*, *SOX2* and *c-MYC*, the transcription factor *KLF4* is not only able to induce pluripotent stem cells from human dermal fibroblast, but also to reprogram non-terminally differentiated mouse B-cells to a pluripotent state. [301,359] Besides, *KLF4* seems to be important for maintaining quiescence of hematopoietic cells, acts as regulator of B-cell number and is involved in activation-induced B-cell proliferation.[310,311,342] These findings indicate that *KLF4* could be an interesting target in the course of CLL. Hence, despite the uncharacterized mechanism of deregulation of *KLF4*, the continued project was aimed to investigate the functional impact of its down-regulation in CLL cells.

As described before, LILRA4 is able to induce ITAM mediated signaling and hence, if present, could activate the BCR signaling pathway in CLL cells. Of note, in previous work from Herishanu and colleagues, *LILRA4* was included in a BCR signature based on expression profiling of IGHV mutated and unmutated CLL cells which were activated by *in vitro* IgM cross-linking.[247] CLL cells are considered to be activated B-cells which are highly dependent on BCR signaling. However, the effects of BCR engagement are diverse depending on IGHV mutations status and presumably IgM and IgD density on the cells.[360] Hence, the impact of LILRA4 on BCR signaling could be of great interest, especially as the ligand of LILRA4 (BST2) is also present on B-cells and other cells after pre-exposure to IFN-I. Unfortunately, functional characterization of LILRA4 was not successful as no commercial antibody nor a custom designed antibody was able to detect LILRA4 on protein level (data not shown). Moreover, no overexpression of *LILRA4* could be obtained by transient transfection in different cell lines.

3. *KLF4* is re-expressed by GSI-I treatment in CLL cells and leukemia cell lines suggesting regulation by NOTCH1 signaling

In previous work, *KLF4* was shown to be a down-stream target of NOTCH1 signaling which can either repress or activate its transcription. *NOTCH1* is currently under

investigation in the course of CLL as it is frequently mutated and constitutively active in CLL cells in contrast to normal B-cells. Furthermore, JAGGED1-ligand increases CLL survival and NFκB activity in the cancer cells while apoptosis is induced after blockage of NOTCH1 receptors.[242,296,297] The exact mechanism, how NOTCH1 signaling impacts on CLL cell survival and development is currently under investigation. Repression of *KLF4* by NOTCH1 could be one potential trigger how NOTCH1 mutations and activity-defects are mediated and sequester effects on CLL cell activation.

Although *KLF4* RNA levels did not correlate with aberrant *NOTCH1* expression in our sample subset, treatment of frozen PBMCs from eight CLL patients and six different lymphoblastoid and leukemia cell lines with GSI-I confirmed the re-expression of *KLF4* on RNA and protein level. However, one has to keep in mind that the inhibition of γ-secretase is not absolutely specific for NOTCH1 inhibition as the protein targets a whole panel of receptors.[361] In CLL, it was shown that GSI-I treatment results not just in apoptosis of the cells but also in the inhibition of the proteasome as well as ER stress which might be NOTCH1-dependent but could also reflect side effects of the treatment on other signaling pathways.[347] This could also explain the divergent results concerning the NOTCH1 downstream targets *MCL-1* and *HES1* and GSI-I responsive genes *NOXA* and *CCND1* which was measured with qRT-PCR here, while investigated on protein level by others.[281,296,347] Nevertheless, γ-secretase inhibitors are a common tool for the inhibition of NOTCH1 and are widely used in literature. The most prominent effects of the GSIs seem to be mediated by NOTCH1 inhibition. This is supported by the finding that a high NOTCH pathway activity could predict response to GSI in glioma tumor-initiating cells, for example.[362] Besides, regulation of *KLF4* by NOTCH1 described in literature supports the assumption that the re-expression of *KLF4* is mediated by NOTCH1 inhibition in the CLL cells.

Furthermore, the effects detected upon treatment of PBMCs freshly isolated from patient samples and immediately exposed to treatment were less pronounced than in the viably frozen cells. GSI-I treatment showed less impact not only on apoptosis induction but also on the transcription of the responsive genes, including *KLF4*. Reduced inhibition of NOTCH1 activity in these cells was also confirmed by western blot. On the one hand, the tumor load of the fresh samples ranging between 47% to 70% $CD5^+CD19^+$ B-cells was reduced in comparison to the frozen samples (over 92% $CD5^+CD19^+$ B-cells). Hence, T-cells, macrophages and monocytes still present in the cell mixture might be less susceptible to the treatment, show different effects or support the CLL cell survival which could influence the read outs. On the other hand it is possible that the previously frozen and subsequently thawed cells are more sensitive to the treatment as they reflect stressed cells.

The exact mechanism how NOTCH1 regulates the activity of the *KLF4* promoter in CLL cells is not clear. In intestinal epithelium and T-cell ALL NOTCH1 inhibits *KLF4* transcription by activation of *HES1* which directly binds to the *KLF4* promoter about -0.5 kb upstream of the TSS and acts as repressor.[282,283] Another NOTCH1 responsive element (NRE) -151 bp upstream of *KLF4* was defined, which mediates *KLF4* repression by a so far unknown but HES1-independent mechanism.[363] In head and neck cancer cells, JAGGED1-induced NOTCH1 signaling activates *KLF4* by direct binding of NICD/CSL to its promoter -1.5 kb upstream.[284] Considering these findings, an indirect effect for example via *HES1* rather than direct binding of NICD/CSL can be expected in CLL, where NOTCH1 inhibits *KLF4* expression. However, besides the regulation by

NOTCH1 and HES1 the expression of *KLF4* can be influenced by a number of other transcription factors like SP1, AP-1 and KLF4 itself.[364] These findings indicate that the regulation of *KLF4* underlies a complex interplay of TFs of which binding might be in addition, influenced by the epigenetic make-up of the promoter region.

Conversely to the regulation of *KLF4* by NOTCH1 signaling, it was shown that KLF4 can regulate the expression of *NOTCH1* as well. In epithelial cells the overexpression of *KLF4* promotes the expression of *NOTCH1*, its ligand *DLL4* and its downstream target *HES1*, which are all direct transcriptional targets of KLF4.[285] In keratinocytes a similar effect on *NOTCH1* after *KLF4* overexpression was seen, however a reduction of *NOTCH1* transcription could only be induced by knock-down of *KLF4* and in addition the transcription factor *SP3* indicating a synergistic control of *NOTCH1* by these proteins.[287] We detected the up-regulation of *NOTCH1* on mRNA level after transient *KLF4* overexpression in MEC1 and MEC2 and to some degree in JeKo-1. This would indicate an activating function of KLF4 on *NOTCH1* in the leukemia cell lines and hereafter a potential negative feedback mechanism.

4. Overexpression of *KLF4* results in the deregulation of genes involved in BCR- and PI3K-signaling in leukemia cell lines

Next, to analyze the effects of *KLF4* repression on the pathomechanism of CLL, several downstream targets were investigated. In other B-cell malignancies and cancer entities *KLF4* was shown to induce apoptosis by changing the *BCL-2/BAX* ratio and by activating the expression of *BAK*.[317] In addition, KLF4 can regulate cell cycle progression amongst other means by repression of *CCND1.*[344] The aberrant expression in CLL and the weak but significant correlation with *KLF4* expression of all four target genes might indicate functional relationship and a regulation by KLF4. However, the transient overexpression of *KLF4* in the three leukemia cell lines MEC1, MEC2 and JeKo-1 did not impact on the transcriptional activity of these genes. Moreover, KLF4 did not act as inducer of apoptosis in these cells, as no decrease of cell viability in comparison to the control could be detected 24h after transfection.

The activation and repression of *KLF4* downstream targets was shown to be dependent to some degree on the interaction of KLF4 with other transcriptional regulators. For example, cooperatively with MEIS2 and PBX1, KLF4 activates *E-cadherin* and *p15* promoters, which is however partly dependent on PBX1 binding sites adjacent to the KLF4 consensus sequences.[365] Furthermore, while acting as transcriptional repressor of *TP53*, KLF4 was shown to increase the DNA-binding affinity of p53 through the formation of a ternary complex on the DNA. This mechanism is again dependent on the presence of neighboring response elements for both proteins and can for example induce the transcription of p21.[304,366] In macrophages KLF4 was shown to interact with the NFκB subunit p65 to cooperatively induce *iNOS* promoter activity.[352] Besides, KLF4 is also suspected to compete with other proteins like SP1 for the same binding sites.[367] In AML, a negative correlation between *KLF4* and histone deacetylase 1 (*HDAC1*) was discovered and knockdown of the latter resulted in increased expression of *KLF4*. Simultaneously, both KLF4 and HDAC1 were shown to competitively bind to the *KLF4* promoter, regulating its transcription.[305] Alongside transcriptional regulation, KLF4 activity can be partly blocked by phosphorylation of the protein by ERK1/2 which results in reinforced degradation, for example triggering the suppression of ESC differentiation.[302] In summary, the regulatory effects of KLF4 in a cell seem to be strongly dependent on the presence of co-regulatory

interaction partners and antagonists. Therefore, to verify the exact impact of KLF4 on target genes in CLL, overexpression of the transcription factor in primary CLL cells might be necessary. Unfortunately, these cells are hard to transfect and neither nucleofection with plasmids nor with *in vitro* produced *KLF4* mRNA resulted in detectable expression of the protein in this work.

Nevertheless, in order to find genes regulated by KLF4 in the cell line models used in this project, expression profiling was conducted with samples taken 3h and 10h after transfection of MEC1, MEC2 and JeKo-1 with *KLF4* coding plasmid. Analysis of the respective data revealed only a small set of genes which are deregulated after 10h in MEC1 and MEC2, while almost no major effects could be detected in JeKo-1 or after 3h in any of the cell lines. Western blots for KLF4 in all three cell lines showed strong induction of its expression in MEC1 and MEC2 after 3h already, while protein levels were reduced at 10h and to even higher degree after 24h. This might be an effect of degradation and the high apoptosis rate of the cells at this time point. In JeKo-1 the dynamics of KLF4 overexpression were different with low protein levels after 3h, which increased after 10h and also after 24h. This could explain the low changes in gene expression patterns seen in the expression array data for JeKo-1 in comparison to MEC1 and -2.

To analyze the genes deregulated by *KLF4* overexpression, Ingenuity pathway analysis was performed with the data obtained 10h after overexpression. As expected from previous analysis results for JeKo-1, ingenuity pathway analysis gave different results in comparison to MEC1 and MEC2 which showed strong overlap in potentially affected pathways and cellular functions. Among the canonical pathways affected by gene expression changes in MEC1 and MEC2 were several which are known to be important for CLL- and B-cells including iNOS-, PI3K- and BCR signaling. It was shown previously that KLF4 can act as mediator of pro-inflammatory signaling in macrophages, amongst others by interaction with p65 and induction of *iNOS*.[352] However, to my knowledge, no studies have been conducted on the impact of KLF4 on B-cell signaling and the effect of deregulation of genes involved in these pathways is hard to predict. Although, most genes deregulated in BCR- and PI3K-signaling in MEC1 and MEC2 showed up-regulation like *BTK*, *SYK* and *VAV1/2*, some were also down-regulated like *IKBKB* in both cell lines or *PIK3R1* in MEC1. Furthermore, not only transcriptional activity but also protein phosphorylation, degradation as well as input signals can influence signaling in the cells.

In CLL, PI3K- and BCR-signaling are tightly related and were shown to play an important role in the pathomechanism as they are activated by the microenvironment over all in the lymph nodes and are a driving factor for CLL tumor-cell survival.[247,368,369] Moreover, CLL cells are believed in general to represent highly activated B-cells with an immunological profile that resembles the one of antigen-experienced B-lymphocytes. Presumably due to their activation status, not all cells respond to BCR stimulation and further the effects of BCR engagement *in vitro* are conflicting, apparently depending on CD38 as well as IgD and IgM density, resulting either in apoptosis or proliferation of the CLL cells.[360] Of note, a number of novel inhibitors including the BTK inhibitor Ibrutinib and the PI3Kδ inhibitor Idelalisib are in use in the clinics and show promising results, highlighting the importance of these pathways for CLL cell survival.[370–372] In addition, it was shown that NOTCH1 activity synergizes with BCR and CD40 signaling to enhance B-cell activation and hence, the suppression of *KLF4* by DNA methylation and NOTCH1 might represent and support the highly activated state of the CLL cells.[373]

5. Future Perspectives

In this project expression- and methylation-array profiling in CLL cells and B-cells of healthy donors was conducted to identify genes deregulated by DNA methylation in CLL which possibly play a role on the pathomechanism of this disease. However, DNA methylation is only one layer of the epigenetic landscape impacting on gene transcription. To further shed light on the epigenetic regulation of the transcriptional profile of CLL cells additional factors including histone modifications, histone variants, chromatin accessibility and chromatin remodelers like polycomb group proteins or CTCF could be investigated. To this end chromatin immunoprecipitation (ChIP) for the respective proteins and modifications could be performed followed by array- or NGS-analysis.

