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EXPLORING THE ROLE OF EPIGENETIC ALTERATIONS IN MYELOID MALIGNANCIES WITH FOCUS ON DRUG RESPONSE

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Exploring the role of epigenetic alterations in myeloid malignancies with focus on drug response

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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Indeed, in the creation of the heavens and the earth, and the alteration of the night and day are signs for those of understanding.

[3:190]

To my beloved family

ABSTRACT

The production of blood cells (hematopoiesis), where the hematopoietic stem cell (HSC) is the cell of origin of all blood cells, is regulated by a complex network of intrinsic and extrinsic signals. Dysregulation of hematopoiesis, including somatic heterozygous mutations, cytogenetic aberrations, or epigenetic aberrations, are found in hematological malignancies. Advances in the technical field have lead to identification of many genetic and epigenetic alterations in myeloid malignancies. However, their exact role in disease pathogenesis and prognosis remains unclear.

The main purpose of this thesis was to explore the role of epigenetic alterations in the myeloid malignancies systemic mastocytosis (SM), the myelodysplastic syndromes (MDS), and acute myeloid leukemia (AML).

In study I, we investigated the overall effect on the epigenome caused by Azacitidine in primary MDS bone marrow CD34+ cells. We observed an increase in genes important for the immune system, ERVs, however with no clear correlation with the changes in the epigenome.

In study II, four different HDACi were found to dose dependently kill KIT D816V mutated mast cells. Primary patient mast cells were selectively targeted compared to healthy mast cells, and the more aggressive disease, the more sensitive to HDACi mediated killing.

In study III, we examined the epigenetic effects of Selenium compounds in AML cell line K562 as well as primary patient AML cells, and found that MSA treatment affected the adhesion capacity of AML cells, which may implicate MSA as a complement to chemotherapy to better target leukemic cells in the bone marrow niche.

In study IV, we further investigated the mechanism of action of the findings in study II, delineating the effects of HDACi treatment on mast cell epigenome and transcriptome. We demonstrate a direct effect of HDACi on KIT as well as KIT downstream signaling in D816V KIT mutated cells.

In conclusion, this thesis provides insight of the mechanism of action of epigenetically active drugs used in the clinic today, findings that may have direct consequences for improved treatment strategies in myeloid malignancies.

LIST OF SCIENTIFIC PAPERS

I. **Comprehensive mapping of the effects of Azacitidine on DNA methylation, repressive/permissive histone marks and gene expression in primary cells from patients with MDS and MDS-related disease**
Tobiasson M*, **Abdulkadir Ali H*,** Lennartsson A, Katayama S, Marabita F, De Paepe A, Karimi M, Krjutskov K, Einarsdottir E, Grövdal M, Jansson M, Ben Azenkoud A, Corddedu L, Lehmann S, Ekwall K, Kere J, Hellström-Lindberg E, Ungerstedt J,
Oncotarget, 2017 Apr 25;8(17):28812-28825.

II. **Histone deacetylase inhibitor SAHA mediates mast cell death and epigenetic silencing of constitutively active D816V KIT in systemic mastocytosis**
Lyberg K, **Abdulkadir Ali H,** Grootens J, Kjellander M, Tirfing M, Arock M, Hägglund H, Nilsson G, Ungerstedt J,
Oncotarget, 2017 Feb 7;8(6):9647-9659

III. **Selenite and methylselenic acid epigenetically affects distinct gene sets in myeloid leukemia: A genome wide epigenetic analysis**
Khalkar P*, **Abdulkadir Ali H***, Codó P, Díaz Argelich N, Martikainena A, Karimi Arzenani M, Lehmann S, Walfridsson J, Ungerstedt J, Fernandes P. A
Free Radic Biol Med. 2018 Mar;117:247-257

IV. **Mechanistic characterization of SAHA mediated D816V KIT downregulation in systemic mastocytosis**
Abdulkadir Ali H, Lyberg K, Martis M, Grootens J, Nilsson G, Månsson R, Luc S, Ungerstedt J *(Manuscript)*

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I. **Mutations in histone modulators are associated with prolonged survival during Azacitidine therapy**
Tobiasson M, McLornan D, Karimi M, Dimitriou M, Jansson M, Ben Azenkoud A, Jädersten M, Lindberg G, **Abdulkadir Ali H**, Kulasekararaj A, Ungerstedt J, Lennartsson A, Ekwall K, Mufti G, Hellström-Lindberg E.
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LIST OF ABBREVIATIONS

PTM	Posttranslational modification
KAT	Lysine acetyl transferase
HAT	Histone acetyl transferase
HDAC	Histone deacetyl transferase
TSS	Transcription start site
KMT	Histone lysine methyl transferase
PRMT	Protein arginine methyl transferase
ES	Embryonic stem cells
SAM	S-adenosylmethionine
DNMT	DNA methyl treansferase
CpGs	Cytosine and guanine bases
CGIs	CpG islands
AID	activation-induced cytosine deaminase
APOBEC	Apolipoprotein B mRNA-editing enzyme
TET	Ten eleven translocation dioxygenases
TDG	thymine-DNA glycosylase
BER	base excision repair
5mC	5-methylcytosine
5hmC	5- hydroxymethylcytosine
5fC	5-formylcytosine
5caC	5-carboxylcytosine
MBD	methyl binding groups
RNA Pol	RNA polymerase
mRNA	Messenger RNA
PIC	pre-initiation complex

TF	Transcription factor
HSC	Hematopoietic stem cell
MPP	Multipotent hematopoietic progenitor cell
LT-HSC	Long-term hematopoietic stem cell
ST-HSC	Short term hematopoietic stem cell
CMP	Common myeloid progenitor
CLP	Common lymphoid progenitor
GMP	Granulocyte/macrophage progenitor
MEP	Megakaryocyte/erythroid progenitor
RBCs	Red blood cells
MDS	Myelodysplastic syndromes
IPSS-R	Revised international prognostic scoring system
AML	Acute myeloid leukemia
HMA	Hypomethylating agents
DAC	Decitabine
AZA	Azacitidine
IC	Intensive chemotherapy
HDACi	Histone deacetylase inhibitor
SM	Systemic mastocytosis
ISM	Indolent systemic mastocytosis
SSM	Smoldering systemic mastocytosis
AHN	Systemic mastocytosis with associated hematological neoplasm
ASM	Aggressive systemic mastocytosis
MCL	MC leukemia (MCL)
2-CDA	2-Chlorodeoxyadenosine
IFN-α	Interferon-alpha (IFN-α)

UCK	Uridine-cytidine kinase
RR	Ribonucleotide reductase
ZBM	Zinc-binding moiety
GPx	Glutathione peroxidases
TrxR	Thioredoxin reductases
DIO	Iodothyronine deiodinases
MBD	Methyl-CpG-binding domain
NGS	Next-generation sequencing
OxBS-Seq	Oxidative bisulfite sequencing
NGS	Next-generation sequencing (NGS)
WGBS	Whole genome bisulfite sequencing
RRBS	Reduced representation bisulfite sequencing
ChIP-seq	Chromatin immunoprecipitation sequencing
ATAC-seq	Assay for transposase-accessible chromatin sequencing
RNA-seq	RNA sequencing
STRT	Single-cell tagged reverse transcription
WHO	World health organisation
FDA	Food and drug administration

1 INTRODUCTION

1.1 EPIGENETICS

Every cell in the human body has identical genetic information, yet a variety of cell types with different phenotype and function are produced. Conrad Waddington first introduced the term “epigenetics” in 1942, in a model describing the influence of different developmental stimuli on the inheritance of acquired phenotypes (Noble 2015). In this model, the developmental landscape contains a series of decisions that he referred to as “valleys” and “forks”. The cell is represented as a ball rolling down this developmental landscape and will be “canalized” to a well-defined valley, which represents a mature cell state. With this model Waddington described epigenetics as the mechanism that gives rise to multiple cellular phenotypes from the same genotype during development (Felsenfeld 2014, Noble 2015).

With the increasing knowledge in molecular biology during the past decades, the definition of epigenetics has been modified to a great extent. In 1996, Riggs and Porter laid forward a more modern description of epigenetics, where they proposed a model including stable changes in gene function during cell division, that cannot be explained by changes in the genetic information (Hurd 2010).

The term epigenetics has been further modified in a model describing the specific mechanisms of epigenetic regulation. In this model (Figure 1) three categories of signals that lead to the establishment of a stably heritable epigenetic state are introduced as: “epigenator”, describing the environment which triggers an intracellular pathway; “epigenetic initiator signal”, which responds to the environmental stimuli and localizes the epigenetic environment; and a signal that maintains the heritable changes defined as “epigenetic maintainer signal” (Berger, Kouzarides et al. 2009).

The term epigenetics is henceforth referring to cellular memories of chromatin states, which are stable during the lifespan of an organism, and are crucial for a stable maintenance of cell type specific gene expression patterns and normal development (Felsenfeld 2014).

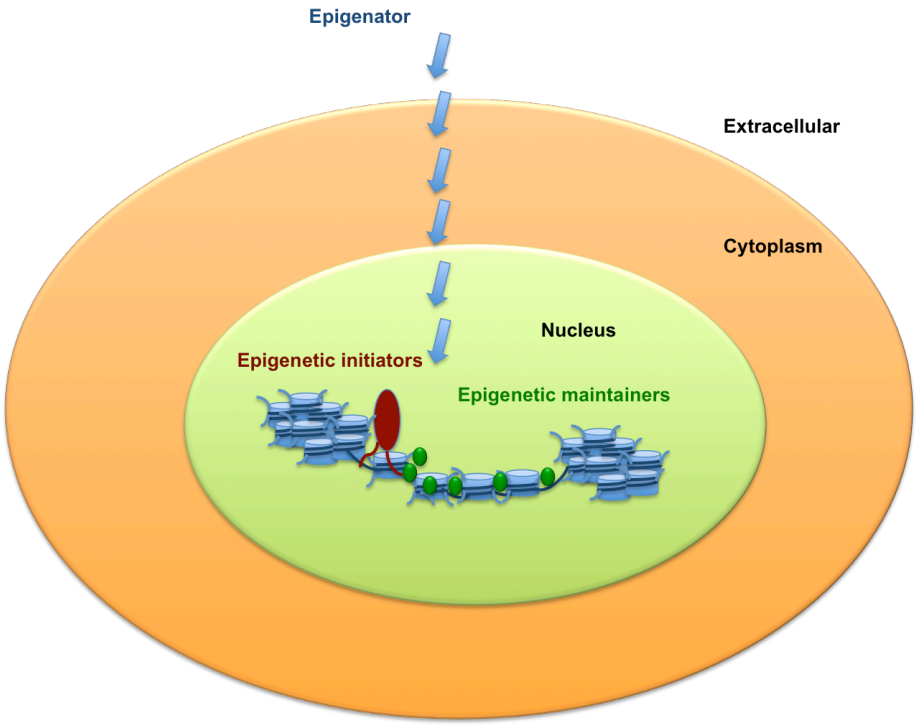

Figure 1. Schematic illustration of heritable changes in a cell. Extracellular stimuli such as differentiation signals or temperature changes that trigger an intracellular signaling cascade are referred to as epigenator. The epigenetic initiators receive the signal, which localizes the signal to the chromatin, through DNA binding factors and non-coding RNAs. The signal is maintained and transmitted to coming generations by the "epigenetic maintainers", by histone and DNA modifiers. Figure adapted with permission from publisher (Berger, Kouzarides et al. 2009).

1.1.1 Chromatin

The eukaryotic cell consists of approximately two meters of DNA, assembled into the nuclei in the form of a compacted spherical structure with the diameter of 10 µm. This macromolecular complex of DNA wrapped around a protein nucleosome in a compact structure, is referred to as chromatin (Kornberg 1977). By organizing the chromatin in structures of different degree of compaction, several specialized chromatin forms are constructed. These can be subdivided into mainly two structural states; heterochromatin, a highly condensed chromatin state, which mainly contains inactive genes and gene-poor regions and replicates in late S-phase; and euchromatin, which is less condensed and contains mostly active genes and replicates in early S-phase (Kornberg and Lorch 1992)

In order for genetic information to be expressed in a controlled way, one function of the chromatin is to regulate DNA accessibility for the many factors involved in gene regulation, such as transcription factors, repair- and replication factors.

1.1.2 The nucleosome and histones

The nucleosome consists of an octameric histone structure with 146 bp of DNA wrapped around it. Two copies of each of the core histones, H2A, H2B, H3 and H4, form the octameric structure. The core histones assemble in H2A-H2B and H3-H4 dimers, forming the octameric structure with two H3-H4 dimers in the center and two flanking H2A-H2B dimers above and below (Kornberg and Lorch 1992, Grimes, Babcock et al. 2004).

Histone proteins are positively charged, due to several positively charged amino acid residues in backbone (lysine, arginine), which will create a tight structure with the negatively charged DNA based on electrostatic interactions. Linker DNA and the linker histone H1 further connect each nucleosome into a compact structure, by changing the entry and exit angle of DNA into each nucleosome. Further twisting and supercoiling compacts the chromatin into a highly condensed form. The stability of the nucleosome is dependent on many factors, such as modifications on the underlying DNA and histone that may disrupt the tight structure.

The core and linker histones, referred to as canonical histones, are synthesized only in the S phase of the cell cycle, and their deposition into the chromatin takes place in a DNA replication-dependent manner (Kurat, Recht et al. 2014). The mRNA of the canonical histones lack introns and contain a specialized stem-loop structure at their 3′ end instead of the polyadenylation tail (Marzluff 2005).

1.1.2.1 Histone variants

In contrast to the canonical histones, there are histones that are synthesized and incorporated into the chromatin in a replication-independent manner through specific chaperones and ATP-dependent chromatin-remodeling factors (Talbert and Henikoff 2017). These are referred to as replacements histones or histone variants, which are produced via alternative splicing.

The histone variants are highly conserved between species, indicating an important functionality (Talbert, Ahmad et al. 2012).

Replacement of the canonical histones with histone variants modifies the arrangement and allocation of nucleosomes as well as DNA-binding proteins. This in turn will change the nucleosome properties and the communication with chromatin remodeler and modifiers (Talbert and Henikoff 2017).

