The role of epigenetic modifiers in Non-Hodgkin B cell lymphoma

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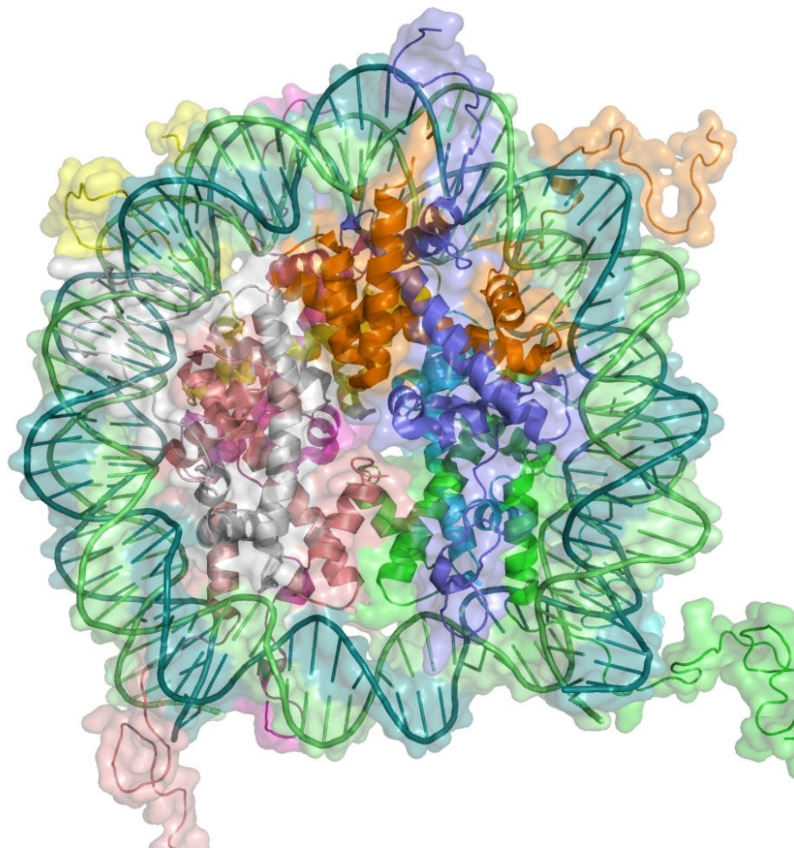

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Abstract

Disruptions in the B cell differentiation process leading to translocations and mutations partly explain the development of Non-Hodgkin lymphomas (NHL). Recently, epigenetic mechanisms were also found to play an important role in NHL, including cytosine methylation, histone modifications and chromatin remodeling. Several of these processes were observed to be deregulation in lymphoma cells due to alterations (mutations or changed expression) in the enzymes involved. This essay gives an overview of the epigenetic modifiers that have been reported to be involved in NHL.

Changes in the function or expression of TET2, DNMT3b, MLL2, EZH2, BMI1, JAK2, CBP/P300, ARID1A and BRG1 have been found in NHL cells. Functional studies indicated that these alterations change the expression of the target genes of the epigenetic modifiers, contributing to B cell malignancy. Most alterations affected histone modifications (MLL2 mutation, EZH2 mutation/overexpression, BMI1 deregulated expression, JAK2 amplification, CBP/P300 mutation). Mutated TET2 and overexpression of DNMT3b deregulated DNA methylation, and ARID1A and BRG1 mutations disrupted the functioning of chromatin remodeling complexes. Most mutations detected lead to a loss of function in the proteins, except for the mutation in EZH2, which resulted in enhanced activity.

Overall, a lot of epigenetic modifiers involved in NHL have already been described, but more research is needed to identify all important epigenetic enzymes, their target genes and the interactions between the epigenetic mechanisms. Ultimately, this knowledge can be used for diagnostic and therapeutic purposes.

Introduction

B cell lymphoma is a collective term for a variety of morphologic and clinical syndromes. Currently 15 different B cell lymphomas are distinguished in the official World Health Organization classification (Campo et al. 2011). The oldest and most important distinction is made between Hodgkin and Non-Hodgkin lymphoma (NHL). In Hodgkin lymphoma typical cancer cells can be found (Reed-Sternberg cells) that are the hallmark of the disease. These cells derive from B cells (Kuppers, Rajewsky 1998). On the other hand, cancer cells in biopsies of NHL patients all have a B cell phenotype and no Reed-Sternberg cells are detected. The age-world-standardized incidence rate (per 100.000) for non-Hodgkin lymphoma (NHL) and Hodgkin lymphoma are 6.7 and 2 respectively, making NHL the 12th most prevalent cancer in Europe (GLOBOCAN 2008).
These two main types can be further divided into multiple subtypes, which can either be recognized by microscopic (Campo et al. 2011) or genetic analysis (Alizadeh et al. 2000). Common subtypes of NHL are for example follicular lymphoma (FL), diffuse large B cell lymphoma (DLBCL) and Burkitt's lymphoma (BL).

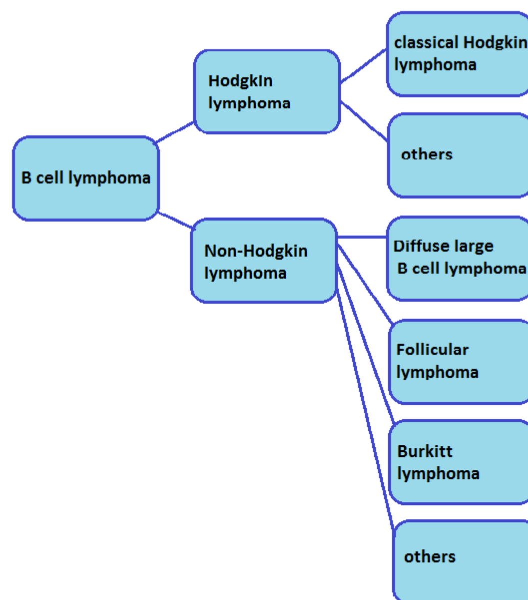

Fig. 1. Simplified overview of B cell lymphoma classification. First, a distinction is made between Hodgkin and Non-Hodgkin lymphomas. Secondly, different subtypes of Hodgkin and Non-Hodgkin lymphomas can be identified. Although a lot of different NHL subtypes are described, in this image only the for this essay important NHL subtypes are shown.