In this work, the transcription and stem cell factor *KLF4* was shown to be silenced by hypermethylation in CLL. The inhibition of NOTCH1 activity by GSI-I treatment resulted in the re-expression of *KLF4* which led to the assumption that in fact *KLF4* is transcriptionally regulated by NOTCH1 in CLL cells. However, y-secretase inhibition is not specific for NOTCH1 and hence to prove this point, *NOTCH1* knock-down should be performed in cell lines and primary CLL cells to test the direct impact on *KLF4* transcription. Another possibility would be to test the effect of NOTCH1 knock-down and overexpression on *KLF4*-promoter-luciferase constructs where firefly luciferase activity represents *KLF4* promoter activation. Using this approach, NOTCH1 responsive elements could be deleted or mutated to identify the effector element of NOTCH1 signaling on *KLF4* expression.

To find downstream targets of KLF4 in CLL, expression profiling was conducted upon transient *KLF4* overexpression in 3 leukemia cell lines. A more elaborate approach would be to directly investigate binding of KLF4 to gene promoters by KLF4 ChIP-Seq analysis. This could be performed in cell lines or in primary cells with or without *KLF4* overexpression, depending on cell material needed for this method. Validation with qRT-PCR would be required subsequently. These data could complement the expression array data and help to distinguish between direct KLF4 targets and secondary transcriptional effects. The overexpression of KLF4 did not induce apoptosis in the cell lines. However, KLF4 is also known as regulator of proliferation which was not investigated in this work. Hence, cell cycle analysis with flow cytometry should be performed after *KLF4* overexpression. Moreover, ingenuity analysis revealed genes involved in BCR signaling to be deregulated after *KLF4* overexpression. The question remains whether these changes really impact on signaling and activation status of the CLL cells. To test this, signaling upon *KLF4* overexpression and BCR stimulation could be measured for example by phosphor-specific flow cytometry which can be used to measure the phosophorylation state of several members of the BCR pathway like SYK, BTK, ZAP70, ERK1/2 and PLCγ in a time resolved manner. Unfortunately, all experiments involving the overexpression or knock-down of proteins in CLL cells and leukemia cell lines require the transfection via electroporation of the cells. This method induces apoptosis in a large proportion of cells and potentially impacts on signaling status and capacity.

Finally, in this work CLL cells were compared to CD19+ B-cells of healthy donors. As the cell of origin in this leukemia is still under debate, comparison to other B-cell subsets could be beneficial and further help to define a potential role of KLF4 in CLL pathogenesis.

V. Appendix

1. Supplemental tables

Tables 1-3: *Summary of ingenuity pathway analysis of top deregulated genes 10h after KLF4 overexpression ($|log_2FC| \geq 0.38$) for MEC1, MEC2 and JeKo-1*

MEC1		
Diseases and Disorders	**p-value**	**#molecules**
Cancer	3.43E-11 - 1.74E-03	374
Inflammatory Response	7.56E-11 - 1.73E-03	207
Hematological Disease	1.51E-09 - 1.69E-03	107
Immunological Disease	1.51E-09 - 1.69E-03	186
Organismal Injury and Abnormalities	1.51E-09 - 1.43E-03	278
Molecular and Cellular Functions		
Cellular Development	6.02E-14 - 1.73E-03	321
Cellular Growth and Proliferation	6.02E-14 - 1.73E-03	347
Cell Death and Survival	1.36E-12 - 1.62E-03	333
Cell- To-Cell Signaling and Interaction	7.56E-11 - 1.38E-03	160
Cellular Movement	7.82E-11 - 1.30E-03	220
Physiological System Development and Function		
Hematological System Development and Function	6.02E-14 - 1.73E-03	224
Hematopoiesis	1.17E-12 - 1.22E-03	124
Lymphoid Tissue Structure and Development	8.99E-12 - 1.22E-03	90
Tissue Morpholgoy	2.33E-11 - 1.38E-03	143
Immunce Cell Trafficking	7.56E-11 - 1.73E-03	141
Top Canonical Pathways	**p-value**	**Ratio**
B Cell Receptor Signaling	4,34E-06	25/167 (0.15)
Leukocyte Extravasation Signaling	6,72E-06	27/193 (0.14)
iNOS Signaling	8,39E-05	10/43 (0.233)
PI3K Signaling in B Lymphocytes	1,24E-04	18/123 (0.146)
Fcy Receptor-mediated Phagocytosis in Macrophages and Monocytes	1,32E-04	15/92 (0.163

MEC2		
Diseases and Disorders	**p-value**	**#molecules**
Inflammatory Response	5.32E-10 - 2.60E-03	202
Cancer	9.53E-10 - 2.24E-03	368
Infectious Disease	4.25E-07 - 2.15E-03	162
Respiratory Disease	4.25E-07 - 2.10E-03	76
Organismal Injury and Abnormalities	5.78E-07 - 2.69E-03	265
Molecular and Cellular Functions		
Cellular Movement	1.44E-15 - 2.69E-03	236
Cell Death and Survival	1.72E-10 - 2.60E-03	307
Cell-To-Cell Signaling and Interaction	5.32E-10 - 2.32E-03	251
Cellular Growth and Proliferation	6.93E-10 - 2.69E-03	332
Cellular Development	1.73E-09 - 2.69E-03	302

Physiological System Development and Function		
Immune Cell Trafficking	1.42E-13 - 2.69E-03	155
Hematological System Development and Function	9.17E-13 - 2.44E-03	213
Hematopoiesis	5.04E-08 - 2.30E-03	94
Lymphoid Tissue Structure and Development	5.04E-08 - 1.53E-03	80
Tissue Morphology	3.76E-07 - 2.44E-03	131

Top Canonical Pathways	p-value	Ratio
PI3K Signaling in B Lymphocytes	2,91E-04	17/123 (0.138)
IL-8 Signaling	3,16E-04	22/183 (0.12)
Tec Kinase Signaling	1,09E-03	18/150 (0.12)
Leukocyte Extravasation Signaling	1,57E-03	21/193 (0.109)
Virus Entry via Endocytic Pathways	2,69E-03	12/89 (0.135

JeKo-1

Diseases and Disorders	p-value	#molecules
Dermatological Diseases and Conditions	7.66E-09 - 1.09E-02	68
Antimicrobial Response	1.51E-08 - 8.60E-03	22
Inflammatory Response	1.51E-08 - 1.13E-02	63
Immunological Disease	7.42E-08 - 1.14E-02	83
Infectious Disease	2.82E-07 - 9.02E-03	83
Molecular and Cellular Functions		
Cellular Growth and Proliferation	2.12E-08 - 7.43E-03	168
Cell Signaling	1.95E-07 - 1.12E-02	41
Cell Death and Survival	8.16E-07 - 1.14E-02	154
DNA Replication, Recombination, and Repair	3.05E-06 - 1.02E-02	47
Cellular Development	4.90E-06 - 1.11E-02	151
Physiological System Development and Function		
Connective Tissue Development and Function	7.40E-06 - 6.98E-03	57
Tissue Development	7.40E-06 - 1.14E-02	78
Hematological System Development and Function	1.35E-05 - 1.07E-02	70
Embryonic Development	1.24E-04 - 1.14E-02	69
Organ Development	1.24E-04 - 1.11E-02	35

Top Canonical Pathways	p-value	Ratio
Mitochondrial Dysfunction	2,98E-07	17/152 (0.112)
Oxidative Phosphorylation	5,09E-07	13/92 (0.141)
Interferon Signaling	1,56E-06	8/34 (0.235)
Tumoricidal Function of Hepatic Natural Killer Cells	2,84E-04	5/24 (0.208)
PDGF Signaling	7,03E-04	8/77 (0.104)

2. References

1. www.cancerresearch.uk.
2. www.cancerresearchuk.org: CS_INFOG_PREVENTABLE CANCERS POSTER IN-DEPTH-1.pdf.
3. Ferlay J, Soerjomataram I, Ervik M, Dikshit R, er S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray, F. E. Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11. *GLOBOCAN 2012 v1.0 [Internet]*
4. Starker, A. & Saß, A. Participation in cancer screening in Germany: results of the German Health Interview and Examination Survey for Adults (DEGS1). *Bundesgesundheitsblatt. Gesundheitsforschung. Gesundheitsschutz* **56,** 858–67 (2013).
5. www.krebsdaten.de; Zentrum für Krebsregisterkarten.
6. *Verbreitung von Krebserkrankungen in Deutschland; Zentrum für Krebsregisterdaten am RKI*. (2010).
7. Wienecke, a, Barnes, B., Lampert, T. & Kraywinkel, K. Changes in cancer incidence attributable to tobacco smoking in Germany, 1999-2008. *Int. J. Cancer* **134,** 682–91 (2014).
8. Kraywinkel, K., Bertz, J., Laudi, A. & Wolf, U. Epidemiologie und Früherkennung häufiger Krebserkrankungen in Deutschland. (2012).
9. Hanahan, D. & Weinberg, R. A. Hallmarks of Cancer: The Next Generation. *Cell* **144,** 646–674 (2011).
10. Hanahan, D., Weinberg, R. A. & Francisco, S. The Hallmarks of Cancer Review University of California at San Francisco. *Cell* **100,** 57–70 (2000).
11. Kandoth, C. *et al.* Mutational landscape and significance across 12 major cancer types. *Nature* **502,** 333–9 (2013).
12. Kool, M. *et al.* Genome sequencing of SHH medulloblastoma predicts genotype-related response to smoothened inhibition. *Cancer Cell* **25,** 393–405 (2014).
13. Nowell P.S. and D.A. Hungerford. A minute chromosome in human chronic granulocytic leukemia. *Science (80-.).* **132,** 1497–1500 (1960).
14. Swerdlow, S. WHO classification of tumours of haematopoietic and lymphoid tissues. (2008).
15. Skorski, T. Transformation of hematopoietic cells by BCR/ABL requires activation of a PI-3k/Akt- dependent pathway. *EMBO …* **16,** 6151–6161 (1997).
16. Reuther, J. Y., Reuther, G. W., Cortez, D., Pendergast, A. M. & Baldwin, A. S. A requirement for NF-kB activation in Bcr-Abl-mediated transformation. *Genes Dev.* **12,** 968–981 (1998).
17. Advani, A. S. & Pendergast, A. M. Bcr–Abl variants: biological and clinical aspects. *Leuk. Res.* **26,** 713–720 (2002).
18. Kusio-Kobialka, M. *et al.* Increased acetylation of lysine 317/320 of p53 caused by BCR-ABL protects from cytoplasmic translocation of p53 and mitochondria-dependent apoptosis in response to DNA damage. *Apoptosis* **17,** 950–63 (2012).
19. Hammond, E. M. & Giaccia, A. J. The role of p53 in hypoxia-induced apoptosis. *Biochem. Biophys. Res. Commun.* **331,** 718–25 (2005).
20. Green, D. R. & Kroemer, G. Cytoplasmic functions of the tumour suppressor p53. *Nature* **458,** 1127–30 (2009).
21. Lasky, T. & Silbergeld, E. P53 mutations associated with breast, colorectal, liver, lung, and ovarian cancers. *Environ. Health Perspect.* **104,** 1324–31 (1996).
22. Damineni, S. *et al.* Germline mutations of TP53 gene in breast cancer. *Tumour Biol.* **35,** 9219–27 (2014).
23. Malcikova, J. *et al.* Detailed analysis of therapy-driven clonal evolution of TP53 mutations in chronic lymphocytic leukemia. *Leukemia* (2014). doi:10.1038/leu.2014.297
24. Dong, P., Xu, Z., Jia, N., Li, D. & Feng, Y. Elevated expression of p53 gain-of-function mutation R175H in endometrial cancer cells can increase the invasive