Eight histone variants of H2A (H2A.X, H2A.Z.1, H2A.Z.2.1, H2A.Z.2.2, H2A.Bbd, macroH2A1.1, macroH2A1.2 and macroH2A2), two variants of H2B (H2BWT, H2B1A) and six variants of H3 (H3.3, CENP-A, H3.1T, H3.5, H3.X and H3.Y) have been characterized in human somatic cells (Talbert, Ahmad et al. 2012). No histone variants for H4 have yet been discovered in higher eukaryotes (Buschbeck and Hake 2017). A histone variant may be associated with a specific chromatin state, for example the H3 variant H3.3 is associated with transcriptionally active chromatin, and the other H3 variant CENP-A is associated with centromeric chromatin leading to a repressed chromatin state (Talbert and Henikoff 2017). The histone variant H2A.Z however, may be linked to both active and repressive chromatin state, depending on its posttranslational modifications. In active gene promoter regions, H2A.Z is acetylated, and in heterochromatin, H2A.Z is ubiquitinylated (Monteiro, Baptista et al. 2014). Phosphorylated H2AX (γ-H2AX) is linked with DNA double strand damage (Pouliliou and Koukourakis 2014).

1.1.3 Histone posttranslational modifications

Histones can be chemically modified on amino acid side chains, at the N-terminal protruding histone tail, by posttranslational modifications (PTM). These may promote either an active or repressive chromatin state. X-ray crystal structure studies have revealed that the histone N-terminal tails are stretching to adjacent nucleosomes, indicating that modifications on these tails may affect the overall chromatin structure and function (Luger, Mader et al. 1997).

The Histone PTMs are diverse and include at least 15 different modifications; *acetylation* of lysine; *methylation* of lysine and arginine; *phosphorylation* of threonine and tyrosine; *ubiquitinylation*, *sumoylation*, *ADPribosylation* and *citrullination* of methyl-arginine residues. Along which histone acetylation, methylation and phosphorylation are the most well described PTMs (Bannister and Kouzarides 2011).

Upon the addition of an acetyl group on the lysine side chain, the positive charge of lysine becomes neutral, which leads to decreased electrostatic interaction with the

bound DNA and less compaction of the chromatin. As a consequence, highly acetylated sites tend to promote a more open chromatin state. The dynamics of histone acetylation is regulated by two categories of enzymes that have the opposing mode of action; acetyl groups are added by lysine- and histone acetyltransferases (KATs/HATs) and removed by histone deacetylases (HDACs) (Marmorstein and Zhou 2014) (Figure 2).

Histone acetylation is a mark of active chromatin, and in transcribed genes, H3K9ac is found enriched in transcription start sites (TSS), H3K12ac is enriched in the gene body and H3K27ac and H3K122ac in enhancer regions (Wang, Zang et al. 2008, Tang, An et al. 2014).

Depending on the cellular localization of the HATs they can be further divided into two classes of groups, cytoplasmic HATs (acetylates free histones) and nuclear HATs (acetylates histones bound to the chromatin) (Parthun 2007, Bannister and Kouzarides 2011). There are three main groups of HATs that are based on the amino acid side chain sequence and structural similarities; these are GNAT, MYST and p300/CREB-binding protein families.

The acetyl groups are removed from the histone through the action of HDACs, which are divided into 4 main classes based on their amino acid side chains (de Ruijter, van Gennip et al. 2003). The members of class I, II and IV HDACs share common catalytic activity, in contrast the members of class III HDACs require NAD+ as a cofactor for the enzymatic reaction and are also referred to as sirtuins. The different HDACs have specific roles in differentiation and development; HDAC1 is involved in embryonic (ES) cell differentiation, HDAC3 is involved in neuronal differentiation and HDAC2 is involved in oligodendrocyte differentiation (Castelo-Branco, Lilja et al. 2014).

The amino acid side chains of histones can also be subjected to methylation, by the addition of a methyl group. In contrast to the acetylation, methylation doesn’t change the positive charge of the histones. Methylation can however take place in the form of mono-, di- or tri-methylation (depending on the number of added methyl groups) on lysine residues, and mono- or di-methylation symmetrically or asymmetrically on arginine residues, which adds another level of complexity (Bannister and Kouzarides 2011).

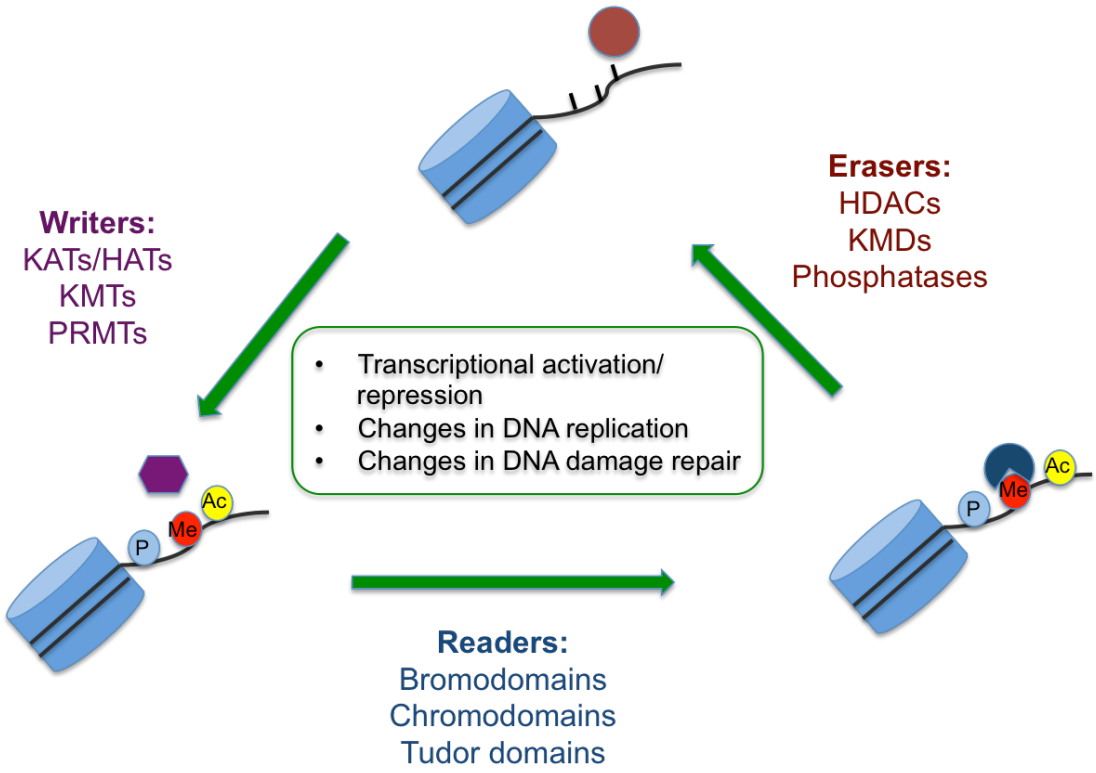

Figure 2: Schematic illustration of interplay between epigenetic maintainers. Activation or repression is controlled through the interplay between writers (histone lysine acetyl transferases (HAT/KAT), histone lysine methyltransferases (KMT) and protein arginine methyl transferases (PRMT)), readers (bromodomain, chromodomain, tudordomain) and erasers (histone deacetylases (HDAC), lysine demethylases (KMD) and phosphatases). Figure adapted from publisher with permission (Ungerstedt 2018).

The enzymes responsible for the histone lysine and arginine methylation are referred to as histone lysine methyltransferases (KMTs) and histone protein arginine methyltransferases (PRMTs). These enzymes catalyze the transfer of a methyl group from S-adenosylmethionine (SAM) to respective amino acid group side chain (Blanc and Richard 2017). Similar to histone acetylation there is an active turnover mechanism for histone methylation, which is performed via specific demethylation enzymes with different substrate specificity depending on associated cofactors (Blanc and Richard 2017, Wesche, Kuhn et al. 2017).

Histone methylation can either be associated with a repressed or active chromatin state depending on the context that they are enriched in, for example; methylation on lysines H3K4, H3K36 and H3K79 are associated with transcriptional activation; while

methylation on lysines H3K9, H3K27 and H4K20 are associated with a transcriptionally repressed state (Bannister and Kouzarides 2005).

The different modifications within each group also diverge in their distribution pattern; H3K4me3 is distributed as a sharp peak around TSS of active genes, compared to H3K36me3 which is enriched throughout the transcribed sites of the active genes (Bannister and Kouzarides 2005). The PTMs linked to heterochromatin formation also show similar difference in enrichment pattern; H3K9me3 is highly enriched in pericentric heterochromatin, while H3K27me3 is enriched in the inactive X-chromosome heterochromatin) (Bernstein, Mikkelsen et al. 2006). In the promoter regions of developmentally regulated genes in ES cells there are both active (H3K4me3) and repressive (H3K27me3) histone marks, so called “bivalent domains”, that seem to promote transcription competence for those genes (Bernstein, Mikkelsen et al. 2006, Li, Wan et al. 2018).

Different enzymes with specialized tasks facilitate the dynamics of epigenetic regulation. The positions to be modified are recognized by the writers, which are site-specific enzymes such as HATs, KMTs and PRMTs. Each modification has to be read and translated into specific function, which is performed by the interaction with enzymes called the readers, such as proteins with bromodomain, chromodomain, and tudordomain. Specialized enzymes mediate removal of each modification, referred as erasers, such as HDACs, KMTs and various phosphatases (Figure 2) (Falkenberg and Johnstone 2014, Feinberg, Koldobskiy et al. 2016).

1.1.4 DNA methylation

DNA can be subjected to chemical modification by the covalent addition of a methyl group, from S-adenosylmethionine on 5’ position of cytosine residues, giving rise to 5mC. There are three DNA methyltransferase (DNMT) enzymes that are responsible for establishing and maintaining DNA methylation; DNMT1, DNMT3A and DNMT3B during cell divisions (Okano, Bell et al. 1999). De novo methylation is performed by DNMT3A and DNMT3B, and maintained during S phase by DNMT1 that methylates the newly synthesized hemimethylated DNA (Okano, Bell et al. 1999). However, it has been demonstrated that all three DNMTs are participating in maintaining the DNA methylation patterns during cell division (Liang, Chan et al. 2002, Jones and Liang 2009).

Regions with high density of cytosine and guanine bases (CpGs) also referred to as CpG islands (CGIs). CGIs that are located in promoter sites are often unmethylated in somatic cells (Lynch, Smith et al. 2012). In contrast regions outside of the CGIs are often methylated, in many cancer cells these regions are hypomethylated (Ehrlich 2009, Wild and Flanagan 2010). Hypermethylation of promoter CGIs is frequently seen in many cancers, often linked with silencing of tumor suppressor genes (Christiansen, Andersen et al. 2003, Agrawal, Hofmann et al. 2007). Genes that should be maintained silenced during a long period of time under normal conditions, for example genes in the inactive X-chromosome, have heavily methylated promoter CGIs (Tanay, O'Donnell et al. 2007).

Promoter DNA methylation is associated with repressed transcription. The consequences of gene body DNA methylation are unclear, however have suggested being associated with active transcription ((Illingworth, Gruenewald-Schneider et al. 2010, Maunakea, Nagarajan et al. 2010).

DNA demethylation occurs via either passive mechanism (replication dependent) or two active mechanisms, (1) deamination via the activation-induced cytosine deaminase (AID) or through the apolipoprotein B mRNA-editing enzyme (APOBEC) and (2) oxidation of the 5mC through ten eleven translocation (TET) dioxygenases (Figure 3). The deamination process results in converts the 5mC to uracil leading to a T-G mismatch that is then cleaved by the thymine-DNA glycosylase (TDG), between the 5mC and the deoxyribose ring which creates an abasic site and leads to a base excision repair (BER) reaction that converts the 5mC to unmethylated cytosine (5C) (Figure 3) (Rai, Huggins et al. 2008, Zhu 2009).

The other mechanism through oxidation via the TET enzymes (TET1-3) is mediated via an oxidation of 5-methyl cytosine (5mC) to form 5- hydroxymethylcytosine (5hmC). This mechanism is a stepwise oxidation process that converts 5hmC to 5-formylcytosine (5fC) and further to carboxylcytosine (5caC), which is excised by TDG and eventually to unmethylated cytosine via the BER reaction (He, Li et al. 2011, Ito, Shen et al. 2011) (Figure 3).

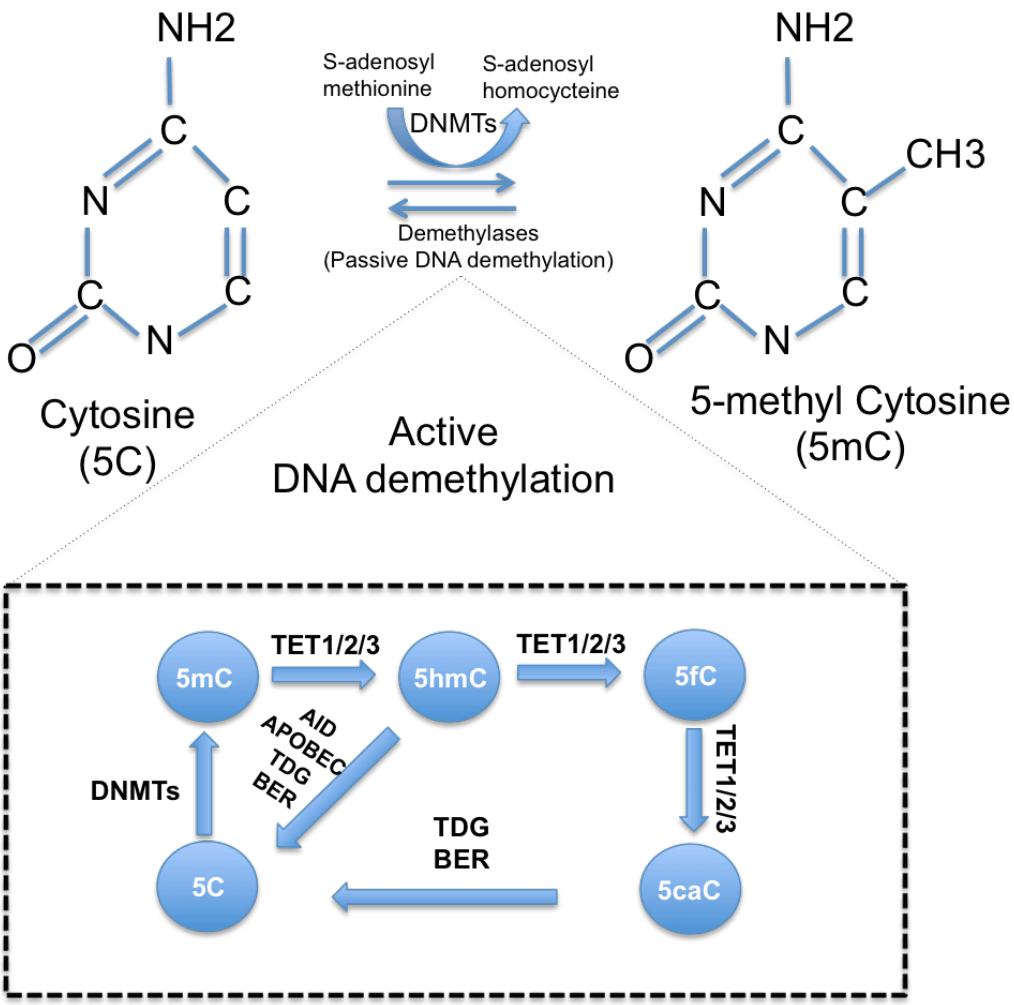

Figure 3: Schematic illustration of DNA methylation and demethylation mechanisms. DNA demethylation can either take place through passive or active mechanisms. Figure illustrated by Hani Abdulkadir Ali.

