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GENETIC AND EPIGENETIC STUDIES OF ACUTE MYELOID LEUKEMIA AND THERAPEUTIC POSSIBILITIES

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To my family

ABSTRACT

Acute myeloid leukaemia (AML) is malignant tumour that forms in the bone marrow and arises from immature myeloid progenitors. Consequently, this leads to excessive accumulation of dysfunctional blast cells and lack of normal blood cells. The molecular and genetic heterogeneity of the disease is substantial which makes the disease challenging to classify and treat. Although the AML classification is updated continuously and more data and research on AML pathophysiology emerges, first line treatment for the vast majority of AML patients remains a combination of cytarabine and an anthracycline. While most patients attain a complete remission, the majority of AML patients relapse and develop drug resistance. Recently, new drugs have been approved for the treatment of specific AML subtypes. However, there is need for better understanding of disease pathogenesis including better genetic and epigenetic risk factors in order to develop more effective treatment regimens to improve the outcome of the disease.

In **Study I**, we studied off-target effects of APR-246, a drug that originally was developed to restore the activity of mutated TP53 protein. Oxidative stress related genes heme oxygenase-1 (*HMOX1*, also termed HO-1), *SLC7A11* and *RIT1* were significantly upregulated. Also, *Nrf2* that induces the expression of *HO-1* was upregulated and depletion of *Nrf2* mRNA resulted in increased cytotoxicity of APR-246. Moreover, blocking Nrf2 from translocating into the nucleus by using PI3K and mTOR inhibitors led to enhanced cell killing. This suggests that a combination of APR-246 with PI3K and mTOR inhibitors improves sensitivity to APR-246 by interfering with the cellular response to ROS activation to achieve better anti-leukemic effects of APR-246.

In **Study II**, we aimed to define the potential of using stroma cells in diagnostic AML samples as a source of germline DNA. To obtain germline DNA, together with DNA from leukemic cells, it is essential to reliably define somatic mutations in AML cells. Consequently, we cultivated and expanded bone marrow stroma cells from vitally frozen mononuclear cells from AML patients with monosomy 7 as well as defined somatic mutations. *In vitro* expanded bone marrow stroma cells were stable after 6 weeks of culture and were able to differentiate into adipocytes or osteocytes. We could also show that cultivated stroma cells do not harbour the somatic mutations found in the malignant cells. Thus, we could conclude that bone marrow stroma cells from diagnostic bone marrow samples could be used as a source of germline DNA in AML patients.

In **Study III**, we studied the binding occupancy of the chromatin organizer CTCF in AML patient cells and compared it to binding in normal CD34+ cells. We found that AML cells display an aberrant increase of CTCF binding. Motif analysis

revealed that gained CTCF sites are enriched for transcription factors such as PU.1, RUNX1 and CEBPA, which is found to be important for normal myeloid development. *TET2* mutated AML patients exhibit a greater gain of CTCF occupancy that is mainly annotated to promoters. Generally, gained CTCF sites were found to be hypomethylated and associated with genes that were upregulated in AML. Knockdown of CTCF in K562 cells resulted in loss of CTCF and decreased gene expression of targeted genes as well as loss of RUNX1 binding at common CTCF and RUNX1 binding sites. Knockdown of CTCF also resulted in increased differentiation of K562 cells. *In vitro* exposure of AML patient cells with azacytidine resulted in major changes in CTCF occupancy where most gained sites restored the binding pattern found in normal CD34+ cells. In conclusion, our results suggest that an aberrant CTCF occupancy in AML can have a role in driving leukemogenic gene expression patterns in AML.

LIST OF SCIENTIFIC PAPERS

I. **Anti-leukaemic effects induced by APR-246 are dependent on induction of oxidative stress and the NFE2L2/HMOX1 axis that can be targeted by PI3K and mTOR inhibitors in acute myeloid leukaemia cells.**

Ali D, Mohammad D.K, Mujahed H, Jonsson-Videsäter K, Nore B, Paul C, Lehmann S. *British Journal of Haematology*, 2016 Mar 15; 174(1):117-26.