phenotypes by activation of the EGFR/PI3K/AKT pathway. *Mol. Cancer* **8,** 103 (2009).
25. Chee, J. L. Y. *et al.* Wild-type and mutant p53 mediate cisplatin resistance through interaction and inhibition of active caspase-9. *Cell Cycle* **12,** 278–288 (2013).
26. Frank, A. K., Pietsch, E. C., Dumont, P., Tao, J. & Murphy, M. E. Wild-type and mutant p53 proteins interact with mitochondrial caspase-3. *Cancer Biol. Ther.* **11,** 740–745 (2011).
27. Vaughan, C. A. *et al.* p53 mutants induce transcription of NF-κB2 in H1299 cells through CBP and STAT binding on the NF-κB2 promoter and gain of function activity. *Arch. Biochem. Biophys.* **518,** 79–88 (2012).
28. Xu, J. *et al.* Heterogeneity of Li-Fraumeni syndrome links to unequal gain-of-function effects of p53 mutations. *Sci. Rep.* **4,** 4223 (2014).
29. Rausch, T. *et al.* Genome sequencing of pediatric medulloblastoma links catastrophic DNA rearrangements with TP53 mutations. *Cell* **148,** 59–71 (2012).
30. Stephens, P. J. *et al.* Massive Genomic Rearrangement Acquired in a Single Catastrophic Event during Cancer Development. *Cell* **144,** 27–40 (2011).
31. Magrangeas, F., Avet-Loiseau, H., Munshi, N. C. & Minvielle, S. Chromothripsis identifies a rare and aggressive entity among newly diagnosed multiple myeloma patients. *Blood* **118,** 675–8 (2011).
32. Hirsch, D. *et al.* Chromothripsis and focal copy number alterations determine poor outcome in malignant melanoma. *Cancer Res.* **73,** 1454–60 (2013).
33. Dahm-Daphi, J. *et al.* Nonhomologous end-joining of site-specific but not of radiation-induced DNA double-strand breaks is reduced in the presence of wild-type p53. *Oncogene* **24,** 1663–72 (2005).
34. Tusell, L., Pampalona, J., Soler, D., Frías, C. & Genescà, A. Different outcomes of telomere-dependent anaphase bridges. *Biochem. Soc. Trans.* **38,** 1698–703 (2010).
35. Sakofsky, C. J. *et al.* Break-induced replication is a source of mutation clusters underlying kataegis. *Cell Rep.* **7,** 1640–8 (2014).
36. Nik-Zainal, S. *et al.* Mutational processes molding the genomes of 21 breast cancers. *Cell* **149,** 979–93 (2012).
37. Alexandrov, L. B. *et al.* Signatures of mutational processes in human cancer. *Nature* **500,** 415–21 (2013).
38. Behnes, C. L. *et al.* Tumor-associated macrophages are involved in tumor progression in papillary renal cell carcinoma. *Virchows Arch.* **464,** 191–6 (2014).
39. Helm, O. *et al.* Tumor-associated macrophages exhibit pro- and anti-inflammatory properties by which they impact on pancreatic tumorigenesis. *Int. J. Cancer* **135,** 843–61 (2014).
40. Cheng, N., Chytil, A., Shyr, Y., Joly, A. & Moses, H. L. Transforming growth factor-beta signaling-deficient fibroblasts enhance hepatocyte growth factor signaling in mammary carcinoma cells to promote scattering and invasion. *Mol. Cancer Res.* **6,** 1521–33 (2008).
41. Zivadinovic, D., Gametchu, B. & Watson, C. S. Membrane estrogen receptor-alpha levels in MCF-7 breast cancer cells predict cAMP and proliferation responses. *Breast Cancer Res.* **7,** R101–12 (2005).
42. Saal, L. H. *et al.* PIK3CA Mutations Correlate with Hormone Receptors, Node Metastasis, and ERBB2, and Are Mutually Exclusive with PTEN Loss in Human Breast Carcinoma. *Cancer Res* **65,** 2554–2559 (2005).
43. Bachman, K. E. *et al.* The PIK3CA Gene is Mutated with High Frequency in Human Breast Cancers. *Cancer Biol. Ther.* **3,** 772–775 (2004).
44. Stemke-Hale, K. *et al.* An integrative genomic and proteomic analysis of PIK3CA, PTEN, and AKT mutations in breast cancer. *Cancer Res.* **68,** 6084–91 (2008).
45. Balko, J.M., I. Mayer, M. Levy, C. A. www.mycancergenome.org/content/disease/breast-cancer/ Molecular Profiling of Breast Cancer. *Internet* (2014).

46. Li, W. *et al.* Merlin/NF2 Loss-Driven Tumorigenesis Linked to CRL4DCAF1-Mediated Inhibition of the Hippo Pathway Kinases Lats1 and 2 in the Nucleus. *Cancer Cell* **26,** 48–60 (2014).
47. Okada, T., Lopez-Lago, M. & Giancotti, F. G. Merlin/NF-2 mediates contact inhibition of growth by suppressing recruitment of Rac to the plasma membrane. *J. Cell Biol.* **171,** 361–71 (2005).
48. Curto, M., Cole, B. K., Lallemand, D., Liu, C.-H. & McClatchey, A. I. Contact-dependent inhibition of EGFR signaling by Nf2/Merlin. *J. Cell Biol.* **177,** 893–903 (2007).
49. Nabetani, A. & Ishikawa, F. Alternative lengthening of telomeres pathway: recombination-mediated telomere maintenance mechanism in human cells. *J. Biochem.* **149,** 5–14 (2011).
50. Bechter, O. E., Zou, Y., Walker, W., Wright, W. E. & Shay, J. W. Telomeric recombination in mismatch repair deficient human colon cancer cells after telomerase inhibition. *Cancer Res.* **64,** 3444–51 (2004).
51. Yu, T.-Y., Kao, Y. & Lin, J.-J. Telomeric transcripts stimulate telomere recombination to suppress senescence in cells lacking telomerase. *Proc. Natl. Acad. Sci. U. S. A.* **111,** 3377–82 (2014).
52. Chin, K. *et al.* In situ analyses of genome instability in breast cancer. *Nat. Genet.* **36,** 984–8 (2004).
53. Artandi, S. E. *et al.* Telomere dysfunction promotes non-reciprocal translocations and epithelial cancers in mice. *Nature* **406,** 641–5 (2000).
54. Ma, H. *et al.* Shortened telomere length is associated with increased risk of cancer: a meta-analysis. *PLoS One* **6,** e20466 (2011).
55. Bojovic, B. & Crowe, D. L. Dysfunctional telomeres promote genomic instability and metastasis in the absence of telomerase activity in oncogene induced mammary cancer. *Mol. Carcinog.* **52,** 103–17 (2013).
56. Ghosh, A. *et al.* Telomerase directly regulates NF-κB-dependent transcription. *Nat. Cell Biol.* **14,** 1270–81 (2012).
57. Listerman, I., Gazzaniga, F. S. & Blackburn, E. H. An investigation of the effects of the core protein telomerase reverse transcriptase on Wnt signaling in breast cancer cells. *Mol. Cell. Biol.* **34,** 280–9 (2014).
58. Masutomi, K. *et al.* The telomerase reverse transcriptase regulates chromatin state and DNA damage responses. *Proc. Natl. Acad. Sci. U. S. A.* **102,** 8222–7 (2005).
59. Kang, H. J. *et al.* Ectopic expression of the catalytic subunit of telomerase protects against brain injury resulting from ischemia and NMDA-induced neurotoxicity. *J. Neurosci.* **24,** 1280–7 (2004).
60. Li, Y. & Tergaonkar, V. Noncanonical functions of telomerase: implications in telomerase-targeted cancer therapies. *Cancer Res.* **74,** 1639–44 (2014).
61. Maida, Y. *et al.* An RNA-dependent RNA polymerase formed by TERT and the RMRP RNA. *Nature* **461,** 230–5 (2009).
62. Van Delft, M. F. & Huang, D. C. S. How the Bcl-2 family of proteins interact to regulate apoptosis. *Cell Res.* **16,** 203–13 (2006).
63. Vela, L., Gonzalo, O., Naval, J. & Marzo, I. Direct interaction of Bax and Bak proteins with Bcl-2 homology domain 3 (BH3)-only proteins in living cells revealed by fluorescence complementation. *J. Biol. Chem.* **288,** 4935–46 (2013).
64. Wallgren, M. *et al.* Reconstitution of the anti-apoptotic bcl-2 protein into lipid membranes and biophysical evidence for its detergent-driven association with the pro-apoptotic bax protein. *PLoS One* **8,** e61452 (2013).
65. Desagher, S. Bid-induced Conformational Change of Bax Is Responsible for Mitochondrial Cytochrome c Release during Apoptosis. *J. Cell Biol.* **144,** 891–901 (1999).
66. Ku, B., Liang, C., Jung, J. U. & Oh, B.-H. Evidence that inhibition of BAX activation by BCL-2 involves its tight and preferential interaction with the BH3 domain of BAX. *Cell Res.* **21,** 627–41 (2011).

67. Del Gaizo Moore, V., Schlis, K. D., Sallan, S. E., Armstrong, S. A. & Letai, A. BCL-2 dependence and ABT-737 sensitivity in acute lymphoblastic leukemia. *Blood* **111,** 2300–9 (2008).
68. Tsujimoto, Y., Finger, L., Yunis, J., Nowell, P. & Croce, C. Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation. *Science (80-.).* **226,** 1097–1099 (1984).
69. Chonghaile, T. N. *et al.* Maturation Stage of T-cell Acute Lymphoblastic Leukemia Determines BCL-2 versus BCL-XL Dependence and Sensitivity to ABT-199. *Cancer Discov.* **4,** 1074–87 (2014).
70. Kim, S.-Y., Song, X., Zhang, L., Bartlett, D. L. & Lee, Y. J. Role of Bcl-xL/Beclin-1 in interplay between apoptosis and autophagy in oxaliplatin and bortezomib-induced cell death. *Biochem. Pharmacol.* **88,** 178–88 (2014).
71. Chen, Z. *et al.* Downregulation of Beclin 1 and impairment of autophagy in a small population of colorectal cancer. *Dig. Dis. Sci.* **58,** 2887–94 (2013).
72. Kwon, O.-J., Zhang, L., Ittmann, M. M. & Xin, L. Prostatic inflammation enhances basal-to-luminal differentiation and accelerates initiation of prostate cancer with a basal cell origin. *Proc. Natl. Acad. Sci. U. S. A.* **111,** E592–600 (2014).
73. Scaffidi, P., Misteli, T. & Bianchi, M. E. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature* **418,** 191–5 (2002).
74. Cao, X., Geradts, J., Dewhirst, M. W. & Lo, H.-W. Upregulation of VEGF-A and CD24 gene expression by the tGLI1 transcription factor contributes to the aggressive behavior of breast cancer cells. *Oncogene* **31,** 104–15 (2012).
75. Wu, Y. *et al.* Hypoxia inducible factor-1 is involved in growth factor, glucocorticoid and hypoxia mediated regulation of vascular endothelial growth factor-A in human meningiomas. *J. Neurooncol.* **119,** 263–73 (2014).
76. Nyberg, P., Xie, L. & Kalluri, R. Endogenous inhibitors of angiogenesis. *Cancer Res.* **65,** 3967–79 (2005).
77. Maeshima, Y. *et al.* Distinct antitumor properties of a type IV collagen domain derived from basement membrane. *J. Biol. Chem.* **275,** 21340–8 (2000).
78. Rodriguez-Manzaneque, J. C. *et al.* Thrombospondin-1 suppresses spontaneous tumor growth and inhibits activation of matrix metalloproteinase-9 and mobilization of vascular endothelial growth factor. *Proc. Natl. Acad. Sci. U. S. A.* **98,** 12485–90 (2001).
79. Ribatti, D. Endogenous inhibitors of angiogenesis: a historical review. *Leuk. Res.* **33,** 638–44 (2009).
80. Shafiee, A. *et al.* Inhibition of Retinal Angiogenesis by Peptides Derived from Thrombospondin-1. *Invest. Ophthalmol. Vis. Sci.* **41,** 2378–2388 (2000).
81. Weis, S. M. & Cheresh, D. A. Tumor angiogenesis: molecular pathways and therapeutic targets. *Nat. Med.* **17,** 1359–70 (2011).
82. Kuang, D.-M. *et al.* Peritumoral neutrophils link inflammatory response to disease progression by fostering angiogenesis in hepatocellular carcinoma. *J. Hepatol.* **54,** 948–55 (2011).
83. Bekes, E. M. *et al.* Tumor-recruited neutrophils and neutrophil TIMP-free MMP-9 regulate coordinately the levels of tumor angiogenesis and efficiency of malignant cell intravasation. *Am. J. Pathol.* **179,** 1455–70 (2011).
84. Jetten, N. *et al.* Anti-inflammatory M2, but not pro-inflammatory M1 macrophages promote angiogenesis in vivo. *Angiogenesis* **17,** 109–18 (2014).
85. Durham, J. T., Surks, H. K., Dulmovits, B. M. & Herman, I. M. Pericyte Contractility Controls Endothelial Cell Cycle Progression and Sprouting: Insights into Angiogenic Switch Mechanics. *Am. J. Physiol. Cell Physiol.* (2014). doi:10.1152/ajpcell.00185.2014
86. Du, R. *et al.* HIF1alpha induces the recruitment of bone marrow-derived vascular modulatory cells to regulate tumor angiogenesis and invasion. *Cancer Cell* **13,** 206–20 (2008).