1.1.5 Crosstalk between chromatin modifiers

The crosstalk between DNA methylation and histone modifications is a dynamic process, utilizing specific proteins and enzymes, and we are only just beginning to understand these complex interactions. Crosstalk between different epigenetic modalities are likely crucial for maintaining the cellular state during cell division and development.

Regions with methylated CpGs attract proteins that contain methyl binding groups (MBD), such as MECP2 and MBD1-4, that will compact the chromatin and reinforce the repression of transcription through the recruitment of transcriptional silencing machinery (Baubec, Ivanek et al. 2013), including recruitment of HDACs but also the

repressive histone mark H3K9me3, which will compact the chromatin further (Wade and Wolffe 2001). Studies of NANOG promoter and OCT4 enhancer repression during differentiation clearly demonstrate an addition of DNA methylation that stabilizes an already repressed chromatin state (You, Kelly et al. 2011).

On the other hand, unmethylated DNA sites are recognized by proteins that contain the CXXC-domain, such as the CXXC finger protein 1 (CFP1), histone methylases (MLL1 and MLL2) and histone demethylases KDM2A and KDM2B) (You, Kelly et al. 2011, Long, Blackledge et al. 2013). The CFP1 protein is directing H3K4methylation to promoters through the recruitment of H3K4 methyltransferases.

The three DNMTs all have the ATRX-DNMT3-DNMT3L (ADD) domain that binds to unmethylated H3K4, which is associated with transcriptional inactivation (Ooi, Qiu et al. 2007, Zhang, Jurkowska et al. 2010).

The crosstalk between H3K4 methylation and DNA methylation is important for the establishment of DNA methylation patterns during embryonic development, which is taking place through the action of H3K4 demethylases ((Ciccone, Su et al. 2009, Wang, Hevi et al. 2009). The histone variant H2A.Z has also been involved in crosstalk with DNA methylation, and H2A.Z is strongly inversely correlated to DNA methylation in actively transcribed genes (Zilberman, Coleman-Derr et al. 2008, Conerly, Teves et al. 2010).

The H3K9 methyltransferases facilitate DNA methylation through the recruitment of DNMTs (Meissner, Mikkelsen et al. 2008). The repressive histone mark H3K27me3 has also been linked to DNA methylation, as promoter CGIs Another repressive histone mark that has been linked to DNA methylation crosstalk is H3K27me3, where promoter CGIs in regions that are enriched with H3K27me3 have a higher probability of acquiring DNA methylation during development (Mohn, Weber et al. 2008).

1.1.6 Transcription

The transcriptional regulation of coding genes is a key mechanism in the establishment of a cell type specific phenotype. Nuclear RNA polymerases (RNA Pol I, RNA Pol II, and RNA Pol III) carry out transcription in eukaryotic cells, where RNA Pol I transcribes ribosomal RNA (rRNA) precursors, Pol III transcribes non-coding

RNAs and Pol II is involved in the transcription of protein-coding genes to produce messenger RNA (mRNA).

Transcriptional initiation involves assembly of the five general transcription factors (GTFs) (TFIIB, TFIID, TFIIE, TFIIF and TFIIH) by Pol II at promoter DNA to form a pre-initiation complex (PIC) (Liu, Bushnell et al. 2013). The double stranded DNA is transformed to a single strand by the action of TFIIH, Pol II can then interact with the open DNA and facilitate the RNA synthesis (Smolle and Workman 2013). The TATA-box binding protein (TBP), present in TFIID, initiates transcription at promoter sites (Liu, Bushnell et al. 2013). However, promoter initiated Pol II transcription is not the only mechanism for transcription of genes. In recent years, regions that are distal from the TSS have been described to be associated with transcription. These regions are referred to as enhancers or insulators ((Butler and Kadonaga 2002), and enhancers can be repressed or themselves enhanced, via the action of several transcription factors (TFs). In enhancer region DNA, DNA binding proteins are found, and these proteins enable DNA to form loops to the target gene promoter (Visel, Blow et al. 2009, Andersson, Gebhard et al. 2014). In the pursuit to identify enhancer elements, increased H3K4me1 and H3K27ac together with the absence of H3K27me3 are associated with active enhancer regions (Pennacchio, Bickmore et al. 2013, Heinz, Romanoski et al. 2015). Bivalent enhancers, which are capable of fast activation or repression of target genes, are defined by the presence of H3K27me3 and H3K4me1 at low levels together with the absence of H3K27ac (Heintzman, Stuart et al. 2007, Visel, Blow et al. 2009).

1.2 HEMATOPOIESIS

Different types of blood cells are responsible for specialized tasks, such as oxygen supply, wound healing and protection from pathogens. These mature blood and immune cells are produced from the multipotent hematopoietic stem cells (HSCs), in the developmental process referred to as hematopoiesis (Jagannathan-Bogdan and Zon 2013, Crisan and Dzierzak 2016). The early research of HSCs demonstrated a self-renewing multipotent potential of the HSCs; that has the ability to generate different lineages determined through both intrinsic and extrinsic factors (Figure 4) (Eaves 2015).

The initial step in the differentiation process requires loss of self-renewal capacity, in order to generate progenitor cell stages with a restricted degree of potency. The HSCs give rise to the multipotent hematopoietic progenitor cells (MPPs), which in turn generates common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs). The CMPs can further produce granulocyte and macrophage progenitors (GMPs) and megakaryocyte and erythrocyte progenitors (MEPs), while the CLPs produce progenitors of B, T and NK cells (Kondo, Weissman et al. 1997, Akashi, Reya et al. 2000, Manz, Miyamoto et al. 2002).

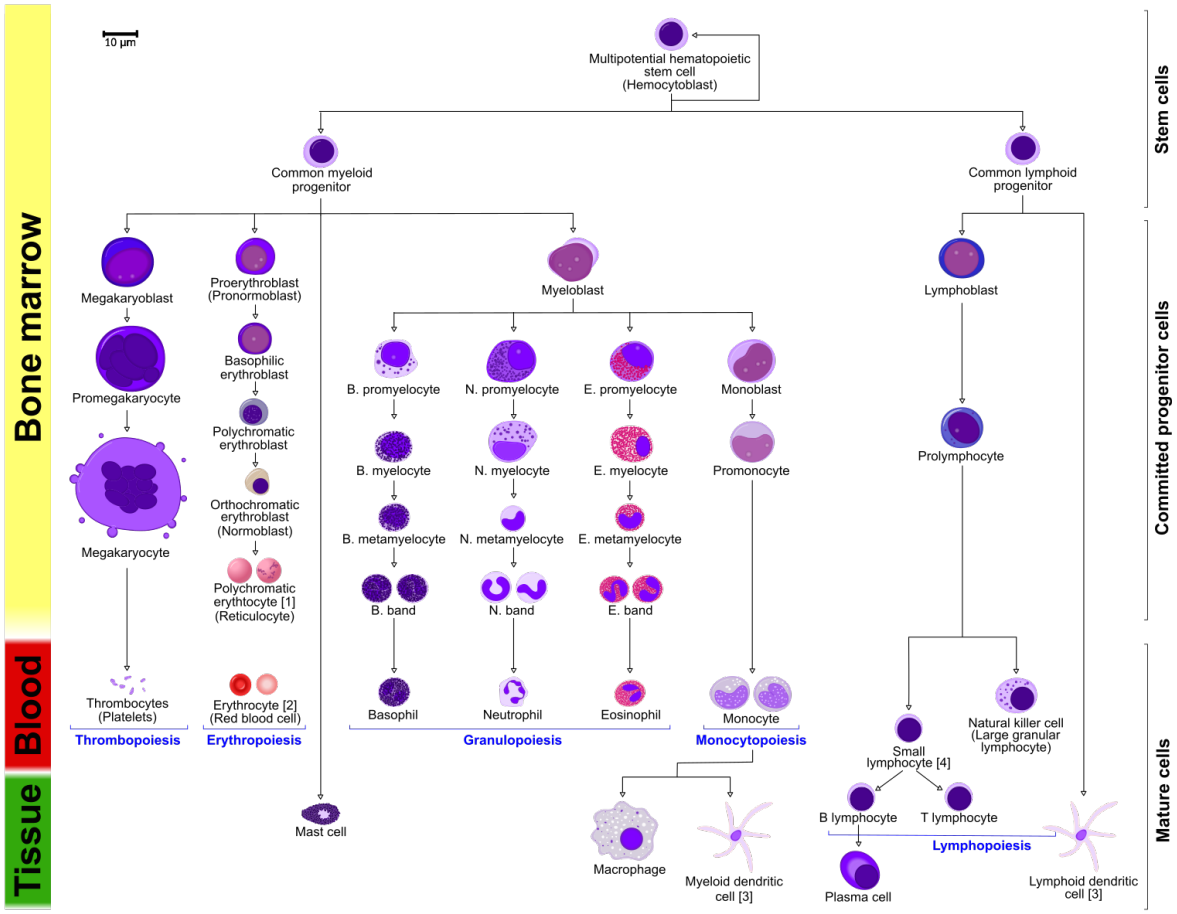

Figure 4: Schematic illustration of hematopoiesis. The differentiation process starts from multipotent self-renewable HSCs, which have the capacity to generate committed progenitor cells and different mature blood cells. Figure reprinted with permission from the publisher (Wikipedia 2018).

1.2.1 Epigenetic regulation of hematopoiesis

Epigenetic regulation has been proven to be important in hematopoiesis, affecting both cellular identity and differentiation (Sharma and Gurudutta 2016). The level of DNA methylation affects myeloid and lymphoid lineage commitment, in that a reduced DNA methylation by reduction of DNMT1 activity causes myeloid biased differentiation (Chavez, Jozefczuk et al. 2010). In contrast, lymphoid lineages depend on acquisition of DNA methylation (Ji, Ehrlich et al. 2010). These differences in the DNA methylome between the two lineages can explain the drive towards myeloid lineages when the DNA methylation machinery is deregulated. Combined reduction of DNMT3A and DNMT3B has however demonstrated an enhanced self-renewal capacity and decreased differentiation potential (Challen, Sun et al. 2014).

Histone modifications also contribute to the regulation of HSC differentiation. Erythroid and myeloid differentiation from CMP has been linked to HDAC1 expression (Wada, Kikuchi et al. 2009), and CMPs have a poised chromatin state with H3K4me3 and H3K27me3 enrichment, and upon further differentiation, H3K4me1 and H2A.Z levels increase (Cui, Zang et al. 2009, Abraham, Cui et al. 2013).

1.2.2 Transcriptional regulation of hematopoiesis

In the differentiation process, networks of growth factors and TFs drive lineage commitment (Gottgens 2015). TFs shape the epigenome and hence provide a crucial regulation by either favoring a certain HSC fate or pushing forward to a lineage commitment, leading to mature blood cells. There are sets of TFs that are important for preserving the stem cell identity as well as production of the HSCs (Orkin and Zon 2008) (Figure 5). The regulatory TFs that are thought to regulate the myeloid lineage commitment are GATA1, PU.1 and RUNX1 (Ferreira, Ohneda et al. 2005, Chou, Khandros et al. 2009). Commitment to the lymphoid lineage and lymphoid cell identity requires activation of E2A, Ebf1 and PAX5 in a linear manner (Nutt and Kee 2007, Zandi, Bryder et al. 2010).