The differences between the NHL subtypes are partly due to varying mechanisms in the normal cause of the B cell differentiation process. Normally, after being exposed to an antigen, activated B cells migrate into a follicle of a lymph node and form a germinal center. First, the B cells proliferate in a part of the germinal centre called the 'dark zone'. After several days the proliferated B cells undergo hypermutation, which is the induction of mutations in the DNA coding for the binding part of the antibody. This leads to diversity in affinity for a specific antigen. After mutations are induced, only B cells that recognize

antigens presented by follicle dendritic cells with high affinity are selected to survive, while other B cells will go into programmed cell death. Then, the surviving B cells undergo further differentiation and selection in the 'light zone' of the germinal centre. The cells undergo isotype switching by recombination of the Ig coding DNA, leading to the production of antibodies with heavy chains of different classes (Abbas et al. 2010).
The DNA recombination and hypermutation mechanisms in the germinal centre are the reason that B cells are prone to transforming into malignant cells. When these events are disrupted they can cause chromosome translocations and mutations in non-Ig genes, leading to the activation of oncogenes, silencing of tumor-suppressor genes, genomic amplification of specific genes or general genetic instability (Kuppers 2005).
When this happens at a particular stage of the differentiation process an accumulation of cells with features specific for this differentiation stage will occur. The malignant B cells seem to be 'frozen' at this specific differentiation stage (Stevenson et al. 2001). This allows determination of the origin of a B cell lymphoma retrospectively. It has been found that most B cell lymphoma derive from the germinal centre or post-germinal centre B cells (Kuppers et al. 1999).

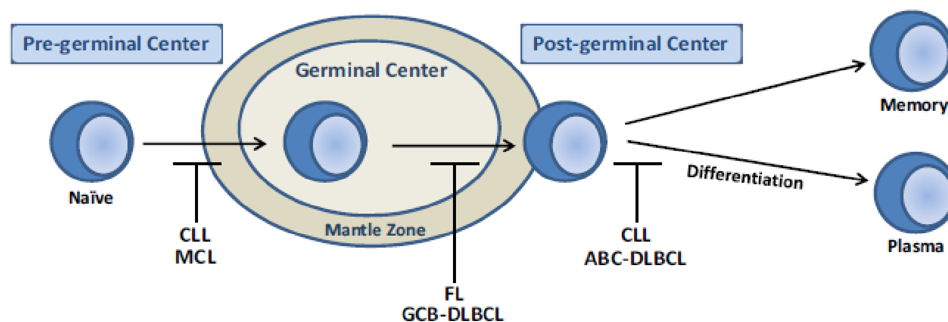

Fig. 2. Origin of different B-cell malignancies. (Epi)genetic alterations may occur at any stage of normal B-cell development and lead to an accumulation of cells at specific stages of differentiation. Abbreviations: CLL, chronic lymphocytic leukemia; MCL, mantle cell lymphoma; FL, follicular lymphoma; GCB-DLBCL, germinal center B-cell-like diffuse large B-cell lymphoma; ABC-DLBCL, activated B-cell-like diffuse large B-cell lymphoma. Image source: Taylor et al. 2013.

Although multiple recurring cytogenetic abnormalities are a characteristic finding in lymphoid malignancies and likely contribute directly to malignant transformation, these abnormalities alone do not always result in transformation. Other mechanisms, including epigenetic alterations, have also been shown to be involved. Epigenetic changes are inheritable changes in gene expression, caused by mechanisms other than changes in DNA sequence (Taylor et al. 2013). This includes the modification of the chromatin architecture by enzymes regulating for example cytosine methylation and histone modifications (explained below), in order to change the DNA accessibility and therefore gene expression. Recently, large scale genomic studies have made it possible to investigate the entire genome of different cell types. This resulted in the discovery of a variety of significantly altered epigenetic regulators in many types of human cancer, including B cell lymphoma.
The aim of this essay is to give an overview of the most important epigenetic modifiers that play a role in NHL as a starting point for further research on the epigenetic mechanisms involved. In this essay the normal function of the selected enzymes in healthy cells will be

discussed, together with the detected changes in these enzymes in malignant B cells and their role in the development of NHL. This essay will mainly focus on three NHL types, namely FL, BL and DLBCL, because these all derive from the germinal centre and are relatively common lymphoma subtypes.

Epigenetic mechanisms and epigenetic modifiers

As mentioned in the introduction, epigenetic mechanisms regulate gene expression. In contrast to gene expression regulation by transcription factors, epigenetic mechanisms provide a form of cell memory which is transmitted from one cell to its daughter cells. The epigenetic mechanisms all influence the chromatin conformation at different levels, thereby either creating an open, activated state or a condensed, inactive state in specific genome regions.
DNA is packed in a highly ordered way in the nucleus. The most fundamental unit of packaging is the nucleosome. It consists of 8 histone proteins, 2 of each of the 4 variants. The DNA helix is coiled around the nucleosome. A lot of nucleosomes together (beads on a string) shorten the DNA in this way with a packaging ratio of 1:6. Besides coiling around nucleosomes the DNA is further packed, depending on the cell phase of the cell.
Epigenetic mechanisms regulate gene expression by targeting the bases of the DNA itself (DNA methylation), by modifying the histones in the nucleosomes (histone modification) and by changing the shape of the chromatin structure (chromatin remodeling) (Strachan, Read 2004).

DNA methylation

Cytosine bases located next to a guanine base (CpG) are often methylated by DNA methyltransferases (DNMT). In human cells, 70%–80% of all CpG dinucleotides in the genome are methylated (Ehrlich 1982). Two types of DNMTs are known. The function of DNMT1 is maintenance methylation, which means that it copies the methylation pattern from the complementary strand along a DNA strand after DNA replication, providing a way of passing epigenetic information between cell generations. DNMT3A and DNMT3B on the other hand facilitate de novo methylation, which plays a role in development and ageing. Despite its name, DNMT2 does not methylate DNA (Goll et al. 2006).
Methylation of cytosines is thought to repel the binding of sequence specific transcription promoting proteins, but also attracts proteins involved in inhibiting transcription. A family of methyl-CpG-binding proteins has been characterized, and four of these proteins (Methyl-CpG-binding domain protein 1, 2 and 3, and methyl CpG binding protein 2) have been implicated in methylation-dependent repression of transcription. Therefore, DNA methylation results in an inhibition of the gene expression at a specific site.
When DNA is unmethylated, genes are expressed. An interesting feature of the DNA methylation pattern are the so called CpG islands, which are unmethylated and are positioned in the promotor region of many human genes (Bird 2002).