87. Chinchar, E. *et al.* Sunitinib significantly suppresses the proliferation, migration, apoptosis resistance, tumor angiogenesis and growth of triple-negative breast cancers but increases breast cancer stem cells. *Vasc. Cell* **6,** 12 (2014).
88. Kakodkar, N. C. *et al.* Sorafenib inhibits neuroblastoma cell proliferation and signaling, blocks angiogenesis, and impairs tumor growth. *Pediatr. Blood Cancer* **59,** 642–7 (2012).
89. De Craene, B. & Berx, G. Regulatory networks defining EMT during cancer initiation and progression. *Nat. Rev. Cancer* **13,** 97–110 (2013).
90. Lamouille, S., Xu, J. & Derynck, R. Molecular mechanisms of epithelial-mesenchymal transition. *Nat. Rev. Mol. Cell Biol.* **15,** 178–96 (2014).
91. Mani, S. A. *et al.* The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* **133,** 704–15 (2008).
92. Wellner, U. *et al.* The EMT-activator ZEB1 promotes tumorigenicity by repressing stemness-inhibiting microRNAs. *Nat. Cell Biol.* **11,** 1487–95 (2009).
93. Kong, D. *et al.* Epithelial to mesenchymal transition is mechanistically linked with stem cell signatures in prostate cancer cells. *PLoS One* **5,** e12445 (2010).
94. Tam, W. L. & Weinberg, R. a. The epigenetics of epithelial-mesenchymal plasticity in cancer. *Nat. Med.* **19,** 1438–49 (2013).
95. McDonald, O. G., Wu, H., Timp, W., Doi, A. & Feinberg, A. P. Genome-scale epigenetic reprogramming during epithelial-to-mesenchymal transition. *Nat. Struct. Mol. Biol.* **18,** 867–74 (2011).
96. Wu, M.-Z. *et al.* Interplay between HDAC3 and WDR5 is essential for hypoxia-induced epithelial-mesenchymal transition. *Mol. Cell* **43,** 811–22 (2011).
97. Ke, X.-S. *et al.* Global profiling of histone and DNA methylation reveals epigenetic-based regulation of gene expression during epithelial to mesenchymal transition in prostate cells. *BMC Genomics* **11,** 669 (2010).
98. Yang, M.-H. *et al.* Bmi1 is essential in Twist1-induced epithelial-mesenchymal transition. *Nat. Cell Biol.* **12,** 982–92 (2010).
99. Fu, J. *et al.* The TWIST/Mi2/NuRD protein complex and its essential role in cancer metastasis. *Cell Res.* **21,** 275–89 (2011).
100. Peinado, H., Ballestar, E., Esteller, M. & Cano, A. Snail Mediates E-Cadherin Repression by the Recruitment of the Sin3A / Histone Deacetylase 1 (HDAC1)/ HDAC2 Complex. *Mol. Cell. Biol.* **24,** 306–319 (2004).
101. Lombaerts, M. *et al.* E-cadherin transcriptional downregulation by promoter methylation but not mutation is related to epithelial-to-mesenchymal transition in breast cancer cell lines. *Br. J. Cancer* **94,** 661–71 (2006).
102. Gregory, P. a *et al.* The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. *Nat. Cell Biol.* **10,** 593–601 (2008).
103. Park, S.-M., Gaur, A. B., Lengyel, E. & Peter, M. E. The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2. *Genes Dev.* **22,** 894–907 (2008).
104. Kim, N. H. *et al.* A p53/miRNA-34 axis regulates Snail1-dependent cancer cell epithelial-mesenchymal transition. *J. Cell Biol.* **195,** 417–33 (2011).
105. Siemens, H. *et al.* miR-34 and SNAIL form a double-negative feedback loop to regulate epithelial-mesenchymal transitions. *Cell Cycle* **10,** 4256–71 (2011).
106. Shimono, Y. *et al.* Downregulation of miRNA-200c links breast cancer stem cells with normal stem cells. *Cell* **138,** 592–603 (2009).
107. Warzecha, C. C. *et al.* An ESRP-regulated splicing programme is abrogated during the epithelial-mesenchymal transition. *EMBO J.* **29,** 3286–300 (2010).
108. Ghigna, C. *et al.* Cell motility is controlled by SF2/ASF through alternative splicing of the Ron protooncogene. *Mol. Cell* **20,** 881–90 (2005).
109. Savagner, P., Valles, A. M., Jouanneau, J., Yamada, K. M. & Thiery, J. P. Alternative Splicing in Fibroblast Growth Factor Receptor 2 Is Associated with

Induced Epithelial- Mesenchymal Transition in Rat Bladder Carcinoma Cells. *Mol Biol Cell* **5,** 851–862 (1994).
110. Yanagisawa, M. *et al.* A p120 catenin isoform switch affects Rho activity, induces tumor cell invasion, and predicts metastatic disease. *J. Biol. Chem.* **283,** 18344–54 (2008).
111. Elinav, E. *et al.* Inflammation-induced cancer: crosstalk between tumours, immune cells and microorganisms. *Nat. Rev. Cancer* **13,** 759–71 (2013).
112. Lesina, M. *et al.* Stat3/Socs3 activation by IL-6 transsignaling promotes progression of pancreatic intraepithelial neoplasia and development of pancreatic cancer. *Cancer Cell* **19,** 456–69 (2011).
113. Lee, J.-K. *et al.* Signal transducer and activator of transcription 3 (Stat3) contributes to T-cell homeostasis by regulating pro-survival Bcl-2 family genes. *Immunology* **140,** 288–300 (2013).
114. Schwitalla, S. *et al.* Intestinal tumorigenesis initiated by dedifferentiation and acquisition of stem-cell-like properties. *Cell* **152,** 25–38 (2013).
115. Suzuki, M. M. & Bird, A. DNA methylation landscapes: provocative insights from epigenomics. *Nat. Rev. Genet.* **9,** 465–76 (2008).
116. Sharp, A. J. *et al.* DNA methylation profiles of human active and inactive X chromosomes. *Genome Res.* **21,** 1592–600 (2011).
117. Jjingo, D., Conley, A. B., Yi, S. V, Lunyak, V. V & King, I. On the presence and role of human gene-body DNA methylation. *Oncotarget* **3,** 462–474 (2012).
118. Kim, J. & Dhanasekaran, S. Deep sequencing reveals distinct patterns of DNA methylation in prostate cancer. *Genome Res.* 1028–1041 (2011).
119. Maunakea, A. K., Chepelev, I., Cui, K. & Zhao, K. Intragenic DNA methylation modulates alternative splicing by recruiting MeCP2 to promote exon recognition. *Cell Res.* **23,** 1256–69 (2013).
120. Gonzalo, S. *et al.* DNA methyltransferases control telomere length and telomere recombination in mammalian cells. *Nat. Cell Biol.* **8,** 416–24 (2006).
121. Gopalakrishnan, S., Sullivan, B. a, Trazzi, S., Della Valle, G. & Robertson, K. D. DNMT3B interacts with constitutive centromere protein CENP-C to modulate DNA methylation and the histone code at centromeric regions. *Hum. Mol. Genet.* **18,** 3178–93 (2009).
122. Su, J. *et al.* Genome-wide dynamic changes of DNA methylation of repetitive elements in human embryonic stem cells and fetal fibroblasts. *Genomics* **99,** 10–7 (2012).
123. Feinberg, A. & Vogelstein, B. Hypomethlyation distinguishes genes of some human cancers from their normal counterparts. *Nature* **301,** 89–92 (1983).
124. Wahlfors, J. *et al.* Genomic hypomethylation in human chronic lymphocytic leukemia. *Blood* **80,** 2074–80 (1992).
125. Soares, J. *et al.* Global DNA Hypomethylation in Breast Carcinoma. *Cancer* **85,** 112–118 (1999).
126. Hernandez-Blazquez, F. J. *et al.* Evaluation of global DNA hypomethylation in human colon cancer tissues by immunohistochemistry and image analysis. *Gut* 689–693 (2000).
127. Hansen, K. D. *et al.* Increased methylation variation in epigenetic domains across cancer types. *Nat. Genet.* **43,** 768–75 (2011).
128. Akalin, A. *et al.* Base-pair resolution DNA methylation sequencing reveals profoundly divergent epigenetic landscapes in acute myeloid leukemia. *PLoS Genet.* **8,** e1002781 (2012).
129. Lander, E. Initial sequencing and analysis of the human genome. *Nature* **409,** (2001).
130. Antequera, F. & Bird, A. Number of CpG islands and genes in human and mouse. *Proc. Natl. Acad. Sci.* **90,** 11995–11999 (1993).
131. Toyota, M. CpG island methylator phenotype in colorectal cancer. *Proc. Natl. Acad. Sci.* **96,** 8681–8686 (1999).

132. Hinoue, T. *et al.* Genome-scale analysis of aberrant DNA methylation in colorectal cancer. *Genome Res.* **22,** 271–82 (2012).
133. Wiestler, B. *et al.* Assessing CpG island methylator phenotype, 1p/19q codeletion, and MGMT promoter methylation from epigenome-wide data in the biomarker cohort of the NOA-04 trial. *Neuro. Oncol.* 1–9 (2014).
134. Doi, A. *et al.* Differential methylation of tissue- and cancer-specific CpG island shores distinguishes human induced pluripotent stem cells, embryonic stem cells and fibroblasts. *Nat. Genet.* **41,** 1350–3 (2009).
135. Irizarry, R. a *et al.* The human colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores. *Nat. Genet.* **41,** 178–86 (2009).
136. Hovestadt, V. *et al.* Decoding the regulatory landscape of medulloblastoma using DNA methylation sequencing. *Nature* **510,** 537–41 (2014).
137. Rodriguez, J. *et al.* Chromosomal instability correlates with genome-wide DNA demethylation in human primary colorectal cancers. *Cancer Res.* **66,** 8462–9468 (2006).
138. Rizwana, R. & Hahn, P. CpG methylation reduces genomic instability. *J. Cell Sci.* **112,** 4513–4519 (1999).
139. Matros, E. *et al.* BRCA1 promoter methylation in sporadic breast tumors: relationship to gene expression profiles. *Breast Cancer Res. Treat.* **91,** 179–86 (2005).
140. Weller, M. *et al.* MGMT promoter methylation in malignant gliomas: ready for personalized medicine? *Nat. Rev. Neurol.* **6,** 39–51 (2010).
141. Agirre, X. *et al.* Methylation of CpG dinucleotides and/or CCWGG motifs at the promoter of TP53 correlates with decreased gene expression in a subset of acute lymphoblastic leukemia patients. *Oncogene* **22,** 1070–2 (2003).
142. Ito, S. *et al.* Role of Tet proteins in 5mC to 5hmC conversion , ES-cell self-renewal and inner cell mass specification. *Nature* **466,** 1129–1133 (2010).
143. Ko, M. *et al.* Impaired hydroxylation of 5-methylcytosine in myeloid cancers with mutant TET2. *Nature* 1–5 (2010). doi:10.1038/nature09586
144. Szwagierczak, A. *et al.* Characterization of PvuRts1I endonuclease as a tool to investigate genomic 5 – hydroxymethylcytosine. *Nucleic Acids Res.* 1–8 (2011).
145. Cortellino, S. *et al.* Thymine DNA Glycosylase Is Essential for Active DNA Demethylation by Linked Deamination-Base Excision Repair. *Cell* **146,** 67–79 (2011).
146. Mariani, C. J., Madzo, J., Moen, E. L., Yesilkanal, A. & Godley, L. a. Alterations of 5-hydroxymethylcytosine in human cancers. *Cancers (Basel).* **5,** 786–814 (2013).
147. Jin, S.-G., Wu, X., Li, A. X. & Pfeifer, G. P. Genomic mapping of 5-hydroxymethylcytosine in the human brain. *Nucleic Acids Res.* gkr120– (2011).
148. Wu, H. *et al.* Genome-wide analysis of 5-hydroxymethylcytosine distribution reveals its dual function in transcriptional regulation in mouse embryonic stem cells. *Genes Dev.* **25,** 679–684 (2011).
149. Ficz, G. *et al.* Dynamic regulation of 5-hydroxymethylcytosine in mouse ES cells and during differentiation. *Nature* **3,** (2011).
150. Song, C.-X. *et al.* Selective chemical labeling reveals the genome-wide distribution of 5-hydroxymethylcytosine. *Nat. Biotechnol.* (2010).
151. Spruijt, C. G. *et al.* Dynamic readers for 5-(hydroxy)methylcytosine and its oxidized derivatives. *Cell* **152,** 1146–59 (2013).
152. Yildirim, O. *et al.* Mbd3/NURD Complex Regulates Expression of 5-Hydroxymethylcytosine Marked Genes in Embryonic Stem Cells. *Cell* **147,** 1498–1510 (2011).
153. Iurlaro, M. *et al.* A screen for hydroxymethylcytosine and formylcytosine binding proteins suggests functions in transcription and chromatin regulation. *Genome Biol.* **14,** R119 (2013).