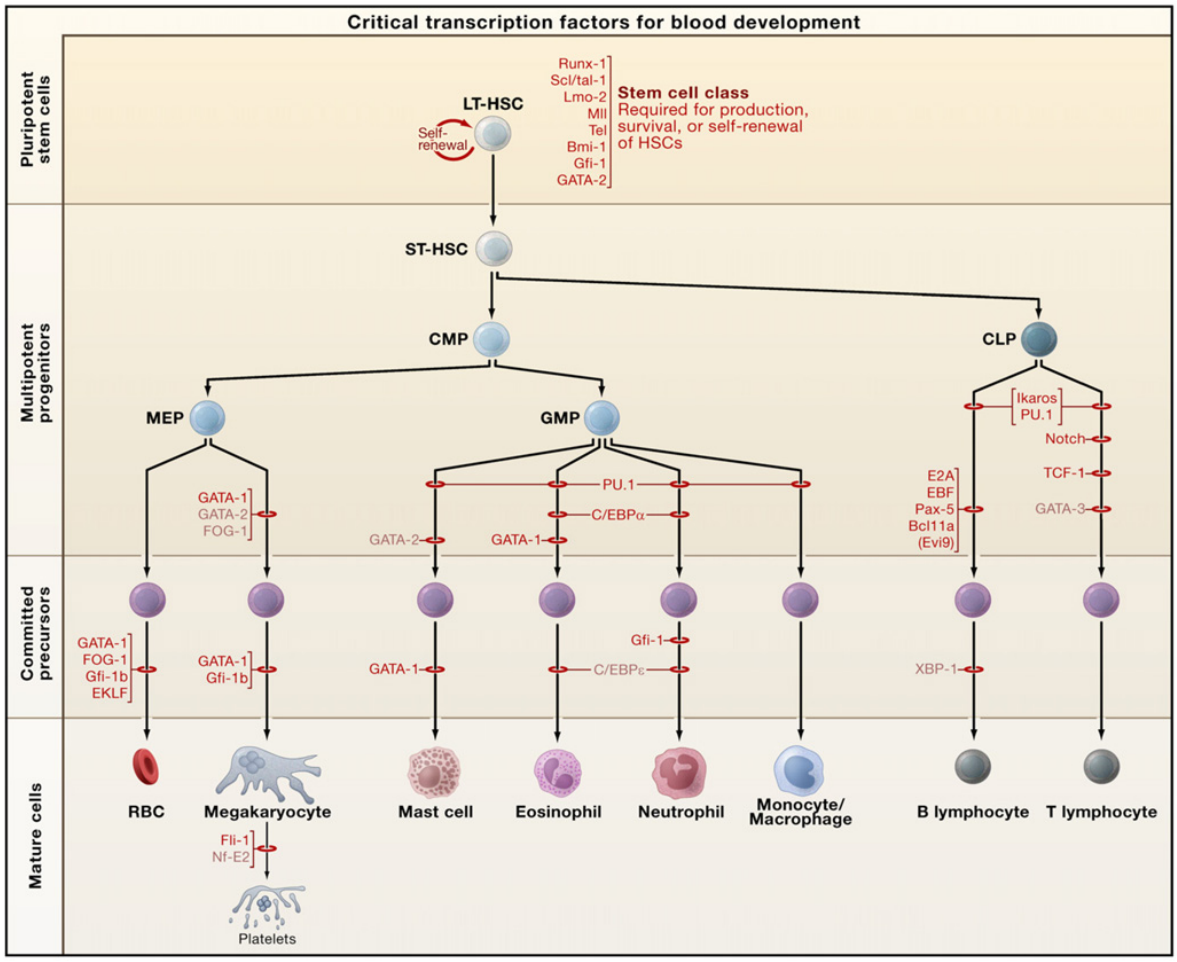

Figure 5. Transcription factors important in hematopoiesis.

TFs active in different stages of the hematopoietic differentiation process are illustrated in red. TFs that have not (yet) been demonstrated to be translocated or mutated in human/mouse are in light font. The remaining TFs, in bold font, have been associated with chromosomal translocations or somatic mutations in hematological malignancies. Figure reprinted with permission from publisher (Orkin and Zon 2008).

1.3 MYELOID MALIGNANCIES

Myeloid malignancies refers to diseases originating from cells of myeloid origin, as opposed to cells of lymphoid origin, all developed from a common HSC.

1.3.1 The myelodysplastic syndromes

The myelodysplastic syndromes (MDS) consist of a heterogeneous group of clonal hematologic stem cell disorders, characterized by ineffective hematopoiesis that leads to low peripheral blood cell counts (Bejar, Stevenson et al. 2011, Montalban-Bravo and Garcia-Manero 2018). In the majority of cases, the bone marrow is hypercellular, however the blood cells produced are dysplastic and the majority of these cells undergo apoptosis before reaching the peripheral circulation, leading to an inefficient hematopoiesis resulting in peripheral blood cytopenia where almost all patients have anemia, sometimes in combination with thrombocytopenia and or neutropenia.

1.3.1.1 Diagnostic tools

The international prognostic scoring system (IPSS) for risk stratification of MDS patients was introduced in 1997 (Greenberg, Cox et al. 1997), and revised in 2012 giving rise to the currently used revised IPSS score (IPSS-R) (Greenberg, Tuechler et al. 2012). The IPSS-R takes into account bone marrow blasts, hemoglobin, platelet and neutrophil count, age and cytogenetic risk and divides the patients into five cytogenetic subgroups (Very good, Good, Intermediate, Poor and Very poor) (Table 1) (Greenberg, Tuechler et al. 2012, Greenberg 2013, Della Porta, Tuechler et al. 2015).

Table 1: Prognostic classification of MDS patients with IPSS-R (adapted from publisher with permission (Greenberg, Tuechler et al. 2012)).

IPSS-R Cytogenetic risk groups

Cytogenetic prognostic subgroups	Cytogenetic abnormalities
Very good	Y, del(11q)
Good	Normal, del(5q), del(12p), del(20q), double including del(5q)
Intermediate	del(7q), +8, +19, i(17q), any other single or double independent clones
Poor	7, inv(3)/t(3q)/del(3q), double including 7/del(7q), Complex: 3 abnormalities
Very poor	Complex: >3 abnormalities

IPSS-R Prognostic Score Values

Prognostic variable	0	0.5	1	1.5	2	3	4
Cytogenetics	Very good		Good		Intermediate	Poor	Very Poor
BM Blast %	<=2		>2-<5%		5-10%	>10%	
Hemoglobin	=>10		8-<10	<8			
Platelets	=>100	50-<100	<50				
ANC	=>0.8	<0.8					

IPSS-R Prognostic Risk Categories/scores clinical outcomes

	No. pts	Very Low	Low	Intermediate	High	Very high
Risk score		<=1.5	>1.5-3	>3-4.5	>4.5-6	>6
Patients (%)	7012	19%	38%	20%	13%	10%
Survival		8.8	5.3	3.0	1.6	0.8
AML/25%		NR	10.8	3.2	1.4	0.7

1.3.1.2 Mutational landscape in MDS

In 2011, several groups described the presence of common somatic heterozygous mutations in MDS patients (Bejar, Stevenson et al. 2011, Malcovati, Papaemmanuil et al. 2011, Papaemmanuil, Cazzola et al. 2011, Thol, Bollin et al. 2014). These mutations are found in genes coding for proteins involved in DNA methylation, chromatin regulation, RNA splicing, transcription regulation, DNA repair, cohesion function or signal transduction (Papaemmanuil, Gerstung et al. 2013, Haferlach,

Nagata et al. 2014). However, as of yet, none of these mutations are included in the diagnostic criteria of MDS, apart from the splicing factor mutation SF3B1 is associated with ring sideroblast anemia and associated with good prognosis (Arber, Orazi et al. 2016).

The clinical implication of these mutations is currently under intense investigation, and likely the mutations have bearing for risk of AML progression, and chance of response to different forms of therapy.

The TET2 gene is frequently mutated in MDS (Figure 6), however the clinical implications of the TET2 mutation remains to be defined, as studies so far report conflicting results. There are studies where TET2 mutations did not have any impact on overall survival, but also reports of TET2 as a favorable prognostic marker (Kosmider, Gelsi-Boyer et al. 2009, Bejar, Stevenson et al. 2011, Bejar, Stevenson et al. 2012).

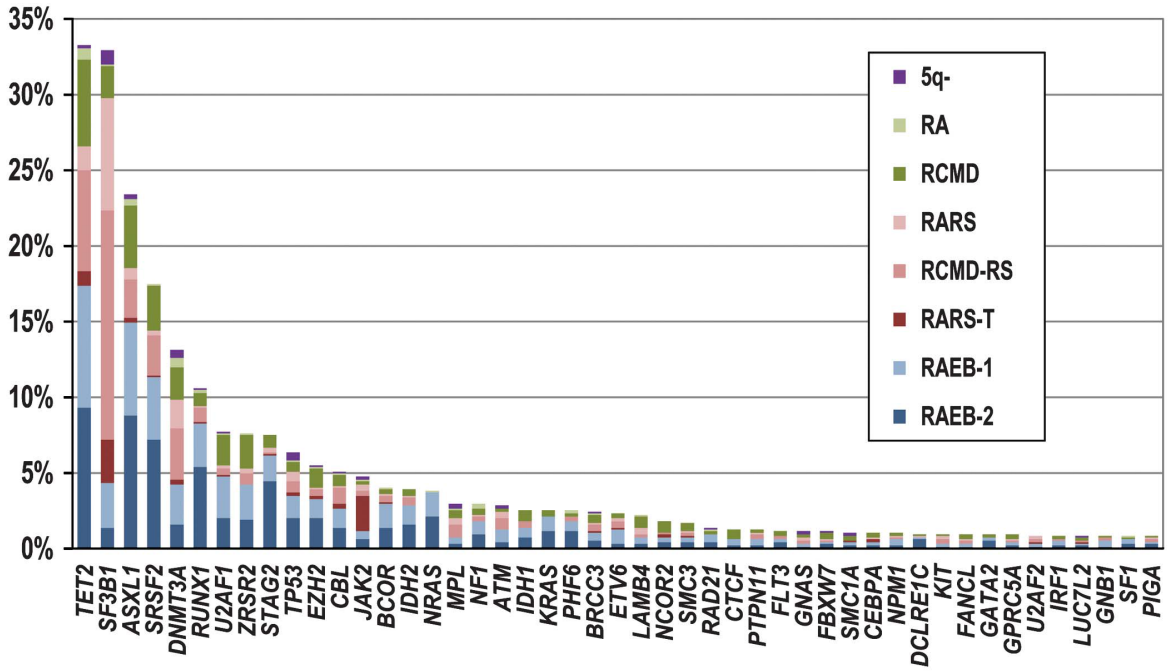

Figure 6. Frequency of common mutations in different subgroups of MDS. Figure reprinted with permission (Haferlach, Nagata et al. 2014).

When it comes to response to therapy, some studies have linked TET2 mutations to better response to the hypomethylating agents Azacitidine (AZA) and Decitabine (DAC) compared to patients without this mutation (Itzykson, Kosmider et al. 2011, Bejar, Lord et al. 2014). Mutations in the histone modulators ASXL1, EZH2 have

been associated with poor prognosis (Abdel-Wahab, Pardanani et al. 2011, Inoue, Kitaura et al. 2013), however a recent study demonstrated an increased survival and good treatment response to AZA (Tobiasson, McLornan et al. 2016). Mutations in TP53 and RUNX1 genes are clearly linked to poor overall survival (Tobiasson, McLornan et al. 2016). However, an individual patient commonly presents with several co-occurring mutations, which makes the prognostic evaluation more difficult (Hasegawa, Oshima et al. 2017).

1.3.1.3 Epigenetic dysregulation in MDS

Aberrant DNA methylation is generally associated with many cancers (Feinberg and Vogelstein 1983, Herman and Baylin 2003, Jones and Baylin 2007). As discussed in the previous section, mutations in the epigenetic machinery are common in myelodysplastic syndromes. With the recent technical advances it has been demonstrated that MDS patients have a global DNA hypermethylation (Figueroa, Skrabanek et al. 2009, Jiang, Dunbar et al. 2009). The aberrant methylation pattern compared to normal cells, has also been linked to progression to acute myeloid leukemia (AML) (Jiang, Dunbar et al. 2009)

Despite the many studies on the MDS methylome, there are not many genes that have been shown experimentally to impact the MDS pathogenesis. One gene that is hypermethylated in MDS is p15, which is involved in cell cycle regulation. Promoter hypermethylation of p15 has been associated with poor prognosis and deletions of chromosome 5q and 7q (Uchida, Kinoshita et al. 1997, Quesnel, Guillerm et al. 1998, Tien, Tang et al. 2001).

Mutations in DNMT3A have been reported to be present in approximately 10 % of the MDS patients with no correlation to any cytogenetic subgroup, and have been associated with poor survival and faster progression to AML (Walter, Othus et al. 2011, Ganguly and Kadam 2016).

Apart from the mutations in the DNA methylation regulators (TET2, DNMT3A) there are also mutations in the histone modifying regulators (ASXL1, EZH2), which have been associated with poor prognosis (Abdel-Wahab, Pardanani et al. 2011, Inoue, Kitaura et al. 2013).

1.3.1.4 Treatment options in MDS

Based on the IPSS-R risk score, treatment choices are made. Therapy options for low risk patients are growth factors, supportive care and if del5q is present, lenalidomide (Montalban-Bravo and Garcia-Manero 2018). Patients in the higher-risk groups are recommended treatment with the hypomethylating agents (HMA), in Sweden Azacitidine is administered in the clinic. If the patient has higher risk disease and is considered eligible for Allogeneic stem cell transplant, this procedure is performed, however the majority of patients are not eligible, due to age or comorbidities (Heuser, Yun et al. 2017). The efficacy of Azacitidine and Decitabine in higher risk MDS is well documented and HMA treatment leads to an increased overall survival, better quality of life and improvements of hemoglobin, platelets and neutrophils (Kantarjian, Issa et al. 2006, Fenaux, Mufti et al. 2009).

However, only 50-60% of patients respond to HMA treatment, and all patients will eventually become treatment resistant. Several studies have investigated the impact of somatic mutations on response rate to HMA treatment, however studies show opposing results and this issue remains to be settled. Some studies have demonstrated that there are no differences in response rate between patients with and without mutations in DNMT3A, IDH1, IDH2, ASXL1 and EZH2 (DiNardo, Jabbour et al. 2016, Welch, Petti et al. 2016). Two other studies reported increased response to HMA with TET2 mutation in the absence of ASXL1 mutation (Itzykson, Kosmider et al. 2011, Bejar, Lord et al. 2014). ASXL1, that is usually associated with bad prognosis, has been linked to a better chance to respond to Azacitidine and increased survival in a recent study (Tobiasson, McLornan et al. 2016).

1.3.2 Acute myeloid leukemia

Acute myeloid leukemia (AML) is a heterogeneous clonal malignancy, with accumulation of immature blood cells in the bone marrow and the peripheral blood (O'Donnell, Abboud et al. 2012, Klepin 2015). The estimated median age of disease onset is around 70 years, making AML primarily a disease of the elderly, however the disease onset can come in any age (Klepin 2015). There are many subtypes of AML, based on morphological and cytogenetic aberrations, and also other genetic alterations like NPM1 and FLT3 mutations (Ref WHO 2016). Heterozygous somatic mutations are also found in AML making the molecular landscape of AML complex

and dynamic. Diagnostic and prognostic clinical methods for mutation detection are rapidly evolving (Dohner, Weisdorf et al. 2015)

1.3.2.1 Diagnostic tools

Bone marrow morphology, immunophenotyping, cytogenetics and molecular genetic testing are routinely used diagnostic tools, and AML is diagnosed if ≥20% blasts are seen in bone marrow smears (Dohner, Estey et al. 2017).