II. **Bone marrow stroma cells derived from mononuclear cells at diagnosis as a source of germline control DNA for determination of somatic mutations in acute myeloid leukemia.**

Mujahed H, Jansson M, Bengtzén S, Lehmann S. *Blood Cancer Journal*, 2017 Oct 06; 7 (e616).

III. **AML Displays Increased CTCF Occupancy Associated to Aberrant Gene Expression and Transcription Factor Binding**

Mujahed H, Miliara S, Neddermeyer A, Bengtzén S, Nilsson C, Deneberg S, Cordeddu L, Ekwall K, Lennartsson A, Lehmann S.

Accepted for publication in Blood.

PAPERS NOT INCLUDED IN THE THESIS

Allele-specific DNA methylation is increased in cancers and its dense mapping in normal plus neoplastic cells increases the yield of disease-associated regulatory SNPs

Catherine Do, Emmanuel Dumont, Martha Salas, Angelica Castano, Huthayfa Mujahed, Leonel Maldonado, Arunjot Singh, Govind Bhagat, Soren Lehman, Angela M. Christiano, Subha Madhavan, Peter L. Nagy, Peter H.R. Green, Rena Feinman, Cornelia Trimble, Karen Marder, Lawrence Honig, Catherine Monk, Andre Goy, Kar Chow, Samuel Goldlust, George Kaptain, David Siegel, and Benjamin Tycko.

Submitted manuscript published in bioRxiv

CONTENTS

LIST OF ABBREVIATIONS

2-HG	2-hydroxyglutarate
5hmC	5-Hydroxymethylcytosine
5mC	5-Methylcytosine
α-KG	Alpha-ketoglutarate
AML	Acute myeloid leukaemia
ASXL1	Additional sex comb-like
BM	Bone marrow
BMS	Bone marrow stroma
CD	Cluster of differentiation
CEBPA	CCAAT enhancer binding protein α
CLPs	Common lymphoid progenitors
CMPs	Common myeloid progenitors
CN-AML	Cytogenetically normal AML
CR	Complete remission
DNMT3A	DNA methyltransferase 3 alpha
ELN	European LeukemiaNet
EZH2	Enhancer of zeste homolog 2
FLT3	FMS-like tyrosine kinase 3
HSCs	Haematopoietic stem cells
HSCT	Hematopoietic stem cell transplantation
IDH	Isocitrate dehydrogenase
LSC	Leukemic stem cell
MDR	Minimal residual disease
MDS	Myelodysplastic syndrome
NGS	Next generation sequencing
NPM1	Nucleophosmin 1
PcG	Polycomb group
RAR-γ	Retinoic acid receptor-γ
RUNX1	Runt-related transcription factor 1
SCF	Stem cell factor
SF3B1	Splicing factor 3b subunit 1
TET	Ten-eleven translocation
TKD	Tyrosine-kinase domain
WHO	World Health Organization

1 INTRODUCTION

1.1 Haematopoiesis

Haematopoiesis is a process that occurs in the bone marrow (BM) where terminally mature blood cells arise from pluripotent haematopoietic stem cells (HSCs) (Bao, Cheng *et al.* 2019). A distinctive characteristic of HSCs is self-renewal as well as multi-lineage differentiation (Weissman 2000). Common progenitors mediate this multistep differentiation process where HSCs divide into common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs). Adaptive immune T-, B- and NK cells and dendritic cells differentiate from CLPs while CMPs give rise to erythrocytes, megakaryocytes and myeloblasts which differentiate into innate immune cells (Fig. 1A). Cell fate is determined by a sequence of growth factor signals that activates the expression of lineage specific genes. On the other hand, transcription factors such as GATA1 and PU.1 are critical for early erythroid and myeloid differentiation, respectively (Arinobu, Mizuno et al. 2007, Suzuki, Shimizu et al. 2011). Ikaros is important for early lymphoid commitment of CLPs (Georgopoulos, Bigby et al. 1994) while EBF, E2A and Pax5 are essential for B-cell development (Nutt and Kee 2007) and GATA3 is required for early differentiation of T-cells (Ting, Olson *et al.* 1996).