154. Haffner, M. *et al.* Global 5-hydroxymethylcytsoine content is significantly reduced in tissue stem/progenitor cell compartments and in human cancers. *Oncotarget* **2,** 627–637 (2011).
155. Lian, C. G. *et al.* Loss of 5-hydroxymethylcytosine is an epigenetic hallmark of melanoma. *Cell* **150,** 1135–1146 (2013).
156. Bhattacharyya, S. *et al.* Genome-wide hydroxymethylation tested using the HELP-GT assay shows redistribution in cancer. *Nucleic Acids Res.* **41,** e157 (2013).
157. Abdel-Wahab, O. *et al.* Genetic characterization of TET1, TET2, and TET3 alterations in myeloid malignancies. *Blood* **114,** 144–7 (2009).
158. Langemeijer, S. M. C., Aslanyan, M. G. & Jansen, J. H. TET proteins in malignant hematopoiesis. *Cell Cycle* **8,** 4044–4048 (2009).
159. Beer, P. A. *et al.* Two routes to leukemic transformation after a JAK2 mutation-positive myeloproliferative neoplasm. *Blood* **115,** 2891–900 (2010).
160. Colaizzo, D. *et al.* New TET2 gene mutations in patients with myeloproliferative neoplasms and splanchnic vein thrombosis. *J. Thromb. Haemost.* **8,** 1142–4 (2010).
161. Flach, J. *et al.* Mutations of JAK2 and TET2, but not CBL are detectable in a high portion of patients with refractory anemia with ring sideroblasts and thrombocytosis. *Haematologica* **95,** 518–9 (2010).
162. Szpurka, H. *et al.* Spectrum of mutations in RARS-T patients includes TET2 and ASXL1 mutations. *Leuk. Res.* **34,** 969–973 (2010).
163. Gelsi-Boyer, V. *et al.* Mutations of polycomb-associated gene ASXL1 in myelodysplastic syndromes and chronic myelomonocytic leukaemia. *Br. J. Haematol.* **145,** 788–800 (2009).
164. Schaub, F. X. *et al.* Clonal analysis of TET2 and JAK2 mutations suggests that TET2 can be a late event in the progression of myeloproliferative neoplasms. *Blood* **115,** 2003–7 (2010).
165. Mohamedali, A. M. *et al.* Novel TET2 mutations associated with UPD4q24 in myelodysplastic syndrome. *J. Clin. Oncol.* **27,** 4002–6 (2009).
166. Nibourel, O. *et al.* Incidence and prognostic value of TET2 alterations in de novo acute myeloid leukemia (AML) achieving complete remission. *Blood* 1132–1135 (2010).
167. Delhommeau, F. *et al.* Mutation in TET2 in myeloid cancers. *N. Engl. J. Med.* **360,** 2289–301 (2009).
168. Smith, A. E. *et al.* Next-generation sequencing of the TET2 gene in 355 MDS and CMML patients reveals low-abundance mutant clones with early origins, but indicates no definite prognostic value. *Blood* **116,** 3923–32 (2010).
169. Smith, Z. D. & Meissner, A. DNA methylation: roles in mammalian development. *Nat. Rev. Genet.* **14,** 204–20 (2013).
170. Li, Z. *et al.* Deletion of Tet2 in mice leads to dysregulated hematopoietic stem cells and subsequent development of myeloid malignancies. *Blood* **118,** 45094518 (2011).
171. Figueroa, M. E. *et al.* Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation. *Cancer Cell* **18,** 553–67 (2010).
172. Feng, J., Guo, X. & Chen, Y. Prognostic significance of IDH1 mutations in acute myeloid leukemia: a meta-analysis. *Am J Blood Res* **2,** 254–264 (2012).
173. Makishima, H. *et al.* CBL, CBLB, TET2, ASXL1, and IDH1/2 mutations and additional chromosomal aberrations constitute molecular events in chronic myelogenous leukemia. *Blood* **117,** e198–206 (2011).
174. Noushmehr, H. *et al.* Identification of a CpG island methylator phenotype that defines a distinct subgroup of glioma. *Cancer Cell* **17,** 510–22 (2010).
175. Ward, P. S. *et al.* The common feature of leukemia-associated IDH1 and IDH2 mutations is a neomorphic enzyme activity converting alpha-ketoglutarate to 2-hydroxyglutarate. *Cancer Cell* **17,** 225–34 (2010).

176. Vardabasso, C. *et al.* Histone variants: emerging players in cancer biology. *Cell. Mol. Life Sci.* **71,** 379–404 (2014).
177. Hondele, M. *et al.* Structural basis of histone H2A-H2B recognition by the essential chaperone FACT. *Nature* **499,** 111–4 (2013).
178. Liu, W. H., Roemer, S. C., Port, A. M. & Churchill, M. E. a. CAF-1-induced oligomerization of histones H3/H4 and mutually exclusive interactions with Asf1 guide H3/H4 transitions among histone chaperones and DNA. *Nucleic Acids Res.* **40,** 11229–39 (2012).
179. Jackson, V. & Chalkley, R. Histone synthesis and deposition in the G1 and S phases of hepatoma tissue culture cells. *Biochemistry* **24,** 6921–30 (1985).
180. Brickner, D. G. *et al.* H2A.Z-mediated localization of genes at the nuclear periphery confers epigenetic memory of previous transcriptional state. *PLoS Biol.* **5,** e81 (2007).
181. Greaves, I. K., Rangasamy, D., Ridgway, P. & Tremethick, D. J. H2A.Z contributes to the unique 3D structure of the centromere. *Proc. Natl. Acad. Sci. U. S. A.* **104,** 525–30 (2007).
182. Meneghini, M. D., Wu, M. & Madhani, H. D. Conserved histone variant H2A.Z protects euchromatin from the ectopic spread of silent heterochromatin. *Cell* **112,** 725–36 (2003).
183. Svotelis, A., Gévry, N., Grondin, G. & Gaudreau, L. H2A.Z overexpression promotes cellular proliferation of breast cancer cells. *Cell Cycle* **9,** 364–370 (2010).
184. Hua, S. *et al.* Genomic analysis of estrogen cascade reveals histone variant H2A.Z associated with breast cancer progression. *Mol. Syst. Biol.* **4,** 188 (2008).
185. Goldberg, A. D. *et al.* Distinct factors control histone variant H3.3 localization at specific genomic regions. *Cell* **140,** 678–91 (2010).
186. Hollenbach, A. D., McPherson, C. J., Mientjes, E. J., Iyengar, R. & Grosveld, G. Daxx and histone deacetylase II associate with chromatin through an interaction with core histones and the chromatin-associated protein Dek. *J. Cell Sci.* **115,** 3319–3330 (2002).
187. Lewis, P. W., Elsaesser, S. J., Noh, K.-M., Stadler, S. C. & Allis, C. D. Daxx is an H3.3-specific histone chaperone and cooperates with ATRX in replication-independent chromatin assembly at telomeres. *Proc. Natl. Acad. Sci. U. S. A.* **107,** 14075–80 (2010).
188. Jin, C. *et al.* H3.3/H2A.Z double variant-containing nucleosomes mark “nucleosome-free regions” of active promoters and other regulatory regions. *Nat. Genet.* **41,** 941–5 (2009).
189. Sturm, D. *et al.* Hotspot mutations in H3F3A and IDH1 define distinct epigenetic and biological subgroups of glioblastoma. *Cancer Cell* **22,** 425–37 (2012).
190. Bender, S. *et al.* Reduced H3K27me3 and DNA hypomethylation are major drivers of gene expression in K27M mutant pediatric high-grade gliomas. *Cancer Cell* **24,** 660–72 (2013).
191. Schwartzentruber, J. *et al.* Driver mutations in histone H3.3 and chromatin remodelling genes in paediatric glioblastoma. *Nature* **482,** 226–31 (2012).
192. Chung, C.-W. & Witherington, J. Progress in the discovery of small-molecule inhibitors of bromodomain--histone interactions. *J. Biomol. Screen.* **16,** 1170–85 (2011).
193. Dubuc, A. M. *et al.* Aberrant patterns of H3K4 and H3K27 histone lysine methylation occur across subgroups in medulloblastoma. *Acta Neuropathol.* **125,** 373–84 (2013).
194. Pugh, T. J. *et al.* Medulloblastoma exome sequencing uncovers subtype-specific somatic mutations. *Nature* **488,** 106–10 (2012).
195. Morin, R. D. *et al.* Frequent mutation of histone-modifying genes in non-Hodgkin lymphoma. *Nature* **476,** 298–303 (2011).
196. Mu, Z. *et al.* EZH2 knockdown suppresses the growth and invasion of human inflammatory breast cancer cells. *J. Exp. Clin. Cancer Res.* **32,** 70 (2013).

197. Yang, Y. A. & Yu, J. EZH2, an epigenetic driver of prostate cancer. *Protein Cell* **4,** 331–41 (2013).
198. Easwaran, H. *et al.* A DNA hypermethylation module for the stem/progenitor cell signature of cancer. *Genome Res.* **22,** 837–49 (2012).
199. Ohm, J. E. *et al.* A stem cell-like chromatin pattern may predispose tumor suppressor genes to DNA hypermethylation and heritable silencing. *Nat. Genet.* **39,** 237–42 (2007).
200. Yap, D. B. *et al.* Somatic mutations at EZH2 Y641 act dominantly through a mechanism of selectively altered PRC2 catalytic activity, to increase H3K27 trimethylation. *Blood* **117,** 2451–9 (2011).
201. Morin, R. D. *et al.* Somatic mutations altering EZH2 (Tyr641) in follicular and diffuse large B-cell lymphomas of germinal-center origin. *Nat. Genet.* **42,** 181–5 (2010).
202. Mar, B. G. *et al.* Sequencing histone-modifying enzymes identifies UTX mutations in acute lymphoblastic leukemia. *Leukemia* **26,** 1881–3 (2012).
203. Van Haaften, G. *et al.* Somatic mutations of the histone H3K27 demethylase gene UTX in human cancer. *Nat. Genet.* **41,** 521–3 (2009).
204. Jankowska, A. M. *et al.* Mutational spectrum analysis of chronic myelomonocytic leukemia includes genes associated with epigenetic regulation: UTX, EZH2, and DNMT3A. *Blood* **118,** 3932–41 (2011).
205. Lu, C. *et al.* IDH mutation impairs histone demethylation and results in a block to cell differentiation. *Nature* **483,** 474–8 (2012).
206. Avantaggiati, M. L. *et al.* Recruitment of p300/CBP in p53-Dependent Signal Pathways. *Cell* **89,** 1175–1184 (1997).
207. Gao, Y., Geng, J., Hong, X. & Qi, J. Expression of p300 and CBP is associated with poor prognosis in small cell lung cancer. *Int J Clin Exp Pathol* **7,** 760–767 (2014).
208. Iyer, N. G., Ozdag, H. & Caldas, C. p300/CBP and cancer. *Oncogene* **23,** 4225–31 (2004).
209. Issa, J.-P. J. *et al.* Phase 1 study of low-dose prolonged exposure schedules of the hypomethylating agent 5-aza-2'-deoxycytidine (decitabine) in hematopoietic malignancies. *Blood* **103,** 1635–40 (2004).
210. Burke, M. J. *et al.* A therapeutic trial of decitabine and vorinostat in combination with chemotherapy for relapsed/refractory acute lymphoblastic leukemia. *Am. J. Haematol.* **89,** 889–895 (2014).
211. Blum, W. *et al.* Phase I study of decitabine alone or in combination with valproic acid in acute myeloid leukemia. *J. Clin. Oncol.* **25,** 3884–91 (2007).
212. Blum, K. a *et al.* Phase I trial of low dose decitabine targeting DNA hypermethylation in patients with chronic lymphocytic leukaemia and non-Hodgkin lymphoma: dose-limiting myelosuppression without evidence of DNA hypomethylation. *Br. J. Haematol.* **150,** 189–95 (2010).
213. Glasspool, R. M. *et al.* A randomised, phase II trial of the DNA-hypomethylating agent 5-aza-2'-deoxycytidine (decitabine) in combination with carboplatin vs carboplatin alone in patients with recurrent, partially platinum-sensitive ovarian cancer. *Br. J. Cancer* **110,** 1923–9 (2014).
214. Nie, J., Liu, L., Li, X. & Han, W. Decitabine, a new star in epigenetic therapy: the clinical application and biological mechanism in solid tumors. *Cancer Lett.* **354,** 12–20 (2014).
215. Lovén, J. *et al.* Selective inhibition of tumor oncogenes by disruption of super-enhancers. *Cell* **153,** 320–34 (2013).
216. Delmore, J. E. *et al.* BET bromodomain inhibition as a therapeutic strategy to target c-Myc. *Cell* **146,** 904–17 (2011).
217. Mertz, J. A. *et al.* Targeting MYC dependence in cancer by inhibiting BET bromodomains. *Proc. Natl. Acad. Sci. U. S. A.* **108,** 16669–74 (2011).
218. Dawson, M. a *et al.* Inhibition of BET recruitment to chromatin as an effective treatment for MLL-fusion leukaemia. *Nature* **478,** 529–33 (2011).