Chromosomal abnormalities are found in approximately 55% of AML patients (Grimwade 2001, Mrozek, Heerema et al. 2004). In the WHO classification “AML with recurrent genetic abnormalities” eight translocations and their variants are represented (Arber, Orazi et al. 2016). Molecular genetic testing are used to screen mutations in disease category defining genes (NPM1, CEBPA and RUNX1), genes of prognostic and therapeutic value (FLT3) and mutations with unfavorable prognostic association (TP53 and ASXL1) (Gale, Green et al. 2008, Bowen, Groves et al. 2009, Pratcorona, Abbas et al. 2012, Schnittger, Eder et al. 2013, Haferlach, Nagata et al. 2014, Devillier, Mansat-De Mas et al. 2015, Tsai, Hou et al. 2016).

1.3.2.2 Mutational landscape

Mutations in the DNA methyltransferase 3A gene (DNMT3A) and isocitrate hydroxygenase genes (IDH1 and IDH2) were one of the first mutations that were discovered in AML (Cancer Genome Atlas Research, Ley et al. 2013, Dohner, Weisdorf et al. 2015). Several studies have investigated the clonal evolution in AML, concluding the presence of a phylogenic tree of mutations in both human and mouse models (Jan, Snyder et al. 2012, Corces-Zimmerman, Hong et al. 2014). Emphasizing that mutations in genes with epigenetic function (DNMT3A, TET2 and ASXL1) occur in preleukemic progenitor cells prior to leukemogenesis. However, these mutations have almost never been found as isolated clones indicating that they are not enough to drive the leukemogenesis (Papaemmanuil, Gerstung et al. 2016).

Tyrosine kinase-RAS receptor pathway mutations were observed to occur late and often more than once in the same patient (Paschka, Du et al. 2013, Corces-Zimmerman, Hong et al. 2014, Groschel, Sanders et al. 2015). The cooperation of mutations in FLT3 and RUNX1 have been associated with induction of AML patients (Behrens, Maul et al. 2017)

Mutations in NMP1 gene occurred usually as a secondary hit after mutations in DNMT3A, IDH1 and NRAS (Papaemmanuil, Gerstung et al. 2016). The different molecular mutations in AML and their frequencies are presented in Figure 7.

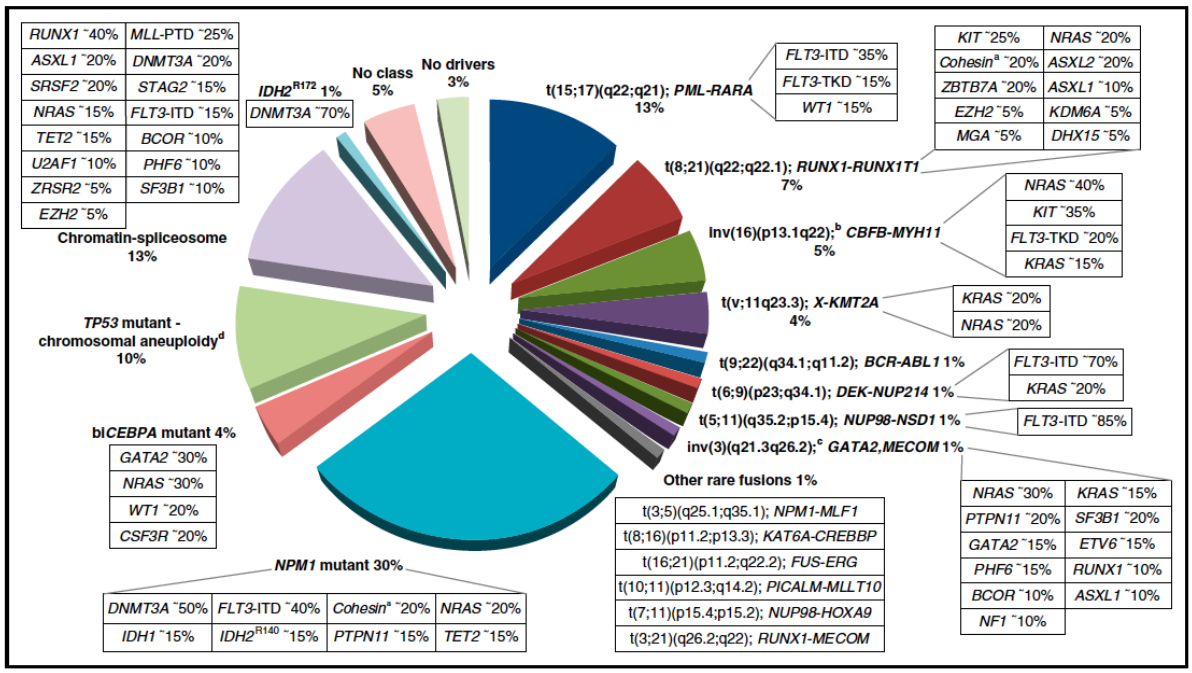

Figure 7: Frequency of common molecular mutations in adult AML. Figure reprinted with permission from publisher (Dohner, Estey et al. 2017).

Together with the advances in the molecular landscape of AML the risk stratification has been updated recently, grouping patients into favorable, intermediate and adverse risk categories (Table 2) (Dohner, Estey et al. 2017).

Table 2. Risk stratification in AML (Dohner, Estey et al. 2017).

Risk category	Genetic abnormality
Favorable	Favorable t(8;21)(q22;q22.1); RUNX1-RUNX1T1 inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11 Mutated NPM1 without FLT3-ITD or with FLT3-ITDlow Biallelic mutated CEBPA
Intermediate	Mutated NPM1 and FLT3-ITDhigh Wild-type NPM1 without FLT3-ITD or with FLT3-ITDlow (without adverse-risk genetic lesions) t(9;11)(p21.3;q23.3); MLLT3-KMT2A Cytogenetic abnormalities not classified as favorable or adverse
Adverse	t(6;9)(p23;q34.1); DEK-NUP214 t(v;11q23.3); KMT2A rearranged t(9;22)(q34.1;q11.2); BCR-ABL1 inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2,MECOM(EVI1) 25 or del(5q); 27; 217/abn(17p) Complex karyotype, monosomal karyotype|| Wild-type NPM1 and FLT3-ITDhigh Mutated RUNX1 Mutated ASXL1 Mutated TP53

1.3.2.3 Epigenetic dysregulation in AML

The methylome in AML is generally hypermethylated in promoter CpG islands (CGI) and hypomethylated in the gene bodies however the methylation aberrations vary to a great extent between the different subtypes in AML (Alvarez, Suela et al. 2010, Bullinger, Ehrich et al. 2010, Deneberg, Guardiola et al. 2011). The epigenetic alterations in AML influence factors involved in regulation of DNA methylation (DNMT3A, IDH1, IDH2, TET), chromatin modulators and histone modifications (MLL fusions, MLL-PZD, EZH2, ASXL1) (Krivtsov and Armstrong 2007, Conway O'Brien, Prideaux et al. 2014). Another component involved in the epigenetic machinery found to be mutated in AML is the cohesion complex, which has the role to organize the chromatin in order to promote intra- and inter-chromosomal interactions (Welch, Ley et al. 2012, Leeke, Marsman et al. 2014, Thol, Bollin et al. 2014). Such mutations have been related with mutations in NPM1 and RUNX1 genes (Thota,

Viny et al. 2014). The clinical outcome of mutations in epigenetically active genes is complex and in need of further investigation. One example of this is that the majority of healthy people in the general population with these mutations (DNMT3A, ASXL1, TET2) do not develop AML (Genovese, Kahler et al. 2014, Jaiswal, Fontanillas et al. 2014, Xie, Lu et al. 2014).

1.3.2.4 Treatment options in AML

The first choice of treatment in AML is induction chemotherapy, with a combination of cytarabine and anthracycline (most often daunorubicin) (Dombret and Gardin 2016). Thereafter, consolidation chemotherapy is administered in order to aggressively target the leukemic cells (Mayer, Davis et al. 1994, Schlenk 2014). Not all patients can tolerate intensive chemotherapy, and patients may be selected for other options like Azacitidine (Dohner, Estey et al. 2017).

Several studies have investigated the clinical outcome of treatment with Azacitidine in AML. Patients with newly diagnosed AML, being both fit and unfit for intensive chemotherapy (IC) (Dombret and Gardin 2016), and patients with relapsed/refractory AML, demonstrated a similar overall survival with Azacitidine compared to IC patients (Ivanoff, Gruson et al. 2013, Itzykson, Thepot et al. 2015). Chemotherapy has often many off-target effects with little overall cure rate in most patients with AML, emphasizing the need of more targeted therapy (Lazarevic, Bredberg et al. 2018).

1.3.3 Systemic mastocytosis

Systemic mastocytosis (SM) is a clonal bone marrow malignancy with an activating point mutation at codon 816 (D816V) in the catalytic domain of the stem cell factor receptor KIT, which leads to increased survival and proliferation of mast cells (Metcalfe and Mekori 2017). Patients with SM suffer from accumulation of clonal mast cells (MCs) in various organs (Valent, Akin et al. 2017).

More than 95% of the patients carry the KIT D816V mutation, and the paradox of SM is that the same KIT D816V mutation may give an indolent or aggressive clinical phenotype. There is a correlation between disease symptoms and the allelic burden of KIT D816V mutation (Kristensen, Broesby-Olsen et al. 2013, Meni, Bruneau et al.

2015), however likely there are additional, unknown factors that determine whether the patient will have an indolent or aggressive SM disease.

The clinical features of SM consist of a broad spectrum of symptoms from various organs (Metcalfe 2008, Teodosio, Garcia-Montero et al. 2010, Jawhar, Schwaab et al. 2016). Symptoms vary from flushing, hypotension, nausea, vomiting, abdominal cramping, headaches, psychiatric symptoms, to anaphylaxis (Metcalfe and Mekori 2017).

1.3.3.1 Diagnostic tools

The major criteria for SM include a multifocal accumulation and clustering of MCs in the bone marrow or other extra cutaneous organ. The clonal nature, abnormal MC morphology, expression of the lymphoid related antigens CD2 and CD25, presence of KIT D816V mutation, and a basal tryptase level over 20 ng/mL are included in the minor criteria of SM (Valent, Akin et al. 2017). When one major criterion and at least one minor criterion, or at least three minor criterions are fulfilled the patient is diagnosed with SM (Valent, Akin et al. 2017).

The different subgroups in systemic mastocytosis consists of indolent systemic mastocytosis (ISM), smoldering systemic mastocytosis (SSM), systemic mastocytosis with associated hematological neoplasm (SM-AHN), aggressive systemic mastocytosis (ASM), and MC leukemia (MCL) (Valent, Akin et al. 2017). Patients with indolent SM may have severe symptoms but have a normal life expectancy (Pardanani 2016). SM-AHN is frequently found, that is patients with indolent or aggressive SM are found to have another hematological non mast cell disease, most frequently MDS of various subtypes. In SM-AHN, survival depends on the AHN disease. Patients with ASM and MCL have the worst prognosis, with usually a survival time of 2 to 4 years for ASM and only months up to one year for MCL.

1.3.3.2 Treatment options in SM

In indolent SM, symptom control is vital, using histamine blockers (H1 and H2) for blocking the effects of mast cell histamine release, and sometimes leukotriene antagonists.

For patients with advanced SM the treatment options include several cytoreductive therapies, such as 2-Chlorodeoxyadenosine (2-CDA), interferon-alpha (IFN-α) in combination with steroids, or sometimes classical chemotherapy agents (such as cytarabine or fludarabine) (Pardanani 2016). However, no chemotherapeutic agent has proven an improved survival in ASM. In the case of SM-AHN, it is recommended to treat the AHN, e.g. hydroxyurea of the AHN is a myeloproliferative neoplasm, and Azacitidine or growth factors if the AHN is MDS.

In recent years, the pan tyrosine kinase inhibitor midostaurin has been approved for ASM and MCL (DeAngelo, George et al. 2018), however despite the promising results, midostaurin has not yet demonstrated an improved survival rate, thus there is still a need for targeted therapy in SM.

1.4 EPIGENETIC DRUGS

1.4.1 Azacitidine

The DNA consists of two polynucleotide strands that form a double helix. The four nucleobases that shape the DNA are adenine (A), guanine (G), cytosine (C) and thymine (T). Azacitidine (AZA) is a structural analog to cytidine. When AZA is taken up in the cell, 80-90% is converted into ribonucleoside triphosphate by uridine-cytidine kinase (UCK) leading to incorporation into RNA and further inhibition of protein synthesis A fraction (10-20%) of the diphosphate forms of AZA is further reduced into deoxy- (DAC)-diphosphates, by ribonucleotide reductase (RR), that can be incorporated into DNA (Aimiuwu, Wang et al. 2012).

Early studies have shown that AZA was preferentially targets malignant cells but not dividing healthy cells (Glover and Leyland-Jones 1987). Additional studies concluded targeting of DNA hypermethylation mainly via DNMT1 depletion (Kaminskas, Farrell et al. 2005, Estey 2013).

1.4.1.1 Molecular mechanisms of action of AZA and DAC

Although AZA and DAC have been used in the clinic for the past three decades the exact mechanism of action is not clear, and several different mechanisms of action have been proposed. Below, three different mechanism of action of AZA and DAC, which can perhaps explain the differences in the clinical outcomes, are being described.

(A) DNMT1 depletion mechanisms dependent on DNA replication

The proposed mechanism of action of histone hypomethylating agents (HMA) is established on the ability of the HMA to mimic normal cytidine and incorporate into DNA, leading to inhibition of methylation by DNMTs. The inhibition is due to the replacement of cytosine carbon in position 5 with a nitrogen group in AZA and DAC analogous, leading to an irreversible covalent bond with DNMT1 that is depleted upon replication. Depletion of DNMT1, which is important for maintaining DNA methylation during replication, leads to reduced overall DNA methylation marks in the daughter cells and an increased gene expression. The idea is that the tumor suppressor genes will be re-activated and hence block the malignant proliferation (Figure 8A).