Histone modification

Another important epigenetic mechanism involved in transcription, replication and repair is histone modification, although the inheritability of these modifications is still under debate. Histones are organized in the nucleosomes in such a way that their tails protrude from the

histone octamer. Specific amino acids in these tails are subject to modification. Today, over 100 distinct histone modifications have been described, and more are being discovered at a rapid pace (Zentner, Henikoff 2013). Some of the modifications are well known, including lysine methylation, lysine acetylation and serine/threonine phosphorylation, others are more unknown modifications such as crotonylations (Tan et al. 2011).
Histone acetylation is the most intensively investigated modification. This acetylation is regulated by histone acetyltransferases (HATs) which add an acetyl group to an amino acid, and histone deacetylases (HDACs), which remove acetyl groups. Hyperacetylation is associated with actively transcribed genes. This can be explained by looking at the chemistry of histone acetylation. Acetylation neutralizes the positive charge of lysine residues, weakening charge-dependent interactions between a histone and the DNA, thus increasing the accessibility of DNA (Hong et al. 1993).
Phosphorylation of amino acids, another type of histone modification, has a comparable effect. Because phosphorylation adds a negative charge, this will create charge repulsion between the histone tail and the negatively charged DNA backbone. Therefore, this will also loosen the bonds between the DNA and the nucleosome (Banerjee et al. 2010).
The third modification is methylation. Interesting here is that one amino acid can be mono-, di- or trimethylated. Methylation of an amino acid does not change its charge, which is why it is thought that this type of modification does not directly affect the accessibility of the DNA (Lenstra et al. 2011).

Chromatin remodeling

Chromatin remodelers are macromolecule assemblies that regulate chromatin structure using the energy of ATP hydrolysis. These chromatin remodeling complexes (CRCs) use energy to disrupt the nucleosome-DNA contacts, in order to remove, replace or move nucleosomes along DNA in order to change the accessibility of the DNA.
Chromatin remodelers can be divided into four families, the SWItch/Sucrose NonFermentable (SWI/SNF) family, the INO80/SWR1 family, the ISWI family and the CHD family. All CRCs contain multiple subunits, vary in structure and function. In addition to ATPase domains, the subunits contain interaction domains that regulate the enzymatic activity of the complex, facilitate binding to transcription factors and other CRCs and target the DNA and modified histones.

A variety of enzymes are involved in the regulation of the different epigenetic marks described above. For the sake of clarity these epigenetic modulators can be categorized in 3 groups. First, 'writers' are the enzymes which establish the epigenetic marks. In contrast, 'erasers' are enzymes that remove the epigenetic marks. The third group is the group of the 'readers'. These proteins are able to recognize the established epigenetic marks and translate the marks for various cellular functions (Lu et al. 2012).
These epigenetic components are essential for normal processes such as development and cell differentiation (Lu et al. 2012). Although there is some evidence that histone modifications are involved in B cell maturation and differentiation, the general knowledge about the role of epigenetic marks in B cell development is still limited (Stevenson et al. 2001).

Deregulation of epigenetic regulation contributes to a variety of human diseases, including cancer. A broad range of frequently observed changes, mainly mutations, have been described in epigenetic modulators in NHL and will be discussed below.

DNA methylation	**Histone modification**	**Chromatin remodeling**
DNMT 1	HAT	SWI/SNF (BAF and PBAF)
DNMT 3a	HDAC	INO80/SWR1
DNMT 3b	HPT (histone phosphotransferase)	ISWI
	HP (Histone phosphatase)	CHD
	HMT (Histone methyltransferase)	
	HDM (Histone demethylase)	

Table 1. Overview of protein complexes involved in the 3 types of epigenetic modification. 3 types of DNA methyltransferases are responsible for DNA methylation. Different types of histone modifications are either placed (transferases) or removed by different proteins. In chromatin remodeling 4 protein families are involved. These proteins within these families form complexes with comparable functions.

Changes in DNA methylation involved in NHL

Tet methylcytosine dioxygenase 2 (TET2)

TET2 is a DNA methylation eraser, belonging to the family of TET proteins (TET1, TET2, TET3). They catalyze the conversion of 5-methyl-cytosine (mC) to 5-hydroxymethyl-cytosine (hmC) (Ko et al. 2010), and thus help to protect against unwanted methylation and as a consequence downregulation of transcription. TET2 requires α-ketoglutarate as a cofactor, which in a normal situation is produced by isocitrate dehydrogenase 1/2 (IDH1/2) from isocitrate (Cimmino et al. 2011).

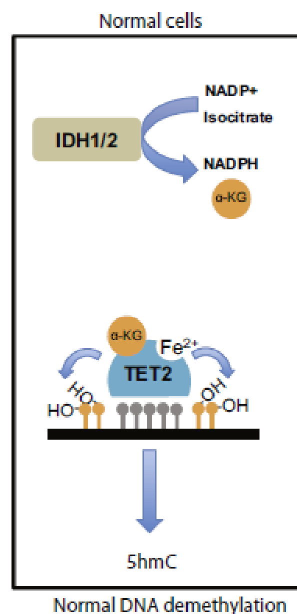

Fig. 3. Normal function of TET2. TET2 erases methylgroups from the DNA by converting it into a hydroxymethylgroup. To do this TET2 needs the cofactor α-ketoglutarate, which is produced by IDH1/2 from isocitrate and NADP+. Image source: Cimmino et al, 2011.

TET2 is essential for normal homeostasis of the hematopoietic system, although the exact target genes of TET2 are not clear. This was shown in studies using comparable *TET2* deficient mouse strains (Moran-Crusio et al. 2011, Li et al. 2011). Analyzing the hematopoietic compartments of these animals showed a progressive enlargement of the whole stem/progenitor compartment (Lineage^{-}Sca1^{+}c-Kit^{+}), without a specific compartment being disproportionally enlarged. Furthermore, *TET2*$^{-/-}$ hematopoietic stem cells have increased self-renewing abilities as proven by both *in vitro* replating assays and *in vivo* competitive transplantation experiments, indicating autonomous control of hematopoietic stem cell homeostasis by TET2 in healthy animals. Interestingly, *TET2*-deficient mice developed myeloid neoplasms.
In humans, a variety of myeloid disorders carry a TET2 mutation, including multiple B cell lymphomas. In a series of 301 B cell lymphoma samples, TET2 mutations were observed in 2.0% of the DLBCL and FL cases. The mutations were mainly insertion/deletions, generating frameshifts and nonsense mutations, either in 1 or 2 alleles, leading to a loss of function. Investigating malignant cells of patients suffering from 2 different types of blood malignancy

showed that it is possible that both malignancies derive from a TET2 mutation in the bone marrow. These data suggest that TET2 mutations predisposes to malignant transformation. Subsequently, secondary mutations acquired later in hematopoietic cell development, including mutation of the second TET2 copy, are thought to cause the phenotype of the disease (Quivoron et al. 2011).