219. A Phase I, Dose-finding Study of the Bromodomain (Brd) Inhibitor OTX015 in Haematological Malignancies. *https://clinicaltrials.gov/ct2/show/NCT01713582* (2012).
220. Hallek, M., Cheson, B. & Catovsky, D. Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia. *Blood* **111,** 5446–5457 (2008).
221. Matutes, E. *et al.* The immunological profile of B-cell disorders and proposal of a scoring system for the diagnosis of CLL. *Leukemia* **8,** 1640–5 (1994).
222. Oscier, D. G. *et al.* Multivariate analysis of prognostic factors in CLL : clinical stage , IGVH gene mutational status , and loss or mutation of the p53 gene are independent prognostic factors. *Blood* **100,** 1177–1185 (2002).
223. Hamblin, T., Davis, Z. & Gardiner, A. Unmutated Ig VH genes are associated with a more aggressive form of chronic lymphocytic leukemia. *Blood* **94,** 1848–1854 (1999).
224. Damle, R., Wasil, T., Fais, F. & Ghiotto, F. Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. *Blood* **94,** 1840–1847 (1999).
225. Stamatopoulos, K. *et al.* Over 20% of patients with chronic lymphocytic leukemia carry stereotyped receptors: Pathogenetic implications and clinical correlations. *Blood* **109,** 259–70 (2007).
226. Minden, M. D. *et al.* Chronic lymphocytic leukaemia is driven by antigen-independent cell-autonomous signalling. *Nature* **000,** 0–4 (2012).
227. Fais, F., Ghiotto, F. & Hashimoto, S. Chronic lymphocytic leukemia B cells express restricted sets of mutated and unmutated antigen receptors. *J. Clin. Invest.* **102,** 1515–1525 (1998).
228. Schroeder, H. W. & Dighiero, G. The pathogenesis of chronic lymphocytic leukemia: analysis of the antibody repertoire. *Immunol. Today* **15,** 288–294 (1994).
229. Seifert, M. *et al.* Cellular origin and pathophysiology of chronic lymphocytic leukemia. *J. Exp. Med.* **209,** 2183–98 (2012).
230. Rassenti, L. Z. *et al.* Relative value of ZAP-70, CD38, and immunoglobulin mutation status in predicting aggressive disease in chronic lymphocytic leukemia. *Blood* **112,** 1923–30 (2008).
231. Wiestner, A. *et al.* ZAP-70 expression identifies a chronic lymphocytic leukemia subtype with unmutated immunoglobulin genes , inferior clinical outcome , and distinct gene expression profile. *Blood* **101,** 4944–4951 (2003).
232. Chen, L. *et al.* ZAP-70 directly enhances IgM signaling in chronic lymphocytic leukemia. **105,** 2036–2042 (2015).
233. Gobessi, S. *et al.* ZAP-70 enhances B-cell – receptor signaling despite absent or inefficient tyrosine kinase activation in chronic lymphocytic leukemia and lymphoma B cells. **109,** 2032–2040 (2015).
234. Pede, V. *et al.* Expression of ZAP70 in chronic lymphocytic leukaemia activates NF-κB signalling. *Br. J. Haematol.* **163,** 621–30 (2013).
235. Richardson, S. J. ZAP-70 expression is associated with enhanced ability to respond to migratory and survival signals in B-cell chronic lymphocytic leukemia (B-CLL). *Blood* **107,** 3584–3592 (2006).
236. Döhner, H. *et al.* Genomic aberrations and survival in chronic lymphocytic leukemia. *N. Engl. J. Med.* 1910–1916 (2000).
237. Garding, A. *et al.* Epigenetic upregulation of lncRNAs at 13q14.3 in leukemia is linked to the In Cis downregulation of a gene cluster that targets NF-kB. *PLoS Genet.* **9,** e1003373 (2013).
238. Austen, B. *et al.* Mutations in the ATM gene lead to impaired overall and treatment-free survival that is independent of IGVH mutation status in patients with B-CLL. *Blood* **106,** 3175–3183 (2005).

239. Döhner, H. *et al.* 11q Deletions Identify a New Subset of B-Cell Chronic Lymphocytic Leukemia Characterized by Extensive Nodal Involvement and Inferior Prognosis. *Blood* **89,** 2516–2522 (1997).
240. Chiaretti, S. *et al.* Evaluation of TP53 mutations with the AmpliChip p53 research test in chronic lymphocytic leukemia: Correlation with clinical outcome and gene expression profiling. *Genes, Chromosom. Cancer* **274,** 263–274 (2011).
241. Zenz, T. *et al.* Monoallelic TP53 inactivation is associated with poor prognosis in chronic lymphocytic leukemia : results from a detailed genetic characterization with long-term follow-up. *Blood* **112,** 3322–3330 (2008).
242. Wang, L. & Lawrence, M. SF3B1 and other novel cancer genes in chronic lymphocytic leukemia. *N. Engl. J. Med.* **365,** 2497–2506 (2011).
243. Landau, D. a *et al.* Evolution and impact of subclonal mutations in chronic lymphocytic leukemia. *Cell* **152,** 714–26 (2013).
244. Puente, X. S. *et al.* Whole-genome sequencing identifies recurrent mutations in chronic lymphocytic leukaemia. *Nature* 1–5 (2011).
245. Quesada, V. *et al.* Exome sequencing identifies recurrent mutations of the splicing factor SF3B1 gene in chronic lymphocytic leukemia. *Nat. Genet.* (2011).
246. Seiffert, M., Stilgenbauer, S., Döhner, H. & Lichter, P. Efficient nucleofection of primary human B cells and B-CLL cells induces apoptosis, which depends on the microenvironment and on the structure of transfected nucleic acids. *Leuk. Off. J. Leuk. Soc. Am. Leuk. Res. Fund, U.K* **21,** 1977–83 (2007).
247. Herishanu, Y. *et al.* The lymph node microenvironment promotes B-cell receptor signaling, NF-kappaB activation, and tumor proliferation in chronic lymphocytic leukemia. *Blood* **117,** 563–74 (2011).
248. Lagneaux, L., Delforge, A. & Bron, D. Chronic lymphocytic leukemic B cells but not normal B cells are rescued from apoptosis by contact with normal bone marrow stromal cells. *Blood* **91,** 2387–2397 (1998).
249. Bhattacharya, N. *et al.* Nurse-like cells show deregulated expression of genes involved in immunocompetence. *Br. J. Haematol.* **154,** 349–56 (2011).
250. Ghia, P. *et al.* Chronic lymphocytic leukemia B cells are endowed with the capacity to attract CD4+, CD40L+ T cells by producing CCL22. *Eur. J. Immunol.* **32,** 1403 (2002).
251. Burger, J. A. *et al.* Blood-derived nurse-like cells protect chronic lymphocytic leukemia B cells from spontaneous apoptosis through stromal cell – derived factor-1. *Blood* **96,** 2655–2664 (2000).
252. Kurtova, A., Balakrishnan, K. & Chen, R. Diverse marrow stromal cells protect CLL cells from spontaneous and drug-induced apoptosis: development of a reliable and reproducible system to assess stromal cell adhesion-mediated drug resistance. *Blood* **114,** 4441–4451 (2009).
253. Tsukada, N., Burger, J. A., Zvaifler, N. J. & Kipps, T. J. Distinctive features of " nurselike " cells that differentiate in the context of chronic lymphocytic leukemia. *Blood* **99,** 1030–1038 (2002).
254. Lutzny, G. *et al.* Protein Kinase C-β-Dependent Activation of NF-κB in Stromal Cells Is Indispensable for the Survival of Chronic Lymphocytic Leukemia B Cells In Vivo. *Cancer Cell* **23,** 77–92 (2013).
255. Pontikoglou, C. *et al.* Study of the quantitative, functional, cytogenetic, and immunoregulatory properties of bone marrow mesenchymal stem cells in patients with B-cell chronic lymphocytic leukemia. *Stem Cells Dev.* **22,** 1329–41 (2013).
256. Burger, J. a *et al.* High-level expression of the T-cell chemokines CCL3 and CCL4 by chronic lymphocytic leukemia B cells in nurselike cell cocultures and after BCR stimulation. *Blood* **113,** 3050–8 (2009).
257. Riches, J. C. *et al.* T cells from CLL patients exhibit features of T-cell exhaustion but retain capacity for cytokine production. *Blood* **121,** 1612–1622 (2013).
258. Görgün, G., Holderried, T. A. W., Zahrieh, D., Neuberg, D. & Gribben, J. G. Chronic lymphocytic leukemia cells induce changes in gene expression of CD4 and CD8 T cells. *J. Clin. Invest.* **115,** 1797–1805 (2005).

259. Ramsay, A. G. *et al.* Chronic lymphocytic leukemia T cells show impaired immunological synapse formation that can be reversed with an immunomodulating drug. *J. Clin. Invest.* **118,** 2427–37 (2008).
260. Fabris, S. *et al.* Biological and clinical relevance of quantitative global methylation of repetitive DNA sequences in chronic lymphocytic leukemia. *Epigenetics* **6,** 188–194 (2011).
261. Claus, R. *et al.* Quantitative DNA methylation analysis identifies a single CpG dinucleotide important for ZAP-70 expression and predictive of prognosis in chronic lymphocytic leukemia. *J. Clin. Oncol.* **30,** 2483–91 (2012).
262. Kanduri, M. *et al.* Differential genome-wide array-based methylation profiles in prognostic subsets of chronic lymphocytic leukemia. *Blood* **115,** 296–305 (2010).
263. Raval, A. *et al.* Downregulation of death-associated protein kinase 1 (DAPK1) in chronic lymphocytic leukemia. *Cell* **129,** 879–90 (2007).
264. Hanada, B. M., Delia, D., Aiello, A., Stadtmauer, E. & Reed, J. C. Gene Hypomethylation and High-Level Expression in B-Cell Chronic Lymphocytic Leukemia. *Blood* **82,** 1820–1828 (1993).
265. Pei, L. *et al.* Genome-wide DNA methylation analysis reveals novel epigenetic changes in chronic lymphocytic leukemia. *Epigenetics* **7,** 1–12 (2012).
266. Chim, C. S., Pang, R. & Liang, R. Epigenetic dysregulation of the Wnt signalling pathway in chronic lymphocytic leukaemia. *J. Clin. Pathol.* **61,** 1214–9 (2008).
267. Gutierrez, A. *et al.* Differentiation of chronic lymphocytic leukemia B cells into immunoglobulin secreting cells decreases LEF-1 expression. *PLoS One* **6,** e26056 (2011).
268. Irving, L. *et al.* Methylation markers identify high risk patients in IGHV mutated chronic lymphocytic leukemia. *Epigenetics* **6,** 300–306 (2011).
269. Baer, C. *et al.* Extensive promoter DNA hypermethylation and hypomethylation is associated with aberrant microRNA expression in chronic lymphocytic leukemia. *Cancer Res.* **72,** 3775–85 (2012).
270. Cahill, N. & Rosenquist, R. Uncovering the DNA methylome in chronic lymphocytic leukemia. *Epigenetics* **8,** 138–48 (2013).
271. Kulis, M. *et al.* Epigenomic analysis detects widespread gene-body DNA hypomethylation in chronic lymphocytic leukemia. *Nat. Genet.* **44,** 1236–1242 (2012).
272. Cahill, N. *et al.* 450K-array analysis of chronic lymphocytic leukemia cells reveals global DNA methylation to be relatively stable over time and similar in resting and proliferative compartments. *Leuk. Off. J. Leuk. Soc. Am. Leuk. Res. Fund, U.K* 150–158 (2012).
273. Landau, D. a *et al.* Locally Disordered Methylation Forms the Basis of Intratumor Methylome Variation in Chronic Lymphocytic Leukemia. *Cancer Cell* **26,** 813–825 (2014).
274. Oakes, C. C. *et al.* Evolution of DNA methylation is linked to genetic aberrations in chronic lymphocytic leukemia. *Cancer Discov.* **4,** 348–61 (2014).
275. Dunwoodie, S. & Henrique, D. Mouse Dll3: a novel divergent Delta gene which may complement the function of other Delta homologues during early pattern formation in the mouse embryo. *Development* **3076,** 3065–3076 (1997).
276. Shawber, C., Boulter, J., Lindsell, C. E. & Weinmaster, G. Jagged 2 : A Serrate-like Gene Expressed during Rat Embryogenesis. *Dev. Biol.* **180,** 370–376 (1996).
277. Jaleco, A. C. *et al.* Differential Effects of Notch Ligands Delta-1 and Jagged-1 in Human Lymphoid Differentiation. *J. Exp. Med* **194,** 991–1001 (2001).
278. Strooper, B. De *et al.* A presenilin-1-dependent g -secretase-like protease mediates release of Notch intracellular domain. *Nature* **398,** 518–522 (1999).
279. Wu, L., Aster, J. & Blacklow, S. MAML1, a human homologue of Drosophila mastermind, is a transcriptional co-activator for NOTCH receptors. *Nat. Genet.* **26,** 484–489 (2000).