(B) DNMT1 depletion mechanisms independent on DNA replication

Several studies have emphasized the need of a DNA replication independent depletion of DNMT1. The rationale behind this is due to observations of decreased DNMT1 activity faster than incorporation of HMA to DNA, depletion of DNMT1 even in the absence of DNA replication and cell division, and the incorporation of AZA into DNA (Creusot, Acs et al. 1982, Aimiuwu, Wang et al. 2012).

The proposed DNA replication independent mechanism occurs through the protein kinase C delta (PKCd) which hyperphosporylates DNMT1 directing it to rapid proteosomal degradation via the ubiquitination system (Pleyer and Greil 2015) (Figure 8B).

(C) RNA dependent inhibition mechanisms of RR

The majority of AZA is incorporated into RNA as described earlier, giving rise to apoptosis through the disruption of mRNA and protein metabolism (Aimiuwu, Wang et al. 2012).

The rate-limiting enzyme ribonucleotide reductase (RR) generates the basic building blocks (deoxyribonucleotides) of DNA synthesis through its two subunits, RRM1 and RRM2. Studies have demonstrated the decrease of RRM2 mRNA stability and inhibition of RRM2 gene expression upon AZA incorporation into RNA (Aimiuwu, Wang et al. 2012). Inhibition of RR leads to decreased intracellular levels of the four deoxyribonucleotides (Figure 8C).

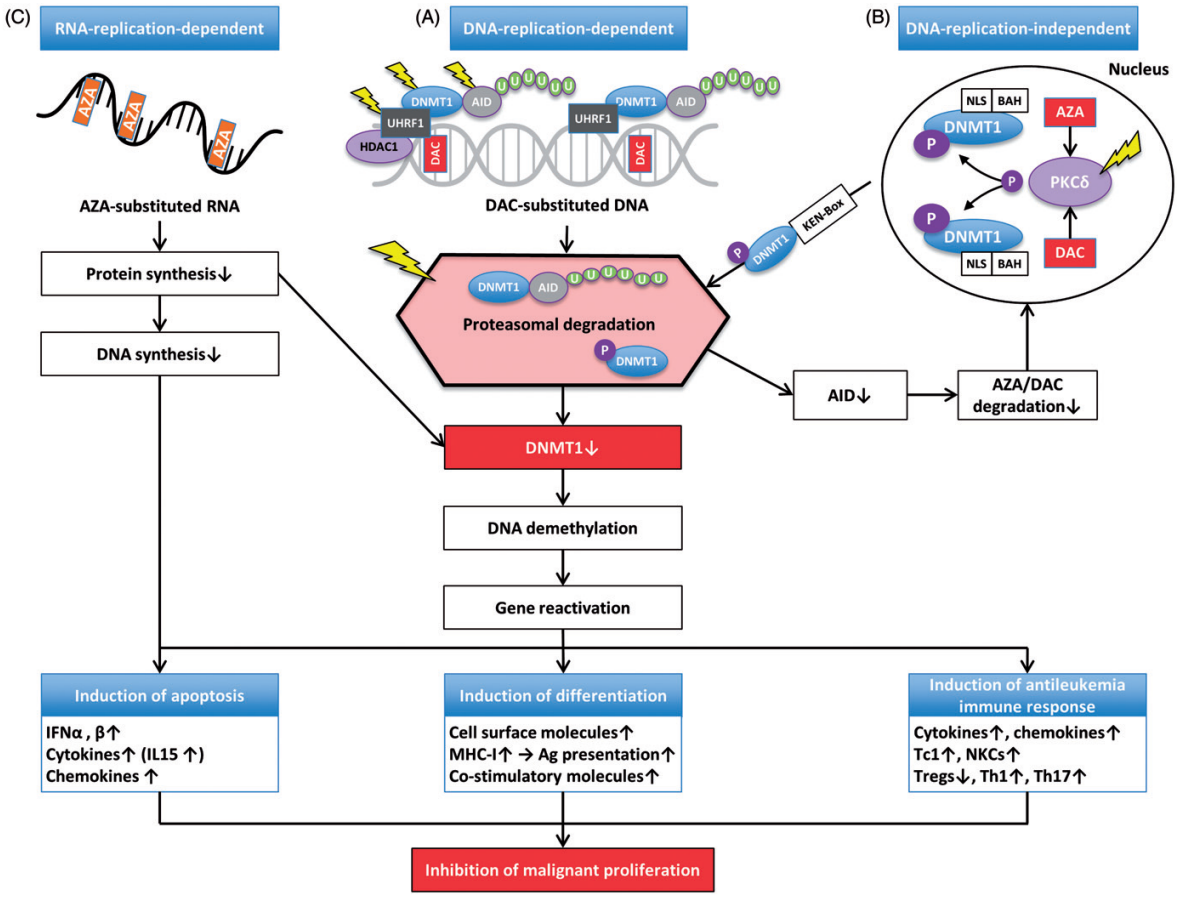

Figure 8: Schematic illustration of different mechanisms of action of AZA and DAC. The three proposed mechanisms of action of AZA/DAC are depicted. (A) DNA replication independent mechanism. (B) DNA independent mechanism. (C) RR replication dependent mechanism. Figure reprinted with permission from publisher (Pleyer and Greil 2015).

1.4.2 Histone deacetylase inhibitors (HDACi)

Histone deacetylation prevents gene expression through removal of acetyl groups on acetylated lysine histones, but histone deacetylases also target non-histone proteins such as α-tubulin, nuclear transporter proteins, chaperones (HSP90 and HSP70) and TFs (p53, c-myc, NFkβ) (Glozak, Sengupta et al. 2005, Minucci and Pelicci 2006). Mutations in many HDACs have been observed in a variety of disorders, making HDACi an attractive treatment option (Mai, Massa et al. 2005). HDACi targets transformed cells via cell cycle arrest, inhibition of angiogenesis and induced apoptosis (Marks, Rifkind et al. 2001, Paris, Porcelloni et al. 2008).

HDACi are classified into different groups depending on their chemical structures, which consists of hydroxamic acids, cyclic peptides, electrophilic ketones, short-

chain fatty acids, benzamides, boronic acid-based compounds, benzofuranone and sulfonamide-containing molecules and α/β peptide structures (Marks and Breslow 2007, Wang, Hevi et al. 2009). There are three functional chemical components of hydroxamic acids; a zinc-binding moiety (ZBM) in the catalytic domain, opposite capping group and a linker group consisting of a straight-chain alkyl, vinyl or aryl. The main task of these functional groups is to interact with the catalytic pocket of HDACs. They are further subdivided into which class of HDACs they inhibit.

SAHA (also referred to Vorinostat) was one of the first HDACi that was approved by the U.S Food and drug Administration (FDA) in treatment of cutaneous T-cell lymphoma (Mann, Johnson et al. 2007). SAHA is a non-selective/pan HDACi, which acts as a chelator for the zinc ions in the catalytic domain of histone deacetylases, resulting in inhibition of the HDACs to remove acetyl groups from lysine histones. This leads to increased expression of genes due to the accumulation of acetylated histones, acetylated proteins and also increasing the expression of TFs important for cell differentiation. SAHA has been demonstrated to induce gene expression of downregulated genes in myeloid malignancies, and suppressed genes that were overexpressed (Silva, Cardoso et al. 2013). Currently there are various HDACi that have either been approved or are in clinical trials (Table 3) (Ungerstedt 2018).

Table 3. HDACis in clinical trials (Ungerstedt 2018).

Drug Type	Compound	Name	Selectivity	Clinical Status	Used in Myeloid Disease
Hydroxamates	MK0653 (SAHA)	Vorinostat	Pan HDACi	Phase II/III. Approved.	Yes. Single and combination
	LBH589	Panobinostat	Pan HDACi	Phase II/III. Approved.	Yes. Single and combination
	PXD101	Belinostat	Pan HDACi	Phase I/ II/III. Approved.	Yes. Combination therapy
	JNJ-26481585	Quisinostat	HDAC1,3,5,8	Phase I/II	MDS and AML. Single therapy
	ITF2357	Givinostat	Class I and II	Phase I/II	MPN. Single and combination
	SB939	Pracinostat	Class I, II, IV	Phase II	Yes. Single and combination
	4SC201	Resminostat	Pan HDACi	Phase II/III	No
	4SC202	Domatinostat	HDAC1,2,3	Phase I/II Approved in melanoma (combination)	Yes. Single therapy
	ACY1215	Ricolinostat	HDAC6	Phase I/II	No
Cyclic tetrapeptides	FK228	Romidepsin	Class I	Phase I/II/III. Approved.	Yes. Single and combination
Benzamides	MS275	Entinostat	HDAC1,2,3	Phase I/II	Yes Combination therapy
	MGCD0103	Mocetinostat	Class I	Phase I/II	Yes Single and combination
Fatty acids	Valproic acid	Valproate	Class I and IIa	Phase I/ II	Yes Combination therapy
	Sodium Butyrate	Butyrate	Class I and IIa	Phase I/II	Mostly non cancer diseases

1.4.3 Selenium compounds

Selenium (Se) is present in a variety of biologically active compounds such as in the antioxidant regulatory system, glutathione peroxidases (GPx), thioredoxin reductases (TrxR) and iodothyronine deiodinases (DIO), the thyroid hormone metabolism and in regulating the endoplasmatic reticulum homeostasis (Papp, Lu et al. 2007).

The epigenetic effects of Se have been reported in several studies. Se has been reported to affect global and gene-specific DNA methylation and the expression of DNMTs (Arai and Kanai 2010). Studies with in vitro human and animal models have demonstrated an inverse correlation of Se with DNA methylation and DNMT activity

(Armstrong, Bermingham et al. 2011, de Miranda, Andrade Fde et al. 2014, Yang, Zhu et al. 2014).

Selenium compounds have also been linked with inhibition of HDAC activity in several studies (Lee, Nian et al. 2009, Desai, Salli et al. 2010, Kassam, Goenaga-Infante et al. 2011, Gowda, Madhunapantula et al. 2012). Two derivatives of HDACi containing Se have demonstrated a higher inhibitory activity against HDACs compared to commercially available HDACi, the clinical implications remains yet to be investigated (Desai, Salli et al. 2010).

2 AIM OF THE THESIS

The overall aim of this thesis is to explore the epigenetic aberrations in the myeloid malignancies myelodysplastic syndromes (MDS), systemic mastocytosis (SM), and acute myeloid leukemia (AML), in order to better understand the pathobiology of these diseases, and understand the mechanism of action of epigenetically active drugs in myeloid malignancies.

The specific aims for papers I-IV were:

Paper I: To evaluate the genome-wide epigenetic status of primary MDS patient stem/early progenitor cells, and to study the treatment effects of Azacitidine.

Paper II: To assess whether HDACi treatment have may have therapeutic potential in D816V KIT mutated SM cells, and assess whether this effect is selective for mutated mast cells compared to healthy.

Paper III: To investigate if the antitumor effects of selenium in AML can be explained by selenium affecting epigenetic regulatory mechanisms.

Paper IV: To delineate the epigenetic mechanisms of action of HDACi in SM.

3 METHODOLOGICAL APPROACHES

3.1 GENOME-WIDE DNA METHYLATION ANALYSIS

During the last decade, DNA methylation analysis has scaled up from locus-specific methods to a genome-wide level due to the recent advances of next-generation sequencing (NGS) platforms, which provide the advantages of single-base resolution (Yong et al., 2016). The various experimental methods to study DNA methylation can be divided into three main methodologies: enzyme digestion, affinity enrichment and bisulfite conversion.

Restriction enzyme-based methods use DNA methylation sensitive enzymes, which cleaves only unmethylated sites, leaving the methylated DNA intact. In *affinity enrichment-based methods* methyl-CpG-binding domain (MBD) proteins or antibodies specific for 5mC are used in order to pull down methylated DNA regions. In *bisulfite conversion-based methods* the DNA is treated with sodium bisulfite, which will deaminate unmethylated C to uracil, and eventually to thymine through a chain of PCR amplification steps, and methylated C will be unaffected.

Since the main principles to study DNA methylation mentioned above can't differentiate between 5mC and 5hmC, there has been a growing field of new experimental modifications to distinguish between these two methylation states. For example the Oxidative bisulfite sequencing (OxBS-seq), in which the DNA is treated with an oxidizing agent prior to bisulfite treatment. Leading to the conversion of 5hmC to 5fmC, which is further converted to uracil during bisulfite treatment and being read as thymine in the sequencing stage. The 5mC will not be oxidized and will be detected as C in the sequencing step (Kirschner, Krueger et al. 2018).

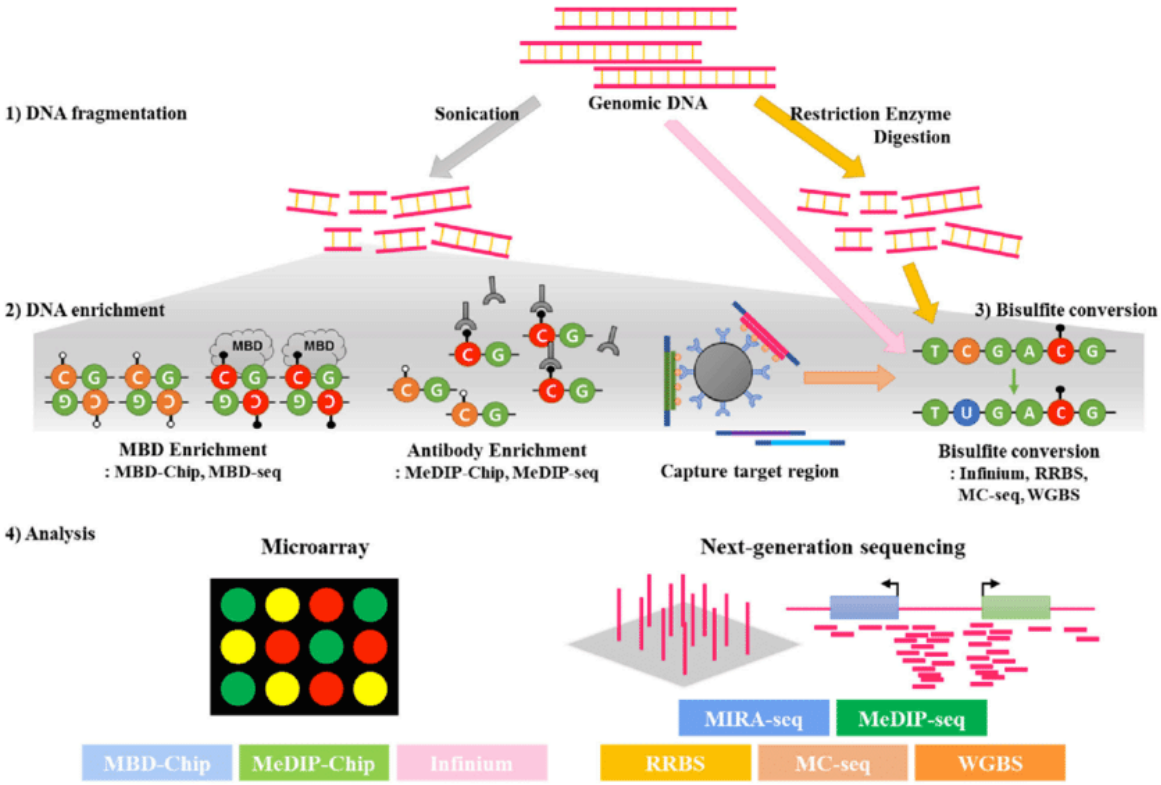

Figure 9: Schematic illustration of different genome-wide DNA methylation assays. The four main steps in genome-wide DNA methylation assays are (1) fragmentation of DNA, (2) enrichment, (3) bisulfite conversion and (4) analysis. Figure reprinted with permission from publisher (Lee, Ryu et al. 2018).