DNMT 3B

Mutations are not always necessary in order to deregulate DNA methylation. It has been shown that overexpression of DNMTs (mainly DNMT1 and DNMT 3b) results in hypermethylation of tumor suppressor genes. Moreover, overexpression of DNMTs has been described in many cancers, including breast cancer (Girault et al. 2003) and leukemia (Mizuno et al. 2001). To elucidate the role of DNMTs in B cell lymphoma, Amara et al. analyzed the expression of DNMTs by immunohistochemistry in samples of DLBCL patients and compared these to the clinicopathological parameters of the patients.
First of all, they found that DNMT1 was overexpressed in 59% of the cases, DNMT3a in 13% of the cases and DNMT3b in 45% of the cases. A significant correlation was found between the co-expression of DNMT1 and DNMT3b and between DNMT1 and DNMT3a.
During the next step the relation between DNMT overexpression and promoter hypermethylation of tumor suppressor genes was investigated. Correlations were found between overexpression of DNMT3b and hypermethylation of Glutathione S-transferase P (*GSTP1),* Src homology region 2 domain-containing phosphatase-1 *(SHP1),* tissue inhibitor of metalloproteinases 3 (*TIMP3)* and *P16*. Overexpression of DNMT1 was correlated with hypermethylation of the genes Von Hippel–Lindau (*VHL)* and retinoblastoma protein 1 (*RB1)*. No correlations were found between overexpression of DNMT3a and hypermethylation of the investigated genes.
To give an indication of the importance of DNMT overexpression, correlations between DNMT overexpression and clinicopathological parameters were investigated in DLBCL. It was found that the expression status of both DNMT 1 and DNMT3b were correlated with advanced clinical stages. Furthermore, concomitant expression of DNMT1 and DNMT3b was significantly correlated with worse therapy response. It was observed that only 26% of patients who achieved complete response showed simultaneous expression of DNMT1 and DNMT3b, whereas 65% of patients who failed to give a complete response showed overexpression of DNMT1 and DNMT3b.
Multivariate analysis showed that DNMT3b overexpression was an independent prognostic factor in predicting shortened overall survival and progression free survival (Amara et al. 2010). These results indicate that DNMTs are commonly overexpressed in DLBCL, and that DNMT overexpression is an important factor in the progression of the disease.

Changes in histone modification involved in NHL

Mixed-Lineage Leukemia 2 (MLL2)

The *MLL2* gene encodes a protein essential for the formation of several multiprotein complexes. Most of these complexes regulate gene transcription. One protein complex has been found to possess histone methyltransferase capacity, by modifying the lysine-4 position of histone 3 (H3K4) resulting in transcription of target genes. This complex was investigated in a study of Issaeva (Issaeva et al. 2007) by mass spectrometry, and consisted of 12 proteins: MLL2, ALR, ASC-2, ASH2, RBQ3 [RbBP5], tubulin α and β, UTX, PTIP, matrin 3, hypothetical protein MGC 4606 and hDPY30. This complex exhibited strong H3K4 methyltransferase activity because of the MLL2 SET domain, an evolutionarily conserved catalytic domain.
Although analysis of its expression suggests that MLL2 might have multiple functions both during development and in the adult organism (Prasad et al. 1997), MLL2 seems to be especially important during development, because deletion of *MLL2* is embryonic lethal (Lubitz et al. 2007).
By comparing gene expression of *MLL2* knockdown cell lines and control HeLa cell lines, Issaeva et al. was also able to identify a list of genes activated by the MLL2 protein complex. Among them were groups of genes involved in cell adhesion, cytoskeleton organization, or transcriptional regulation. Direct targets were for example Cysteine-rich secretory protein 2 (*CRIP2)*, Chondroitin sulfate proteoglycan 4 (*CSPG*4), Beta-enolase (*ENO3)*, and Dickkopf-related protein 1 (*DKK1)*.
Recently, 2 studies concluded independently from each other that MLL2 mutations are one of the most frequent mutations in DLBCL and FL (Pasqualucci et al. 2011b, Morin et al. 2011). Morin et al. found this mutation in 32% of the DLBCL cases and 89% of the FL cases. Pasqualucci et al. investigated DLBCL samples and cell lines and detected 33 sequence variants distributed in 28 samples, including 21 out of 92 biopsies and 7 out of 23 cell lines. Both studies showed that almost all mutations were inactivating mutations. They detected mainly nonsense mutations, frameshift insertions or deletions, consensus splice site mutations and truncation mutations. These mutations will lead to truncated proteins lacking the C-terminal cluster of conserved domains (including the SET domain) or substantial portions of it.
The fact that *MLL2* was mainly monoallelic inactivated in DLBCL suggests a role for MLL2 as a haploinsufficient tumor suppressor, which means that inactivation of one of both alleles already contributes to tumorigenisis. However, Morin et al. detected mutations in both alleles in 8 FL patients. These findings show that deregulation of histone modification due to inactivating mutations in the MLL*2* gene, which leads to decreased activation of its target genes, is an important factor in the development of NHL.

Polycomb group proteins

The polycomb group of epigenetic gene repressors maintain, together with the activating Trithorax proteins, the correct gene expression level of several key developmental regulators. Originally, these proteins were identified in *D. Melanogaster* as repressors of the Hox genes (Ringrose, Paro 2004). Polycomb group proteins can be classified according to their association with the multimeric complex, called Polycomb repressive complex (PRC). The 2 main complexes are PRC1 and PRC2, although recently a third PRC (PHORC) was identified. The PRC2 complex has multiple ways to influence gene expression, but its main function is methylation of lysine 27 and to a lesser extent lysine 9 of histone H3 (H3K27 and H3K9). It consists of multiple mammalian homologues of *D. Melanogaster* proteins, including Enhancer of zeste (E(Z)), which is the catalytic active component of the complex. The more complex PRC1 comprises other main components, most important being B lymphoma Mo-MLV insertion region 1 homolog (BMI-1) and E3 ubiquitin-protein ligase (RING1). This complex is able to recognize the H3K27 mark and target PRC1 to specific genomic regions resulting in ubiquitination of H2AK199. Although these compositions of these PRC complexes were described, it is now suggested that the PRC core components can form various functionally distinct sub-complexes depending on the environment (Sparmann, van Lohuizen 2006).

The formation of multiple core proteins of the PRC complexes has been described to be deregulated in different manners in pathological settings, including NHL. A change in these core proteins will influence the overall function of the PRC complex, and can therefore change the gene expression of target genes.