280. Nam, Y., Sliz, P., Song, L., Aster, J. C. & Blacklow, S. C. Structural basis for cooperativity in recruitment of MAML coactivators to Notch transcription complexes. *Cell* **124,** 973–83 (2006).
281. Ronchini, C. & Capobianco, A. Induction of cyclin D1 transcription and CDK2 activity by Notchic: implication for cell cycle disruption in transformation by Notchic. *Mol. Cell. Biol.* **21,** 5925–5934 (2001).
282. Ghaleb, A. M., Aggarwal, G., Bialkowska, A. B., Nandan, M. O. & Yang, V. W. Notch inhibits expression of the Krüppel-like factor 4 tumor suppressor in the intestinal epithelium. *Mol. Cancer Res.* **6,** 1920–7 (2008).
283. Real, P. J. *et al.* Gamma-secretase inhibitors reverse glucocorticoid resistance in T cell acute lymphoblastic leukemia. *Nat. Med.* **15,** 50–8 (2009).
284. Chen, H.-F. *et al.* Twist1 induces endothelial differentiation of tumour cells through the Jagged1-KLF4 axis. *Nat. Commun.* **5,** 4697 (2014).
285. Hale, A. T. *et al.* Endothelial Kruppel-like factor 4 regulates angiogenesis and the Notch signaling pathway. *J. Biol. Chem.* **289,** 12016–28 (2014).
286. Liu, Z. *et al.* Epithelial transformation by KLF4 requires Notch1 but not canonical Notch1 signaling. *Cancer Biol. Ther.* **8,** 1840–1851 (2009).
287. Lambertini, C., Pantano, S. & Dotto, G. P. Differential control of Notch1 gene transcription by Klf4 and Sp3 transcription factors in normal versus cancer-derived keratinocytes. *PLoS One* **5,** e10369 (2010).
288. Miyamoto, S., Nakanishi, M. & Rosenberg, D. W. Suppression of colon carcinogenesis by targeting Notch signaling. *Carcinogenesis* **34,** 2415–23 (2013).
289. Zang, S. *et al.* RNAi-mediated knockdown of Notch-1 leads to cell growth inhibition and enhanced chemosensitivity in human breast cancer. *Oncol. Rep.* **23,** 893–899 (2010).
290. Weng, A. P. *et al.* Activating Mutations of NOTCH1 in Human T Cell Acute Lymphoblastic Leukemia. *Science (80-.).* **306,** 269–272 (2004).
291. O'Neil, J. *et al.* FBW7 mutations in leukemic cells mediate NOTCH pathway activation and resistance to gamma-secretase inhibitors. *J. Exp. Med.* **204,** 1813–24 (2007).
292. Lin, Y., Nichols, R. A., Letterio, J. J. & Aplan, P. D. Notch1 mutations are important for leukemic transformation in murine models of precursor-T leukemia / lymphoma. *Blood* **107,** 2540–2544 (2006).
293. Zou, J. *et al.* Notch1 is required for hypoxia-induced proliferation, invasion and chemoresistance of T-cell acute lymphoblastic leukemia cells. *J. Hematol. Oncol.* **6,** 3 (2013).
294. Rao, S. S. *et al.* Inhibition of NOTCH signaling by gamma secretase inhibitor engages the RB pathway and elicits cell cycle exit in T-cell acute lymphoblastic leukemia cells. *Cancer Res.* **69,** 3060–8 (2009).
295. Xu, Z.-S. *et al.* Constitutive activation of NF-κB signaling by NOTCH1 mutations in chronic lymphocytic leukemia. *Oncol. Rep.* 1–6 (2015). doi:10.3892/or.2015.3762
296. Rosati, E. *et al.* Constitutively activated Notch signaling is involved in survival and apoptosis resistance of B-CLL cells. *Blood* **113,** 856–65 (2009).
297. Nwabo Kamdje, a H. *et al.* Role of stromal cell-mediated Notch signaling in CLL resistance to chemotherapy. *Blood Cancer J.* **2,** e73 (2012).
298. Fabbri, G. *et al.* Analysis of the chronic lymphocytic leukemia coding genome: role of NOTCH1 mutational activation. *J. Exp. Med.* **208,** 1389–401 (2011).
299. Willander, K. *et al.* NOTCH1 mutations influence survival in chronic lymphocytic leukemia patients. *BMC Cancer* **13,** 274 (2013).
300. Rossi, D. *et al.* Molecular history of Richter Syndrome: origin from a cell already present at the time of CLL diagnosis. *Int. J. Cancer* 1–18
301. Takahashi, K. *et al.* Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131,** 861–72 (2007).
302. Kim, M. O. *et al.* ERK1 and ERK2 regulate embryonic stem cell self-renewal through phosphorylation of Klf4. *Nat. Struct. Mol. Biol.* **19,** 283–90 (2012).

303. Pandya, A. Y. *et al.* Nuclear Localization of KLF4 Is Associated with an Aggressive Phenotype in Early-Stage Breast Cancer Nuclear Localization of KLF4 Is Associated with an Aggressive Phenotype in Early-Stage Breast Cancer. *Clin. Cancer Res.* 2709–2719 (2004).
304. Rowland, B., Bernards, R. & Peeper, D. The KLF4 tumour suppressor is a transcriptional repressor of p53 that acts as a context-dependent oncogene. *Nat. Cell Biol.* **7,** 1074–82 (2005).
305. Huang, Y., Chen, J., Lu, C., Han, J. & Wang, G. HDAC1 and Klf4 interplay critically regulates human myeloid leukemia cell proliferation. *Cell Death Dis.* **5,** e1491 (2014).
306. Feinberg, M. W. *et al.* The Kruppel-like factor KLF4 is a critical regulator of monocyte differentiation. *EMBO J.* **26,** 4138–48 (2007).
307. Alder, J. & Georgantas, R. Kruppel-like factor 4 is essential for inflammatory monocyte differentiation in vivo. *J. Immunol.* **180,** 5645–5652 (2008).
308. Wen, X., Liu, H., Xiao, G. & Liu, X. Downregulation of the transcription factor KLF4 is required for the lineage commitment of T cells. *Cell Res.* **21,** 1701–10 (2011).
309. Yamada, T., Park, C. S., Mamonkin, M. & Lacorazza, H. D. Transcription factor ELF4 controls the proliferation and homing of CD8+ T cells via the Krüppel-like factors KLF4 and KLF2. *Nat. Immunol.* **10,** 618–26 (2009).
310. Good, K. & Tangye, S. Decreased expression of Krüppel-like factors in memory B cells induces the rapid response typical of secondary antibody responses. *PNAS* **104,** 13420–13425 (2007).
311. Li, H. *et al.* Krüppel-like factor 4 promotes differentiation by transforming growth factor-beta receptor-mediated Smad and p38 MAPK signaling in vascular smooth muscle cells. *J. Biol. Chem.* **285,** 17846–56 (2010).
312. Tai, S.-K. *et al.* Persistent Krüppel-like factor 4 expression predicts progression and poor prognosis of head and neck squamous cell carcinoma. *Cancer Sci.* **102,** 895–902 (2011).
313. Yoon, O. & Roh, J. Downregulation of KLF4 and the Bcl-2/Bax ratio in advanced epithelial ovarian cancer. *Oncol. Lett.* **4,** 1033–1036 (2012).
314. Yang, W.-T. & Zheng, P.-S. Promoter hypermethylation of KLF4 inactivates its tumor suppressor function in cervical carcinogenesis. *PLoS One* **9,** e88827 (2014).
315. Li, H. *et al.* Epigenetic inactivation of KLF4 is associated with urothelial cancer progression and early recurrence. *J. Urol.* **191,** 493–501 (2014).
316. Faber, K., Bullinger, L. & Ragu, C. CDX2-driven leukemogenesis involves KLF4 repression and deregulated PPARγ signaling. *J. Clin. Invest.* **123,** 299–314 (2013).
317. Guan, H. *et al.* KLF4 is a tumor suppressor in B-cell non-Hodgkin lymphoma and in classic Hodgkin lymphoma. *Blood* **116,** 1469–78 (2010).
318. Kharas, M. G. *et al.* KLF4 suppresses transformation of pre-B cells by ABL oncogenes. *Blood* **109,** 747–55 (2007).
319. Garding, A. Genetic and Epigenetic Lesions in Chronic Lymphocytic Leukemia (CLL): The impact of aberrations in single genes, complex loci and whole genomes onthe pathomechanism of CLL. 191 (2010).
320. Gebhard, C. *et al.* Genome-wide profiling of CpG methylation identifies novel targets of aberrant hypermethylation in myeloid leukemia. *Cancer Res.* **66,** 6118–28 (2006).
321. Ehrich, M. *et al.* Quantitative high-throughput analysis of DNA methylation patterns by base-specific cleavage and mass spectrometry. *Proc. Natl. Acad. Sci. U. S. A.* **102,** 15785–90 (2005).
322. Smyth, G. K. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat. Appl. Genet. Mol. Biol.* **3,** Article3 (2004).
323. Göbel, M. *et al.* Progranulin is a novel independent predictor of disease progression and overall survival in chronic lymphocytic leukemia. *PLoS One* **8,** e72107 (2013).

324. Stilgenbauer, S. *et al.* Subcutaneous alemtuzumab in fludarabine-refractory chronic lymphocytic leukemia: clinical results and prognostic marker analyses from the CLL2H study of the German Chronic Lymphocytic Leukemia Study Group. *J. Clin. Oncol.* **27,** 3994–4001 (2009).
325. Goede, V. *et al.* Interactions between comorbidity and treatment of chronic lymphocytic leukemia: results of German Chronic Lymphocytic Leukemia Study Group trials. *Haematologica* **99,** 1095–100 (2014).
326. Hallek, M. *et al.* Addition of rituximab to fludarabine and cyclophosphamide in patients with chronic lymphocytic leukaemia: a randomised, open-label, phase 3 trial. *Lancet* **376,** 1164–1174 (2010).
327. Bichi, R. *et al.* Human chronic lymphocytic leukemia modeled in mouse by targeted TCL1 expression. *Proc. Natl. Acad. Sci. U. S. A.* **99,** 6955–60 (2002).
328. Pekarsky, Y., Zanesi, N., Aqeilan, R. I. & Croce, C. M. Animal models for chronic lymphocytic leukemia. *J. Cell. Biochem.* **100,** 1109–18 (2007).
329. Pepper, C., Hoy, T. & Bentley, D. Bcl-2/Bax ratios in chronic lymphocytic leukaemia and their correlation with in vitro apoptosis and clinical resistance. *Br. J. Cancer* **76,** 935–938 (1997).
330. Martinez-Lostao, L. *et al.* Role of the STAT1 pathway in apoptosis induced by fludarabine and JAK kinase inhibitors in B-cell chronic lymphocytic leukemia. *Leuk. Lymphoma* **46,** 435–442 (2005).
331. Bhattacharya, N. *et al.* Loss of cooperativity of secreted CD40L and increased dose-response to IL4 on CLL cell viability correlates with enhanced activation of NF-kB and STAT6. *Int. J. Cancer* **136,** 65–73 (2015).
332. Spaner, D. E. *et al.* Immunomodulatory effects of Toll-like receptor-7 activation on chronic lymphocytic leukemia cells. *Leukemia* **20,** 286–95 (2006).
333. Jelinek, D. F. *et al.* Identification of a Global Gene Expression Signature of B-Chronic Lymphocytic Leukemia. *Mol. Cancer Res.* **1,** 346–361 (2003).
334. Jr, A. G., Tschumper, R. & Wu, X. LEF-1 is a prosurvival factor in chronic lymphocytic leukemia and is expressed in the preleukemic state of monoclonal B-cell lymphocytosis. *Blood* **116,** 2975–2984 (2010).
335. Dai, Z., Chen, Q., Lu, H. & Xie, Y. Defective expression and modulation of B7-2/CD86 on B cells in B cell chronic lymphocytic leukemia. *Int. J. Hematol.* **89,** 656–63 (2009).
336. Hutterer, E. *et al.* CD18 (ITGB2) expression in chronic lymphocytic leukaemia is regulated by DNA methylation-dependent and -independent mechanisms. *Br. J. Haematol.* **18,** 10–13 (2014).
337. Cao, W. *et al.* Regulation of TLR7/9 responses in plasmacytoid dendritic cells by BST2 and ILT7 receptor interaction. *J. Exp. Med.* **206,** 1603–14 (2009).
338. Cao, W. *et al.* Plasmacytoid dendritic cell-specific receptor ILT7-Fc epsilonRI gamma inhibits Toll-like receptor-induced interferon production. *J. Exp. Med.* **203,** 1399–405 (2006).
339. Cho, M. *et al.* SAGE library screening reveals ILT7 as a specific plasmacytoid dendritic cell marker that regulates type I IFN production. *Int. Immunol.* **20,** 155–64 (2008).
340. Rosén, A. *et al.* Lymphoblastoid cell line with B1 cell characteristics established from a chronic lymphocytic leukemia clone by in vitro EBV infection. *Oncoimmunology* **1,** 18–27 (2012).
341. Sellick, G. S. *et al.* Scan of 977 nonsynonymous SNPs in CLL4 trial patients for the identification of genetic variants influencing prognosis. *Blood* **111,** 1625–33 (2008).
342. Klaewsongkram, J. *et al.* Krüppel-like factor 4 regulates B cell number and activation-induced B cell proliferation. *J. Immunol.* **179,** 4679–84 (2007).
343. Li, Z. *et al.* KLF4 promotes hydrogen-peroxide-induced apoptosis of chronic myeloid leukemia cells involving the bcl-2/bax pathway. *Cell Stress Chaperones* **15,** 905–12 (2010).