Each of the above-mentioned approaches could then either be analyzed through gel-based methods, array-based methods or NGS based methods (Figure 9) (Lee, Ryu et al. 2018). As with all methods there are strengths and limitations to take into consideration when choosing a method to analyze genome-wide DNA methylation. One important aspect to take into consideration is the CpG coverage throughout the genome and resolution. Both restriction enzyme- and affinity-based approaches have a poor coverage in regions with low CpG density, the restriction enzyme sites and the antibody affinity also limits the coverage. The bisulfite conversion-based methods have the best coverage, being more sensitive in regions with low CpG density. Whole-genome bisulfite sequencing (WGBS) gives rise to the best coverage and a single-base resolution, however this method requires large amount of DNA material due to extensive sequencing and is very expensive (Soozangar, Sadeghi et al. 2018). Reduced-representation bisulfite sequencing (RRBS) is a good alternative to WGBS, providing an accurate and cost effective method for genome-wide DNA

methylation mapping. In this thesis the Illumina human methylation 450K-array method was used to evaluate the global DNA methylation maps.

3.1.1 Illumina 450K array

The Illumina human methylation 450K arrays is an affordable alternative to WGBS; it covers approximately 480,000 CpG sites and 99% of the RefSeq genes (hg19) (Sun, Cunningham et al. 2015). With this method it is possible to study genome-wide 5mC DNA methylation patterns. However, it is not possible to distinguish between 5hmC and 5mC, unless the OxBS treatment is implemented prior to sequencing.

Illumina replaced the 450K beadchip to the Epic BeadChip (>850,000 CpG sites), covering approximately 90% of the CpG sites represented on the 450K beadchip (Logue, Smith et al. 2017). A recent correlation study of same sample sets run on both Epic BeadChip and 450K array, showed 94% of overlapping differentially methylated probes (Pidsley, Zotenko et al. 2016).

The Illumina methylation array utilizes probes with specificity towards individual CpG sites across the genome, and the methylation level is quantitatively measured by genotyping the bisulfite treated genomic DNA. The 450K arrays use the chemistry of two assays, Infinium I and II. The difference between the two technologies lies in the probe detection of methylated and unmethylated DNA; Infinium I uses two different probes to detect methylated and unmethylated DNA, giving rise to one color (blue) in the readout, whereas Infinium II only uses one bead type which can detect methylated signal in green and unmethylated signal in red (Bibikova, Barnes et al. 2011, Sandoval, Heyn et al. 2011). The signal is then reported as β-value, which resembles a percentage of methylated probe intensity versus the sum of overall intensity (methylated and unmethylated probe intensities) (Dedeurwaerder, Defrance et al. 2011). β-value ranges from 0 (unmethylated) to 1 (fully methylated). M-value, another method to measure methylation level, is a log2 ratio of the intensities of methylated versus unmethylated probes (Du, Zhang et al. 2010). There are pros and cons in using any of the methods; the β-value is easier to interpret from a biological point of view, but the interpretation is an approximation and is strongly influenced by the normalization process. The M-value on the other hand is a better statistically valid method, however is does not have a straightforward biological interpretation. It is recommended to use M-value in differential methylation analysis, and also to report the β-value statistics in the final report (Du, Zhang et al. 2010). M-value was used in paper I to analyze the differential methylation levels after treatment.

3.2 CHROMATIN ACCESSIBILITY AND MODIFICATION ANALYSIS

There are several methods available to study the accessibility of the chromatin; one such method is the Assay for transposase-accessible chromatin using sequencing (ATAC-seq) (Buenrostro, Giresi et al. 2013). With this approach regions that are accessible for transcription, will be cut by the Tn5 transposase, and adapters for high-throughput sequencing will be ligated to the fragments. Subsequent sequencing will give rise to genome-wide mapping of the chromatin accessibility (Buenrostro, Wu et al. 2015).

Another way to study the chromatin dynamics is to study the posttranslational modifications (PTMs) on the histone proteins, which will either give an active or repressed chromatin state. Chromatin immunoprecipitation followed by next generation sequencing (ChIP-seq) is one such technique that enables to study the histone PTMs (Kidder, Hu et al. 2011).

3.2.1 Chromatin immunoprecipitation sequencing

The first step in a ChIP-seq experiment is formaldehyde fixation to crosslink DNA-binding protein to DNA; thereafter the chromatin is fragmented by sonication, into fragments of 200-600 bp, thereafter the region of interest is pulled down with an antibody that targets the modification of interest. After the immunoprecipitation step, DNA is released and purified, and the DNA-protein fragments of interest are prepared for sequencing. The preparation step is also referred to as library construction, which involves ligation of sequencing adapters and barcodes, in order to be able to pool several samples, and PCR amplification steps before eventually being analyzed by sequencing. Since there will be a lot of noise in the ChIP-seq data, a region that has not been immunoprecipitated, input, is also sequenced as a background control.

A first step in the analysis of the ChIP-seq data is an evaluation of the quality of the sequencing data, and filtering out bad quality reads. The filtered reads are then mapped to a reference genome by the use of different algorithms (Bailey, Krajewski et al. 2013).

One of the main tasks in the bioinformatics analysis of ChIP-seq data is to predict regions that are bound by a specific histone modification or transcription factor, this is done by calling peaks. There are several algorithms that can perform peak calling

(Bailey, Krajewski et al. 2013); the overall aim is to define regions with tags (reads) higher than the background (input).

There are many factors to consider when performing ChIP-seq experiments, the choice of antibody is one of the most important factor when it comes to generating high quality data. It is therefore important to choose an antibody that is specific and sensitive both for the assay and for the protein of interest (Kidder, Hu et al. 2011).

In paper I the histone modifications H3K9me3 and H3K18ac were studied by ChIP-seq. In paper II, H3K9me3, H3K27ac and H3K18ac were analyzed by ChIP-qPCR. In paper III, the epigenetic effects caused on the histone marks H3K9me3, H3K9ac and H3K4me3 were investigated using ChIP-seq. In paper IV H3K9me3, H3K27ac and H3K4me3 were studied by ChIP-seq. The quality metrics of the raw data was checked with the FASTQC tool, and Bowtie2 was used to align the reads to the human reference genome. Peak calling was performed with HOMER algorithm.

3.2.2 Assay for transposase-accessible chromatin using sequencing (ATAC-seq)

Profiling nucleosome occupancy and chromatin accessibility is of great importance when performing genome-wide epigenetic studies. As mentioned earlier ATAC-seq is one approach that can be used in chromatin accessibility studies (Bailey, Krajewski et al. 2013). ATAC-seq experiments consists of Tn5 transposase cleavage on unfixed nuclei from 50 000 sorted cells. After the transposition reaction the DNA is purified, and libraries are prepared through PCR amplification with sequence specific adapters. Amplified libraries are then purified, quantitatively and qualitatively controlled before being analyzed by high-throughput sequencing.

ATAC-seq data processing is of great importance, and there are several different algorithmic tools available (Miskimen, Chan et al. 2017). The analysis workflow involves quality assessment of raw data, removal of bad quality reads and adapter sequences, alignment to reference genome and peak detection.

In paper III ATAC-seq was used as a method of choice to investigate the chromatin accessibility in mutated mast cells upon HDACi treatment.

3.3 TRANSCRIPTOME PROFILING

The total amount of transcripts and their quantity in a cell at a specific developmental stage or condition is defined as the transcriptome (Wang, Fang et al. 2010). In order to understand the phenotypically or functionally specific characteristics of a cell type it is of great importance to study the transcriptome of that specific cell. With the increasing access to affordable high-throughput sequencing platforms, it is possible to map and quantify transcriptomes on a genome-wide level (McGettigan 2013). The method applied to both map and quantify transcriptomes is referred to as RNA sequencing (RNA-seq). In this thesis, messenger RNA (mRNA) sequencing was applied in paper I, paper III and paper IV.

3.3.1 Messenger RNA Sequencing

The first step in the RNA-seq experimental workflow is to extract the RNA of interest, in this case the mRNA. Since ribosomal RNA (rRNA) is highly abundant in a cell, constituting of approximately 90% of the total RNA content, it is therefore crucial to use good extraction protocols yielding pure mRNA. The RNA quality is assessed with a technique called bioanalyzer, which measures the RNA integrity number (RIN). The RIN value is ranging from fully degraded RNA to intact RNA (1-10), a RIN value of at least 8 is required for a good library construction and sequencing. The next step is to synthesize and purify cDNA libraries, thereafter ligation of sequencing adapters and amplification of the cDNA libraries are performed before analysis by NGS sequencing.

The sequencing workflow generally starts with hybridizing the libraries on a flow-cell; thereafter clusters are generated in a stepwise synthesis to generate identical DNA strands that are covalently bound to the flow-cell surface. The amount of starting material that is loaded on the flow-cell is the limiting factor for the clusters density.

After quality metric assessment of the sequencing data, the reads are mapped to the reference genome, and genes can be annotated. Gene expression is then quantitatively measured by counting tags in each annotated gene, similar to peak calling algorithms. The signal is then reported as normalized expression such as reads per kilobase of exon model per million mapped reads (RPKM), fragments per kilobase of exon model per million mapped reads (FPKM) or trimmed mean of M-values (TMM) or median count ratio (Mutz, Heilkenbrinker et al. 2013). Differential

expression analysis is then performed with tools such as EdgeR, Noiseq and DEseq (Conesa, Madrigal et al. 2016).

In paper I a modified version of mRNA-seq was used, referred to as single-cell tagged reverse transcription (STRT) RNA-seq. This method measures transcription initiation at the 5′end of polyA+ transcripts starting from 10 ng of mRNA as template (Islam, Kjallquist et al. 2012, Krjutskov, Katayama et al. 2016). In paper III and IV traditional quantitative mRNA sequencing was used.

3.4 INTEGRATIVE BIOINFORMATIC ANALYSIS

The advances in technical approaches in data generation from several layers of biological systems have lead to a growing field of bioinformatic tools, that can assist in the pursue of understanding the complex biological networks. An integrative bioinformatic approach can be more informative of the genotype-phenotype associations than analyzing a single type of data. However, it is important to keep in mind the different layers of biological regulation, in order for the integrative approach to be successful as a biological model (Holzinger and Ritchie 2012). It is also crucial to consider the limitations with each single data type before combining them in a meta-dimensional analysis.

The integration process can be divided into multi-stage analysis, where only two types of data is integrated stepwise, or meta-dimensional analysis, simultaneous integration of all types of data. The multi-stage analysis is based on a biological hypothesis that a phenotype is driven by a hierarchical variation, meaning changes in genome leads to changes in the transcriptome which gives rise to changes in the proteome. The meta-dimensional analysis on the other hand is driven by the hypothesis that variation in all of the different omics (genome, epigenome, transcriptome and proteome) gives rise to the phenotype (Ritchie, Holzinger et al. 2015).

The limitations with using statistical prediction models to understand complex biological systems is first and foremost the estimation of the power; some methods calculate power from theoretical distributions while others require simulation observations (Ritchie, Holzinger et al. 2015). Finding meaning and true significance in the different data sets also creates another level of difficulty in the translational

interpretation of the integrative analysis (Davis-Turak, Courtney et al. 2017)).

In the studies conducted in this thesis integration of DNA methylation data, RNA-seq data and ChIP-seq data were performed in paper I in a multi-stage approach, to investigate the correlation of changes in DNA methylation landscape to gene expression and histone PTMs. In paper III, ChIP-seq and RNA-seq was integrated. In paper IV, chromatin accessibility and modification was correlated to gene expression through the integration of ATAC-seq, ChIP-seq and RNA-seq.

4 RESULTS AND DISCUSSION

The four studies included in this thesis investigate the effect on the epigenome after treatment with epigenetically active drugs, in three different myeloid malignancies. In paper I, genome-wide DNA methylation was evaluated and correlated to gene expression and histone PTMs (H3K9me3 and H3K18ac), after treatment of MDS bone marrow progenitors with the DNA hypomethylating agent Azacitidine. In paper II, the effect of HDACi on D816V KIT mutated mast cell line and primary mast cells was investigated. Paper IV is a follow-up study of paper II, where the mechanism of action of the HDACi SAHA in D816V KIT mutated mast cells was further characterized. In paper III, the epigenetic effects of selenium compounds on AML patient cells and a leukemic cell line, was investigated.

4.1 PAPER I: COMPREHENSIVE MAPPING OF THE EFFECTS OF AZACITIDINE ON DNA METHYLATION, REPRESSIVE/PERMISSIVE HISTONE MARKS AND GENE EXPRESSION IN PRIMARY CELLS FROM PATIENTS WITH MDS AND MDS-RELATED DISEASE

In this study, bone marrow samples were collected from a previously untreated patient cohort consisting of 6 higher-risk MDS, 4 AML with multilinear dysplasia and 20–29% blasts (MDS/AML), and one patient with chronic myelomonocytic leukemia, CMML-2. All of the patients had a clinical indication to start Azacitidine (AZA) treatment. Mononuclear cells were isolated from bone marrow samples, and CD34+ progenitor cells were sorted using magnetic beads. CD34+ cells were incubated with and without AZA for 24h and 48h. Genome-wide methylation was profiled using Illumina 450K array, transcriptome analysis with STRT RNA-seq, and the histone modification mapping with ChIP-seq, using H3K18ac and H3K9me3 antibodies.