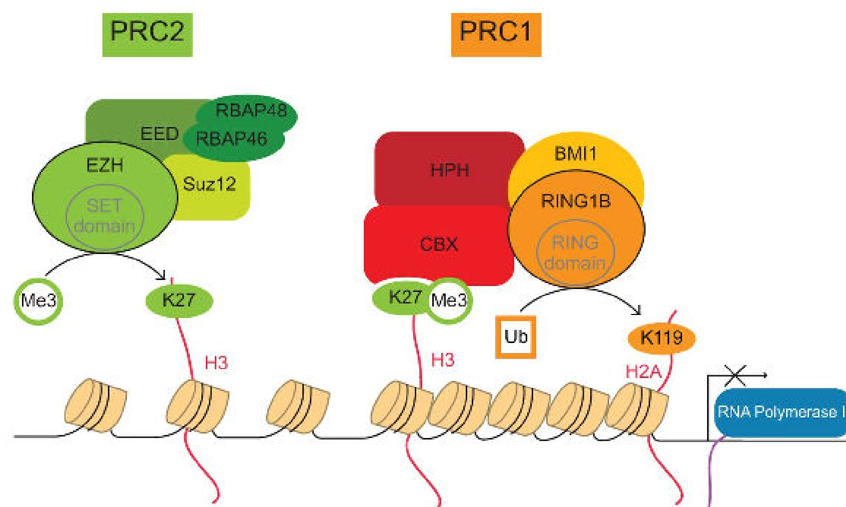

Fig. 4. PRC mediated epigenetic gene silencing. The catalytic site in the EZH2 protein of the PRC2 complex mediates H3K27 trimethylation. This recruits PRC1, which recognizes the trimethylated spot by CBX. RING1 and its cofactor BMI1 in turn ubiquitinate the nucleosome at the H2AK119 position. Together these protein complexes promote silencing of the target gene. Image adapted from Vissers et al, 2012.

EZH2

Because EZH2 contains the catalytic site of the PRC2 complex, changes in the expression or sequence of this protein may have a huge impact on the working of PRC2. Indeed, changes in EZH2 have been described in the context of many cancers including NHL.

First, Bracken et al. showed that overexpression of EZH2 occurred in about 30% of the investigated lymphomas (not further classified). This was either caused by amplification of the *EZH2* gene (> 4 copies) or by a deregulation of the retinoblastoma pathway (pRB-E2F pathway), which is often seen in human cancers. By expressing the transcription factor E2F and its upstream regulators p16 and pRB in different cell lines and measuring the gene expression of EZH2 they found that EZH2 is a physiological target of the pRB-E2F pathway. Downregulation of this pathway in cancer leads to an upregulation of the EZH2 expression. By inhibiting and stimulating the expression of EZH2 it was found that EZH2 is required for proliferation in both transformed and normal cells, and that overexpression of EZH2 leads to a proliferative advantage. This can be understood by looking at the target genes of EZH2. In the same study these target genes were revealed by inhibiting EZH2 expression using siRNA. This resulted in a decrease of all cyclin molecules tested, including D1, E1, B1 and A2, a group of proteins that control the cell cycle progression. Therefore, it was suggested that PRC2 may be required for either the activation or the maintenance of the activated state of a number of these genes. Myeloblastosis (M*yb),* Myb-related protein B *(B-Myb),* cell division cycle 6 (*CDC6),* transcription factor E2Fl *(E2F1)* and Cyclin-dependent kinase 1 (*CDC2)* were also identified as target genes of EZH2 (Bracken et al. 2003).

In accordance with the described overexpression of EZH2 in lymphoma, next-generation sequencing of follicular lymphoma and diffuse-large B-cell lymphoma has revealed frequent somatic, heterozygous gain of function mutations in the *EZH2* gene, occurring in 21.7% of the DLBCLs and 7.2% of the FLs investigated (Morin et al. 2010). The mutations were all missense mutations that changed a single highly evolutionarily conserved tyrosine residue in the catalytic SET domain (Tyr641) to phenylalanine (Y641F), asparagine (Y641N), histidine (Y641H), or serine (Y641S). Because this mutation does not affect the stability of the protein, cells containing an Y641 mutation contain an equal amount of wildtype and mutated EZH2. The fact that in DLBCL cell lines and in tissue containing the EZH2 mutation higher levels of trimethylated H3K27 were observed compared to cells without the mutation, indicated hyperactivity of the PRC2 complexes when the mutation was present. However, the mutated EZH2 showed an increased substrate preference for dimethylated peptide and is inactive on non-methylated histone peptides. Therefore it can only work in the presence of wildtype EZH2 (Yap et al. 2011). This might explain why in a cell free assay PRC2 containing any of the detected mutations showed a reduced trimethylation of H3K27 (Morin et al. 2010).

BMI1

Overexpression of BMI1, a cofactor of the enzymatic subunit RING1 in PRC1, is also associated with the development of NHL. Normally, the expression of the different polycomb complexes is highly regulated during the germinal centre reaction in B cells. It was observed that in a healthy situation the expression of BMI1 and the expression of EZH2 are mutually exclusive, when PRC1 is expressed PRC2 is absent and the other way around.

By analyzing the expression of EZH2 and BMI1 in lymph node tissue of NHL patients using immunofluorescence, coexpression of EZH2 and BMI1 was detected in neoplastic centroblasts. Furthermore, the detection of EZH2 and BMI1 also overlapped with the presence of proliferation marker Mib-1/Ki67, while BMI1 expression is absent from normal dividing cells. This indicates a disturbed balance between EZH2 and BMI1 expression, which is thought to be an early event in lymphomagenesis (van Kemenade et al. 2001). BMI1 (over)expression was mainly detected in mantle cell lymphoma (MCL) and BL (Bea et al. 2001).
Other studies focused on the question how BMI1 exactly contributes to tumorigenesis. Since 1993 it has been known that one mechanism of BMI1 is cooperation with Myc. This was shown in *BMI1/Myc* transgenic mice that died from massive leukemia (Haupt et al. 1993, Haupt et al. 1991, Jacobs et al. 1999). Later on studies were performed to find the target genes of BMI1. Now it is known that BMI1 functions as a negative regulator of the INK4A-ARF locus. The INK4A-ARF locus contains 2 tumor suppressor genes, *P16* and p19 ADP-ribosylation factor (*P19ARF)*. This means that an upregulation of BMI1 leads to a downregulation of these tumor suppressor genes, which in turn leads to an increased tumorigenesis rate. It was also concluded that this is at the basis of the collaboration between BMI1 and Myc, because Myc normally induced apoptosis via the INK4A-ARF locus (Jacobs et al. 1999).