344. Wei, D., Kanai, M., Jia, Z., Le, X. & Xie, K. Krüppel-like factor 4 induces p27Kip1 expression in and suppresses the growth and metastasis of human pancreatic cancer cells. *Cancer Res.* 4631–4640 (2008).
345. Tolia, A. & De Strooper, B. Structure and function of gamma-secretase. *Semin. Cell Dev. Biol.* **20,** 211–8 (2009).
346. Schroeter, E., Kisslinger, J. & Kopan, R. Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. *Nature* **393,** 382–386 (1998).
347. Rosati, E. *et al.* γ-Secretase inhibitor I induces apoptosis in chronic lymphocytic leukemia cells by proteasome inhibition, endoplasmic reticulum stress increase and notch down-regulation. *Int. J. Cancer* **132,** 1940–53 (2013).
348. Cohen, B. *et al.* Cyclin D1 is a direct target of JAG1-mediated Notch signaling in breast cancer. *Breast Cancer Res. Treat.* **123,** 113–24 (2010).
349. Nefedova, Y., Cheng, P., Alsina, M., Dalton, W. S. & Gabrilovich, D. I. Involvement of Notch-1 signaling in bone marrow stroma – mediated de novo drug resistance of myeloma and other malignant lymphoid cell lines. *Blood* **103,** 3503–3510 (2004).
350. Nefedova, Y., Sullivan, D. M., Bolick, S. C., Dalton, W. S. & Gabrilovich, D. I. Inhibition of Notch signaling induces apoptosis of myeloma cells and enhances sensitivity to chemotherapy. *Neoplasia* **111,** 2220–2229 (2008).
351. Hammadi, A., Billard, C., Faussat, A.-M. & Kolb, J.-P. Stimulation of iNOS expression and apoptosis resistance in B-cell chronic lymphocytic leukemia (B-CLL) cells through engagement of Toll-like receptor 7 (TLR-7) and NF-kappaB activation. *Nitric Oxide* **19,** 138–45 (2008).
352. Feinberg, M. W. *et al.* Kruppel-like factor 4 is a mediator of proinflammatory signaling in macrophages. *J. Biol. Chem.* **280,** 38247–58 (2005).
353. Kaisho, T. *et al.* Ikb Kinase a Is Essential for Mature B Cell Development and Function. *J. Exp. Med.* **193,** 417–426 (2001).
354. DiDonato, J. A., Hayakawa, M., Rothwarf, D. M., Zandi, E. & Karin, M. A cytokine-responsive IkB kinase that activates the transcription factor NF-kB. *Nature* **388,** (1997).
355. Li, Z.-W. *et al.* The IKK Subunit of I B Kinase (IKK) is Essential for Nuclear Factor B Activation and Prevention of Apoptosis. *J. Exp. Med.* **189,** 1839–1845 (1999).
356. Issa, J.-P. J. DNA Methylation As a Clinical Marker in Oncology. *J. Clin. Oncol.* **30,** 2566–2568 (2012).
357. Kulis, M., Queirós, A. C., Beekman, R. & Martín-Subero, J. I. Intragenic DNA methylation in transcriptional regulation, normal differentiation and cancer. *Biochim. Biophys. Acta* **1829,** 1161–74 (2013).
358. Ezkurdia, I. *et al.* Multiple evidence strands suggest that there may be as few as 19 000 human protein-coding genes. *Hum. Mol. Genet.* **23,** 5866–78 (2014).
359. Hanna, J. *et al.* Direct Reprogramming of Terminally Differntiated Mature B Lymphocytes To Pluripotency. *Cell* **133,** 250–264 (2008).
360. Chiorazzi, N. & Ferrarini, M. B CELL CHRONIC LYMPHOCYTIC LEUKEMIA : Lessons Learned from Studies of the B Cell Antigen Receptor Biases in V Gene Use. *Annu. Rev. Immunol.* **21,** 841–94 (2003).
361. Barthet, G., Georgakopoulos, A. & Robakis, N. K. Cellular mechanisms of γ-secretase substrate selection, processing and toxicity. *Prog. Neurobiol.* **98,** 166–75 (2012).
362. Saito, N. *et al.* A High Notch Pathway Activation Predicts Response to y Secretase Inhibitors in Proneural Subtype of Glioma Tumor-Initiating Cells. *Stem Cells* **32,** 301–312 (2014).
363. Zheng, H. *et al.* KLF4 gene expression is inhibited by the notch signaling pathway that controls goblet cell differentiation in mouse gastrointestinal tract. *Am. J. Physiol. Gastrointest. Liver Physiol.* **296,** G490–8 (2009).

364. Mahatan, C. & Kaestner, K. Characterization of the structure and regulation of the murine gene encoding gut-enriched Krüppel-like factor (Krüppel-like factor 4). *Nucleic Acids Res.* **27,** 4562–4569 (1999).
365. Bjerke, G. a, Hyman-Walsh, C. & Wotton, D. Cooperative transcriptional activation by Klf4, Meis2, and Pbx1. *Mol. Cell. Biol.* **31,** 3723–33 (2011).
366. Brandt, T., Townsley, F. M., Teufel, D. P., Freund, S. M. V & Veprintsev, D. B. Molecular Basis for Modulation of the p53 Target Selectivity by KLF4. *PLoS One* **7,** e48252 (2012).
367. Shie, J. L., Chen, Z. Y., Fu, M., Pestell, R. G. & Tseng, C. C. Gut-enriched Krüppel-like factor represses cyclin D1 promoter activity through Sp1 motif. *Nucleic Acids Res.* **28,** 2969–76 (2000).
368. Cuní, S. *et al.* A sustained activation of PI3K/NF-kappaB pathway is critical for the survival of chronic lymphocytic leukemia B cells. *Leukemia* **18,** 1391–400 (2004).
369. Ringshausen, I. *et al.* Constitutively activated phosphatidylinositol-3 kinase (PI-3K) is involved in the defect of apoptosis in B-CLL : association with protein kinase Cd. *Blood* **100,** 3741–3748 (2002).
370. Hoellenriegel, J. *et al.* The phosphoinositide 3'-kinase delta inhibitor , CAL-101 , inhibits B-cell receptor signaling and chemokine networks in chronic lymphocytic leukemia. *Blood* **118,** 3603–3613 (2011).
371. Brown, J. R. *et al.* Idelalisib , an inhibitor of phosphatidylinositol 3-kinase p110d , for relapsed / refractory chronic lymphocytic leukemia. *Blood* **123,** 3390–3397 (2014).
372. Byrd, J. C. *et al.* Targeting BTK with ibrutinib in relapsed chronic lymphocytic leukemia. *N. Engl. J. Med.* **369,** 32–42 (2013).
373. Thomas, M. *et al.* Notch activity synergizes with B-cell – receptor and CD40 signaling to enhance B-cell activation. *Blood* **109,** 3342–3351 (2007).

3. List of figures

4. Publications

Epigenetic upregulation of lncRNAs at 13q14.3 in leukemias linked to the In Cis downregulation of a gene cluster that targets NF-kB. **PloS Genet. 2013 Apr;9(4)**
Garding A, Bhattacharya N, Claus R, Ruppel M, Tschuch C, **Filarsky K**, … Mertens D.

Stem cell factor KLF4 is inactivated by promoter DNA-methylation and repressed by NOTCH signaling in CLL. **submitted**
Filarsky K, **Garding A**,… Mertens D.

5. Danksagung

Zunächst ein großes Dankeschön an Dr. Daniel Mertens, für die Möglichkeit meine Doktorarbeit in seiner Abteilung zu machen. Ich danke ihm für die Unterstützung bei meinen Projekten, die zahlreichen Diskussionen und den wissenschaftlichen Austausch. Aber auch für die schönen Anekdoten von „damals“ und für seine unzerstörerische positive Einstellung gegenüber seinen permanent pessimistischen Doktorandinnen ;-) Des Weiteren ein großes Dankeschön an die gesamte Kooperationseinheit „Mechanismen der Leukämogenese“ in Ulm und in Heidelberg.

Vielen Dank an Prof. Peter Lichter für seine große wissenschaftliche Expertise, die Diskussionen in zahlreichen Seminaren und TAC Meetings und nicht zuletzt dafür das er unsere kleine B061-Heidelberg Truppe so herzlich in seine Abteilung aufnimmt. Auch an alle anderen Mitglieder der Abteilungen B060 und B062 ein großes Dankeschön für die gute Zusammenarbeit und die spaßigen Retreats, viele lustige und amüsante Mittagessen, Weihnachtsfeiern und Betriebsausflüge.

Bei Dr. Karsten Rippe und Dr. Claudia Scholl möchte ich mich für die hilfreichen Diskussion und den starken Input bei meinen TAC Meetings bedanken.

Danke auch an Prof. Elmar Schiebel und Prof. Harald Herrmann-Lerdon für die Bereitschaf als Prüfer an meiner Verteidigung teilzunehmen.

Tine, an dich ein riesiges Danke für die tolle Zusammenarbeit, zahlreiche Diskussionen, Korrekturen und Zellkultur-Kooperationen. Wichtig waren natürlich vor allem die gemeinsamen korreli-korrelas und Wonderfulplots, all der Obstsalat (manchmal sogar mit Grießbrei) sowie final der Lettitor. Aber natürlich auch unsere tollen Urlaube, Wandertouren, Pizza-Nights, Feierabendbierchen und Ausflüge mit den beiden Mädels. Vielen Dank auch an Mama Wolf für die Verköstigung mit Kuchen, Bärlauchbutter, Marmelade, Zucchini…

Bei meinen Mädels Tine, Elle, Sabrina, Susanne und Sonja bedanke ich mich für den Spaß im Labor und auch außerhalb. Vor allem die zahlreichen, sonntagnachmittäglichen BBQs inklusive unzähliger Flaschen Sekt haben mir über die stressigsten Zeiten geholfen.

Außerdem danke ich allen unseren tollen TAs, Sybille, Petra, Frauke, Verena und Magdalena und allen anderen, die mir so oft mir Rat und Tat zur Seite standen.

Steffi und Daniela, euch danke ich für eine jahrelange wunderbare Freundschaf, die vielen Telefonate und und und…

Stefan, du warst die beste Entdeckung die ich in Heidelberg gemacht habe. Danke für deine Unterstützung in den letzten Monaten und dafür dass du meine Launen so tapfer ertragen hast!

Und nicht zuletzt, von ganzem Herzen ein großes Dankeschön an meine Eltern und meine Geschwister Michael und Sabine. Während meinem gesamten Werdegang habt ihr mich immer auf ganzer Linie und bei all meinen Entscheidungen unterstützt. Ihr hattet immer ein offenes Ohr für meine Sorgen. Ohne euch wäre ich nie so weit gekommen.