We identified 65 769 differentially methylated probes (DMPs) after 24h treatment, when comparing samples cultured with and without AZA. The majority (n=65 664) was less methylated in the AZA treated samples. The distribution of DMPs across the genome was lower in the CpG islands and higher in the open sea probes, when compared to the Illumina 450k reference. Moreover, we observed lower DMPs in regions close to the TSS, and higher probes in regions located in gene bodies or non-gene related probes and regions defined as heterochromatin. However, the overall mean reduction in β-value was 0.018, which indicates a modest demethylation.

A significant increase in global gene expression was observed for all patients after 24h AZA treatment. The upregulated genes were involved in translation, RNA processing and ribosomal function gene ontology pathways. When we correlated the global changes in DNA demethylation with the increased gene expression, we observed a weak correlation. This might be linked with the observation of demethylation mainly taking place in gene bodies and non-gene related probes and regions, emphasizing alternative triggers for the increased gene expression.

In order to understand the changes in gene expression, we assessed if AZA treatment affected active (H3K18ac) or repressive (H3K9me3) histone marks. When we correlated DNA methylation changes with the changes in the repressive histone mark we observed a strong correlation. This supports the hypothesis that AZA may have a great effect in heterochromatin regions defined by H3K9me3. However, the correlation with the increased gene expression and histone marks showed weak correlation for H3K9me3, and no correlation at all for H3K18ac.

Since the epigenetic changes could only partly explain the increase in gene expression we performed TF binding site analysis, by looking for enriched motifs in the significantly upregulated genes. We found motifs for eight transcription factors (*ELK1, STAT 1, STAT3, RUNX1, GABPA, ERG, NRF1* and *PU.1*) that were significantly enriched, and also upregulated in expression. Hence, the increased gene expression may partly be explained by increase of the above mentioned TFs, many of which have important regulatory functions in hematopoietic differentiation. Hypothetically, the AZA effect may somewhat be through interfering with the differentiation process. The increased expression of the observed TFs could however not be explained by our epigenetic data, and the upstream regulation needs to be investigated.

Correlation of gene expression data with regions defined as repetitive elements, illustrated significant increase of 16 transcript far 5'-ends (TFEs) overlapping with repetitive elements in AZA treated samples, and significant decrease in only three. Among the upregulated TFEs, 15 were members of the endogenous retroviruses (ERVs) family. Since there were no probes in the Illumina 450K array we could not assess the DNA methylation changes in those regions. Assessment of the changes of H3K9me3 peaks in the AZA samples compared to the control samples over the ERV regions, demonstrated no general reduction.

An activation of the 15 ERV containing transcripts was observed in the AZA samples, which is in line with previous studies on cell lines (Chiappinelli, Strissel et al. 2015, Roulois, Yau et al. 2016). However, with this study we demonstrate for the first time that AZA treatment results in ERV activation also in primary CD34+ MDS cells. Due to the short incubation time we were not able to investigate the response of the immune genes.

Previous studies have demonstrated that DNA methylation during mammalian gametogenesis and early embryonic development give rise to activation of ERVs, which correlates with pluripotency (Macfarlan, Gifford et al. 2012). Therefore, it is possible that the observed increase in ERV expression in MDS cells could participate in cell differentiation upon AZA treatment.

4.2 PAPER II: HISTONE DEACETYLASE INHIBITOR SAHA MEDIATES MAST CELL DEATH AND EPIGENETIC SILENCING OF CONSTITUTIVELY ACTIVE D816V KIT IN SYSTEMIC MASTOCYTOSIS

In this study, we assessed the sensitivity of primary mast cells and mast cell lines, D816V mutated HMC1.2 and ROSA cells and KIT WT ROSA cells, to treatment with histone deacetylase inhibitors (HDACi), SAHA, Panobinostat, Romidepsin and Valproic acid. We observed a dose and time-dependent growth arrest and cell death of the KIT mutated HMC1.2 cells, with the greatest effect upon SAHA treatment. A global acetylated histone level was observed already after 2 hours of incubation with SAHA, and a decrease of active phosphorylated KIT at 6 hours. We observed decrease levels of cell surface KIT, KIT mRNA and total protein levels after 24 hours of treatment, which was followed by mast cell apoptosis.

When assessing the sensitivity of SAHA on the primary mast cells, we observed a decrease in cell surface KIT and major cell death in the SM patient MCs compared to the age-matched healthy controls. The sensitivity was higher in the primary cells from patients with more aggressive SM disease compared to cells from ISM. Moreover, MCs from a patient with highly aggressive MCL were completely dead after 48h of SAHA treatment. Our findings demonstrate that the malignant mast cells with KIT mutation are more susceptible of the SAHA mediated killing compared to healthy mast cells.

In order to investigate the mechanism of action of SAHA in this context we assessed the changes of histone PTMs (H3K9me3, H3K27ac and H3K18ac) in the

KIT transcription start site, and a gene upstream (PDGFRα) and downstream (KDR) of KIT by ChIP-qPCR. The first observation was a general increase of the acetylated histone marks, as anticipated upon SAHA treatment. However, a significant global increase of the histone H3 density in the KIT gene and the adjacent genes was observed after SAHA treatment. Taking this into consideration we calculated the enrichment for the histone marks relative to the total H3 density in each gene. The active chromatin marks (H3K27ac and H3K18ac) showed a significant decrease in the promoter region of KIT upon SAHA treatment, compared to the gene upstream and downstream which had unchanged levels. These findings are indicating that there might be an epigenetic response that closes the chromatin in the KIT gene, and leads to the SAHA mediated downregulation of KIT mRNA and protein levels.

Altogether the conclusion from the findings in this study is that mast cells from aggressive SM show high sensitivity to SAHA, which may be clinically applicable as treatment for SM.

The exact mechanism of action of SAHA as well as the selectivity towards mutated mast cells is investigated in paper IV.

4.3 PAPER III: SELENITE AND METHYLSELENIC ACID EPIGENETICALLY AFFECTS DISTINCT GENE SETS IN MYELOID LEUKEMIA: A GENOME WIDE EPIGENETIC ANALYSIS

The proposed epigenetic effects as well as cytotoxic effects of selenite and methylseleninic acid (MSA) were assessed in a leukemic cell line (K562) as well as primary AML patient cells. The activities of both active (H3K4me3 and H3K9ac) and repressive (H3K9me3) histones were assessed with ChIP-seq, and the gene expression effects were evaluated with RNA-seq. Both selenium compounds affected the histone marks, however the effect caused by MSA was more pronounced. Gene ontology analysis revealed that selenite and MSA affected different gene sets. The pathways affected by selenite, involved response to hypoxia and oxygen, while MSA on the other hand affected cell adhesion and glucocorticoid receptors. This indicates that selenite and MSA have different mechanism of actions.

The findings were functionally confirmed by plating K562 cells and AML primary cells on fibronectin-coated plates; which demonstrated a decreased expression of integrin β and a loss of cellular adhesion upon MSA treatment, in AML cell line K562 as well as in primary patient AML cells. We postulate that this effect of MSA on adhesion

capacity may be utilized therapeutically, to release AML cells from protected stem cell niche into the circulation, where cells are more susceptible to chemotherapeutic drugs.

4.4 PAPER IV: MECHANISTIC CHARACTERISATION OF SAHA MEDIATED D816V KIT DOWNREGULATION IN SYSTEMIC MASTOCYTOSIS

The purpose of this study is to investigate the exact mechanism of action of HDACi (SAHA) targeting of D816V mutated HMC-1.2 mast cells.

We treated SM cell line HMC-1.2 with SAHA and performed ChIP-seq, for both active (H3K27ac and H3K4me3) and repressive histone marks (H3K27me3), and ATAC-seq, to study the nucleosome accessibility. We also investigated the HDACi mediated effects on the transcriptome by performing RNA-seq, in order to pinpoint the mechanism of action of HDACi in SM.

SAHA treatment induced global transcriptional and chromatin alterations on mutated mast cells. The repressive chromatin mark was however not significantly changed on a global level, but both the active marks were significantly decreased globally upon SAHA treatment. When assessing the global effects on the transcriptome, gene ontology analysis revealed that many strongly downregulated genes were in the downstream signaling cascade of KIT. There were only modest global effects observed on the nucleosome positioning after treatment.

We moved on to investigate changes in the promoter region of KIT and a region up to 1000 kb upstream of the KIT gene. The active mark H3K27ac had a significant decrease in the KIT promoter region upon SAHA treatment. The repressive mark and the other active mark didn’t demonstrate profound changes. ATAC-seq did not demonstrate significant changes on the nucleosome positioning in the KIT promoter region.

In our quest to understand the upstream regulatory network of KIT promoter region we overlapped our ChIP-seq data from H3K27ac with the enhancer defined ChIP-seq data from the FANTOM5 project. We then performed transcription binding site (motif) analysis for the peaks/regions that had an exact overlap between the two datasets. This resulted in identifying six transcription factors (TAL1, MZF-1, NFIC, NR2F2, ZNF24 and ZNF354C) that had a decreased gene expression level after SAHA treatment. When analyzing the nucleosome accessibility pattern in the

promoter region of these six transcription factors, TAL1 was significantly altered upon SAHA treatment.

Since TAL1 is part of the TAL1/SCL complex we assessed the effect on this complex, by analyzing the transcription levels of the different components of the complex. GATA1 and TAL1 were significantly decreased on transcript levels already after 6 hours of treatment. HDAC1 has been demonstrated in several studies to be part of this complex. Altogether our findings indicate a direct targeting of SAHA on the TAL1/SCL complex, and we are currently studying the dynamics and composition of this complex before and after HDACi treatment.

In conclusion, we speculate that the mechanism of SAHA mediated killing of D816V mutated mast cells occurs partly via targeting HDAC1 which further affects TAL1 binding in the TAL1/SCL complex.

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The advances in the technical field have lead to increased insight to the molecular landscapes of several myeloid malignancies. Identification of mutations that are leading to development of a myeloid malignancy, as well as which mutations are drivers, is important both for finding optimal treatment strategies and giving right diagnosis and care in the clinic. The overall goal is to improve the quality of life and increase survival of these patients. Despite the increased knowledge in the mutational landscape of the different myeloid malignancies discussed in this thesis, the current treatment strategies have little overall effect and cure rate. A reason behind this is partly due to the heterogeneity of the disease population, and the lack of understanding of the exact mechanism of action of the current drugs used in the clinic.

In this thesis, epigenetic modulators have been studied in order to mechanistically investigate their effect on the epigenome of mutated cells in myeloid malignancies. Understanding the effects of these epigenetic modifiers is important in optimizing the treatment strategies, as well as development of novel targeted therapies.

Combinatorial treatment strategies of HMA and HDACi have been studied in several clinical trials (Griffiths and Gore 2013, Stahl, Gore et al. 2016, Ball, Zeidan et al. 2017) with little beneficial results. A key parameter that is lacking in order to better optimize the treatment strategies is how to best combine the drugs, and how are they interacting with each other. Preclinical studies are therefore important to understand the combinatorial effects, and how they interact on molecular level.
Another issue that requires further knowledge is the optimal drug for a specific patient population regarding selectivity and inhibition of clonal and mutated proteins, and the pattern of patient specific mutations and chromosomal alterations.

There is a lack of a good readout method with the current therapeutic strategies that would give information about how the drugs are interacting, and how effective they are in different patient populations. This would help in characterizing which patient population, with regards to mutational landscape and chromosomal aberrations, would benefit from a specific drug.

Our studies have contributed with knowledge of how the epigenetic modifiers act to modulate the epigenetic landscape in different myeloid malignancies. We found profound changes on the epigenetic landscape, however not always corresponding to changes on the transcriptome. This emphasizes the complexity in the epigenetic machinery, with different factors that either promote or counteract each other. Hence, gaining better understanding of how the different drugs modulate the epigenetic landscape in the different malignancies provides important knowledge in designing future biomarkers to monitor the therapeutic response and obtain beneficial clinical outcome.

For future studies in the field of epigenetic modifying drugs, it is important to choose the right method to measure the effects on the epigenome. Since the sensitivity, coverage, the amount of required biological material, and the bioinformatic data processing differ immensely between them, and can affect the interpretation of the data.

6 POPULÄRVETENSKAPLIG SAMMANFATTNING

Produktionen av olika blodkroppar sker genom det hematopoetiska systemet som regleras via flera interna och externa signaler. Den hematopoetiska stamcellen (HSC) som finns i benmärgen är ursprungscellen för alla blodkroppar, och kan via självförnyelse och multipotenta förmåga generera olika blodkroppar med olika funktioner. Det genereras nästan 12 miljoner nya blodkroppar varje sekund från denna kompetenta stamcell. När något går fel i denna noggrant reglerade process, kan det leda till myeloida maligniteter som MDS, AML och SM, som studeras i denna avhandling.

Denna avhandling ger en mekanistisk inblick i hur de olika läkemedlen som används idag för behandling av olika myeloida maligniteter, påverkar arvsmassan.

De viktigaste fynden är:

- Vi hittade ett ökat uttryck av immunförsvarsgener, i primära stamceller från benmärgen vid behandling av MDS med Azacitidin.

- Vi har visat att histonmodifierande läkemedel kan vara en selektiv, riktad behandling vid systemisk mastocytos, en patientgrupp där vi idag saknar bra behandling.

- Seleninnehållande läkemedel påverkar leukemiceller epigenetiskt, och leder till fästning i benmärgen. Detta skulle kunna fungera som ett komplement till befintlig kemoterapi för att bättre komma åt leukemiceller i benmärgen.

Sammanfattningsvis så har vi kunnat påvisa en ökad förståelse för hur olika läkemedel påverkar det epigenetiska landskapet i myeloida maligniteter. Detta är en viktig kunskap som behövs vid utformningen av framtida biomarkörer för att övervaka responsen till behandlingen, samt optimera behandlingsstrategierna så att fler patienter gynnas.

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