Janus kinase 2 (JAK2)

In many ways JAK2 is involved in tumorigenesis. For example a JAK2 V617F mutation has been reported in high proportions of chronic myeloproliferative disorders. However, this mutation was found to be very uncommon in NHL, which suggests that this mutation does not play a role in the development of this type of lymphoma (Lee et al. 2006). Another mutation, a DNA copy-number gain or amplification affecting chromosome band 9p24, was found to occur in multiple NHL types including DLBCL, FL, and Primary mediastinal B-Cell Lymphoma (PMBL). By investigating the functions of the genes located at this chromosome band, JAK2 was suggested to be a potent oncogene. First, this was mainly thought because JAKs are known to be important components of receptor mediated signal transduction of growth factors, hormones and cytokines. In this JAK-STAT pathway phosphorylated JAK serves as a binding ligand for signal transducers and activators of transcription (STAT), which in turn migrates to the nucleus to induce transcription of target genes (Meier et al. 2009). Later, Dawson et al. discovered another function of JAK2 which might be related to lymphogenesis. They showed that JAK2 is present in the nucleus of haematopoietic cells and directly phosphorylates Tyr 41 (Y41) on histone H3. This phosphorylation inhibits the binding of Heterochromatin Protein 1α (HP 1α) to the H3 histone, which is known to repress the transcription of heterochromatic genes. While the displacement of HP1α by JAK2 is likely to be tightly regulated in normal cells, amplification of the *JAK2* gene will disturb this balance in lymphoma (Dawson et al. 2009).
The exact mechanism involved in the (partly) JAK2 dependent tumorigenesis was further investigated by Rui et al, using PMBL biopsies (Rui et al. 2010). In 45% of the biopsies of this lymphoma type an amplification of the 9p24 locus was detected. By looking closer at

this region it was found that besides *JAK2* another potent oncogene was located here. This gene was also amplified in PMBL and was identified as another histone modifier, namely Lysine-specific demethylase 2C (JMJD2C). Because inhibition of both genes was more toxic for a PMBL cell line compared to inhibition of *JAK2* or *JMJD2C* alone, a functional cooperation was proposed. Indeed, further analysis of apoptosis and cell cycle using flow cytometry revealed that *JAK2* inhibition caused apoptosis but did not influence proliferation. In contrast, inhibition of *JMJD2C* decreased proliferation but did not affect apoptosis.
By measuring gene expression profiles in PMBL cell lines after inhibition of *JAK2* and/or *JMJD2C, Myc* and its transcriptional network were found to be targets of JAK2 and/or JMJD2C activation. Furthermore, Myc was found to regulate cell survival by reducing apoptosis.
Based on studies on histone modifications in general and the *Myc* gene in particular, a working model of JAK2 and JMJD2C regulation was proposed. Both regulators control recruitment of the heterochromatin protein HP1α to histone tails, but by different mechanisms. HP1α normally recognizes and binds histone H3K9me3, but demethylation of this residue by JMJD2C removes this HP1α binding site. HP1α also binds to a second region of the histone H3 tail, and phosphorylation of tyrosine 41 by JAK2 in turn blocks this binding. Because HP1α is a heterochromatin marker, JAK2 and JMJD2C create a more accessible and active chromatin at their target regions, including *Myc*. An amplification of the *JAK2* and *JMJD2C* genes, will therefore prevent HP1α even more from binding, which results in enhanced activity of the target genes.

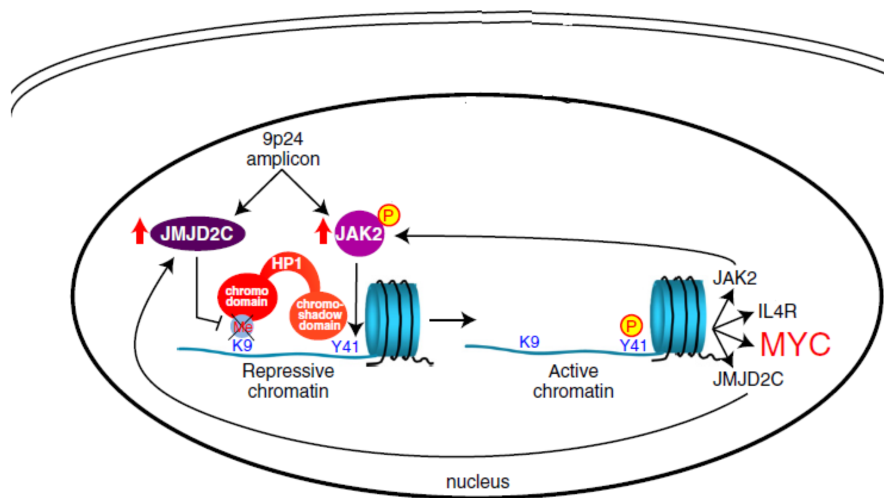

Fig. 5. Model of cooperation in epigenetic regulation of JAK2 and JMJD2C. In a normal situation JAK2 and JMJD2C regulate the binding of HP1α to the DNA, which is a protein associated with repressive chromatin. They do this by modifying different parts of the H3 histone tail. An amplification of the 9p24 region of the genome leads to an upregulation of both JAK2 and JMJD2C. This in turn causes more histone modifications, and HP1α binding is prevented. This results in an increased expression of target genes, including the oncogene *Myc*. Image adapted from Rui et al, 2010.

CREB-binding protein (CBP) and P300

The last histone modifiers that are widely recognized to be involved in lymphogenesis are the homologs CBP and P300. Earlier on it was already known that p300 and CBP were global transcriptional coactivators involved in the regulation of various DNA-binding transcriptional factors, with mainly the same structure and function. In 1996 Ogryzko et al discovered that these proteins also had intrinsic histone acetyltransferase (HAT) activity, modifying lysine residues on both histone and non-histone nuclear proteins (Ogryzko et al. 1996). This means that CBP and P300 are not just transcriptional adaptors, but they are also able to directly change the chromatin structure by acetylation of histone tails.

Further investigation of their histone acetyltransferase function lead to the identification of the catalytic site in both CBP and P300, which showed 91% similarity between these proteins. However, comparing these residues with the catalytic site of previously discovered HATs (PCAF and GCN5) showed significant differences. Thus, CBP and P300 were found to be a novel class of HATs (Kat3 family). The specificity of CBP and P300 was also investigated, and it was found that these proteins are able to acetylate all 4 histone types present in nucleosomes, but preferred H3 and H4. Furthermore, the acetylation capacity was quite strong, CBP and P300 were able to bring the DNA in a hyperacetylated state, related to active gene transcription.

In a recent study that used next-generation whole exome sequencing analysis and a genome-wide high-density single nucleotide polymorphism (SNP) array in order to detect mutations in DLBCL and FL cells, mutations in *CREBBP* (gene coding for CBP) and *EP300* (gene coding for P300) were found to be the most frequent involved regions in these lymphoma types (Pasqualucci et al. 2011a). On the other hand, these aberrations are rare in BL. In DLBCL *CREBBP* was mutated in 29% of the cases and EP300 in 10% of the cases. In FL 32,6% of the cases showed a mutation in *CREBBP* and 8,7% had a mutation in *EP300*.

50 of the mutations found were inactivating events, including nonsense mutations, frameshift insertions/deletions and mutations at consensus splice donor/splice acceptor sites. The remaining variants included three in-frame deletions and 14 missense mutations within the catalytic HAT domain, suggesting that they also may influence the protein function.

Because these mutations were monoallelic, in most both the mutant and wildtype protein were present in the cell.

Important target genes of CBP and P300 are already known for a longer time. The tumor suppressor *P53* depends on acetylation by these HATs in order to stay transcriptional active (Lill et al. 1997, Pasqualucci et al. 2011a). In contrast, acetylation of the proto oncoprotein B-cell lymphoma 6 protein (BCL6) by CBP or P300 leads to inactivation of its transcriptional repressor function (Bereshchenko, Gu & Dalla-Favera 2002). Furthermore, both targets are known to be involved in germinal centre reaction and lymphogenesis (Klein, Dalla-Favera 2008).

When testing the acetylation capacity of CBP and P300 for those 2 targets in NHL cells, it was found that the acetylation at these sites was impaired, leading to a changed expression of both targets. By using different manufactured CBP and P300 mutated proteins it was found that the impaired acetylation of *P53* and BCL6 loci was due to a decreased affinity for acetyl-CoA, which normally provides the acetyl group needed for the acetylation of a histone by HATs.

Changes in chromatin remodelers involved in NHL

Of all protein families involved in chromatin remodeling, the SWI/SNF proteins were associated with cancer formation in particular. The proteins belonging to this family form large multiprotein complexes, consisting of 9 to 12 subunits per complex. In humans 2 main complexes have been described, termed BRG1 associated factor (BAF) and polybromo BRG1 associated factor (PBAF). The complexes consist of one of the 2 catalytic ATP-ase subunits, Brahma protein (BRM) and Brahma-related gene 1 (BRG1), a set of core proteins and a couple variable proteins. BRG1 and BRM can be present in both BAF and PBAF, but are mutually exclusive (Wilson, Roberts 2011). Their chromatin remodeling properties have been confirmed *in vitro* (Kim, Bresnick & Bultman 2009). Other important proteins are the AT rich interactive domain containing proteins 1A and 1B (ARID1A and ARID1B). These proteins contain a domain which is involved in the binding of the protein complex to the DNA (Wilsker et al. 2004), although its exact role in chromatin remodeling is not understood. These proteins are only present in the BAF complex.

Although mutations were found in a lot of SWI/SNF complex proteins in a variety of cancers, including, BAF180, BRM and Bromodomain-containing protein 7 (BRD7) (Wilson, Roberts 2011), a few were suggested to be especially important in NHL.

ARID1A

Multiple independent studies detected mutations in the *ARID1A* gene in BL, pediatric BL (pBL) and DLBCL using comparable genomic sequencing methods (Giulino-Roth et al. 2012, Love et al. 2012, Zhang et al. 2013). Guilino-Roth et al. found a mutation in *ARID1A* in 17,2% of the investigated cases of pBL (Giulino-Roth et al. 2012). Love et al. detected an ARID1A mutation in 13,5% of the BL cases and in 9% of the DLBCL samples (Love et al. 2012). Mutations were found through the whole gene. Most mutations resulted in a truncation of the protein, which indicates that ARID1A is a tumor suppressor. Furthermore, it was found that the mutation was heterozygous, because the mutation was present in about 50% of the reads. When looking at the ARID1A protein expression using immunohistochemistry a decrease in protein expression was detected in the cases with a truncating mutation compared to wildtype ARID1A cases. In contrast, a group of pBL lymphoma cases without an ARID1A mutation showed an increased expression of this protein, which suggests that a changed protein expression can be involved in lymphomagenesis in multiple ways (Giulino-Roth et al. 2012).

Although ARID1A seems to be important, its role in the development of NHL is not known yet. ARID1A is suggested to be play a role in the regulation of cell cycle arrests. One study showed that knocking down of ARID1A in an osteoclast cell line resulted in abrogation of the cell cycle, from which they concluded that the loss of ARID1A may promote proliferation, possibly by promoting ARID1B containing protein complexes (Nagl et al. 2005). However, more research on this topic is needed.

BRG1

Another recurrently mutated gene is *BRG1*, which codes for one of the catalytic protein in the SWI/SNF complexes. Mutations in *BRG1* and *ARID1A* are mutually exclusive, which means that the mutations alone are sufficient to deregulate a SWI/SNF complex. Mutations in the BRG1 gene occurred in about 15% of the investigated BL cases. The same amount of mutations were detected in DLBCL samples (Love et al. 2012).

Besides in the BRG1 gene itself, mutations in another gene, nuclear factor of activated T cells c1 (*NFATC1*), influence the function of the BRG1 protein as well. Overexpression of this protein is also associated with the development of DLBCL (Lenz et al. 2008).

NFATc1 is a member of the NFAT family of transcription factors and coactivators, and plays an important role in chromatin remodeling, although the protein itself shows no remodeling capacities. In fact, NFATc1 helps to promote transcription by making the DNA accessible for other transcription factors and other proteins by interacting with BRG1.

Pham et al. investigated the function of NFATc1 in this context (Pham et al. 2010). By comparing the transcription of genes and performing luciferase reporter essays, they discovered that the oncogene *Myc* is a target of NFATc1, and that NFATc1 increased promoter activity of *Myc* in DLBCL cells. By looking further into the mechanism it was found that NFATc1 recruits BRG1 to the promoter site of *Myc* with a high affinity and a direct interaction was shown using immunoprecipitation. Although NFATc1 also recruits other proteins, the chromatin remodeling associated with NFATc1 is now thought to be mediated by BRG1.

Summary

In the table below an overview of the proteins discussed is given. For each protein the function (writer/eraser/reader), detected modifications, known targets and the lymphoma type in which the mutations are observed are listed. It should be noted that the lists of targets and lymphoma types are likely to be incomplete. Only the in this essay discussed target genes and lymphoma are listed.

Protein	Function	Type	Modification	Known targets	Lymphoma
TET2	Methylation eraser	eraser	Inactivating mutations	Homeostasis hematopoietic cells (target genes not known)	DLBCL FL
DNMT 3b	methylation	writer	overexpression	*GSTP1, SHP1*, *TIMP*3 and *P16*	DLBCL
MLL2	Histone methyltransferase	writer	Inactivating mutations	i.a. *CRIP*2, *CSPG*4, *ENO*3, AND *DKK*1	DLBCL FL
EZH2	Histone methyltransferase	writer	Overexpression/ amplification of gene/activating mutations	cyclins (E1, B1 and A2) and *Myb, B-Myb, CDC6,E2F1* and *CDC2*	DLBCL FL Possibly other types
BMI1	ubiquitination	writer	Overexpression/ deregulated expression	INK4A-ARF locus including P*16* and *P19ARF*	Mainly MCL and BL
JAK2	Histone phosphorylation	writer	Amplification of gene	*Myc*	DLBCL FL PMBL
CBP/P300	Histone acetyltransferase	writer	Inactivating mutations	*P53* BCL*6*	DLBCL FL
ARID1A	Binding to DNA	Reader	Inactivating mutations	*ARID1B?*	DLBCL BL pBL
BRG1	Chromatin remodeling	writer	Inactivating mutations	*Myc?*	DLBCL BL

Table 2. Overview of the proteins discussed in the previous sections. The function, type of epigenetic modifier, modification observed in NHL, known target genes and the lymphoma types in which the mutations are observed are listed for every protein.

Discussion

The aim of this thesis was to give an overview of the epigenetic modifiers playing a role in the development of NHL. The literature search performed revealed that changes in the function or expression of many epigenetic modifiers (TET2, DNMT3b, MLL2, EZH2, BMI1, JAK2, CBP/P300, ARID1A and BRG1) have been found in NHL cells. This, together with follow-up studies investigating the effects of these changed epigenetic proteins, indicate that these alterations in enzyme function and expression contribute to the transformation of B cells into cancer cells.
Epigenetic modifiers involved in all 3 epigenetic mechanisms, DNA methylation, histone modification and chromatin remodeling, were found to be altered in NHL. Most changed proteins were detected in the group of histone modifiers.
When looking at the specific function of the proteins, it can be concluded that most changed proteins are writers, except for the eraser TET2 and the reader ARID1A. In most cases these proteins lost this writing capacity due to a mutation disturbing the catalytic site. However, studies performed on EZH2 showed that a gain of function can occur as well.
Besides the presence of mutations in the gene coding for the epigenetic modifiers, also the expression of the gene should be kept in mind. The importance of this became clear in for example DNMT3b overexpression. Expression can be upregulated due to amplification of a gene, but can also be changed by upstream alterations in the pathway the protein is involved in (both options also cause EZH2 upregulation).
The epigenetic modifiers involved in NHL mentioned above where first identified using genome wide sequencing, a technique that became available recently. It is likely that in the near future more mutated epigenetic proteins will be detected in NHL cells.
However, just the detection of these mutations in NHL cells is not enough to prove its role in the transformation of the cells. With the development of the sequencing technique came a new challenge, proving that the mutations detected are important in lymphogenesis, and not coincidences or consequences of the transformation of the cells. Therefore, it is important to identify the target genes of the epigenetic modifiers, investigate the effects of the changed epigenetic regulation on the target gene expression, and ultimately investigate the effects of this changed target gene expression on proliferation and apoptosis mechanisms in the cell. For most of the epigenetic modifiers discussed before this has been done, but it can not be ruled out that the epigenetic modifiers have more, still unknown targets. This means that the exact effects of a mutated epigenetic modifiers are not fully understood yet.
Another complicating factor is the interaction between different epigenetic modifications. This occurs within one modification type, but also between different modification types. Methylation and histone acetylation patterns are often seen as a code. This code is created and maintained by multiple proteins working together, for example methyltransferases and demethylases or histone acetyltransferases and deacetylases. In case of a mutation in one of the proteins involved, this might cause a disequilibrium in modification events and therefore change the whole epigenetic code. For example a disequilibrium in histone methylation

caused by mutations in EZH2 or MLL2 is thought to be a key factor in lymphogenesis (Shaknovich, Melnick 2011).
Furthermore, DNA methylation, histone modification and chromatin remodeling are also interdependent. Many studies report the importance of histone modifications to target DNA methylation (McCabe, Brandes & Vertino 2009). For example, EZH2 was found to be needed for DNMT functioning at specific EZH2 target sites (Vire et al. 2006). In the same manner histone modification interacts with chromatin remodeling. DNA methylation in turn also influences histone modification and chromatin remodeling (Costa 2008). These interactions make it even more complex to predict or investigate the effects of an alteration in a specific epigenetic protein.
Another very interesting research area is the specificity of the epigenetic modifiers. It is still unclear why epigenetic modifiers target specific parts of the genome, while other parts are not modified. It is now thought that non-coding RNAs (ncRNAs), which are RNAs without any protein-coding potential, are involved in directing epigenetic modification. NcRNAs can bind to several proteins forming ribocomplexes and associate with specific DNA sequences, which suggests a regulatory role in epigenetic modifications. So far, studies showed that ncRNAs are involved in all types of epigenetic modification. For example, Jost et al. showed that demethylation of DNA in a chicken embryo both required proteins and RNA (Jost 1997). Polyhomeotic 1, a PRC1 subunit, was shown to bind RNA (Zhang 2004). Further studies on its *D. Melanogaster* homolog (Sop2) showed that the protein contained 3 RNA binding domains, and that these regions are essential for the localization of the protein. In the fruit fly the localization and histone acetylation capacity of another protein complex, dosage compensation complex (DCC), depends on 2 ncRNAs (RoX 1 and 2) as well (Gu et al. 1998, Meller and Rattner 2002).
More research is needed to fully elucidate the epigenetic mechanisms and their contribution to the development of NHL. However, it has already been shown that knowledge about the epigenetic state of the genome is useful to the clinic.
For example, by investigating the methylation and gene expression of DLBCL cases 239 genes were identified that showed distinct methylation in different subtypes of DLBCL. This information was then successfully used to distinguish between different types of DLBCL according to their DNA methylation profile (Shaknovich et al. 2010). This shows that DNA methylation profiles are potentially useful in diagnostics, for example as biomarkers to distinguish between specific NHLs.
Furthermore, epigenetic marks are likely to be interesting targets for treatment against NHL, because these marks are thought to be reversible. Studies are already testing agents that target chromatin remodelers and epigenetic readers (Cerchietti et al. 2010, Filippakopoulos et al. 2010).
For now, this study provided an overview of the current knowledge about the epigenetic modifiers involved in NHL in order to serve as a starting point for new research on this topic.

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