1.1.1 Bone marrow microenvironment

The genesis of multi-lineage blood cells takes place in the bone marrow. This is a heterogeneous and complex microenvironment that consists of various cell populations, primarily with the role to support and regulate haematopoiesis. Non-haematopoietic bone marrow stroma (BMS) cells together with HSCs forms a so called niche which govern the fate of HSCs (Pinho and Frenette 2019). However, BMS consist of different cell types such as mesenchymal stem cells, osteolineage cells, adipolineage cells, endothelial cells and neurons which provide a sophisticated framework of regulatory mechanisms that drive haematopoiesis and maintains the balance between self-renewal and differentiation of HSCs (Kfoury and Scadden 2015, Tikhonova, Dolgalev *et al.* 2019). This happens directly though cell-bound molecules or indirectly by secreted molecules. For instance, the two soluble factors CXC-chemokine ligand 12 (CXCL12) (Sugiyama, Kohara *et al.* 2006), stem cell factor (SCF) (Asada, Kunisaki *et al.* 2017) and cell-bound vascular cell adhesion molecule 1 (VCAM-1), also known as cluster of differentiation 106 (CD106) (Dutta, Hoyer *et al.* 2015), are required for maintenance of HSC. Other factors like notch ligands and fibroblast growth factor 1 (FGF1) promote HSC proliferation (Calvi, Adams *et al.* 2003, Zhao, Perry *et al.* 2014). Overall, it is a complex process in which multiple factors are required to maintain normal haematopoiesis and haemostasis of HSC populations. Deregulation of this cross-talk between

HSCs and BMS cells can drive neoplasia. For instance, deficiency of retinoic acid receptor-γ (RARγ) can lead to development of a myeloproliferative syndrome (Walkley, Olsen et al. 2007). Additionally, knockout of RNA-processing enzyme Dicer 1 gene in mesenchymal-derived stromal cells leads to the development of myelodysplastic syndrome (MDS) which can progress to acute myeloid leukaemia (AML) (Raaijmakers, Mukherjee *et al.* 2010). This shows that deregulated signals from the microenvironment can cause malignant transformation.

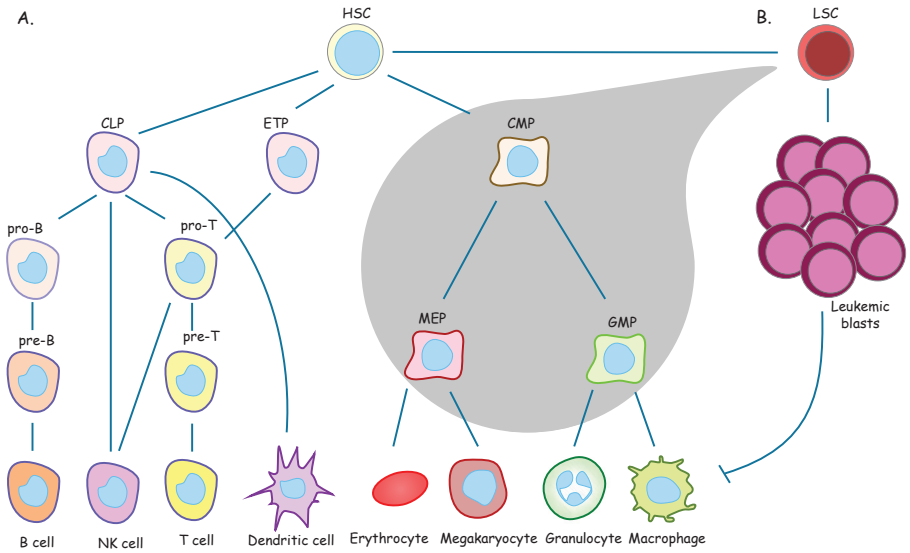

Figure 1. Haematopoiesis and LSCs formation. A) Normal haematopoiesis shows different stages of differentiation from HSC to mature blood cells. B) Development of AML and accumulation of LSCs in bone marrow.

1.2 Acute myeloid leukaemia (AML)

Acute myeloid leukaemia (AML) is a type of blood cancer in which myeloblasts fail to differentiate into mature functional cells. This results in accumulation of aberrant myeloid blasts in the bone marrow and deficiencies in innate immune cells, red blood cells and platelets. Leukemic stem cells (LSC) acquire early mutations that retain the ability of self-renewal and this might occur in HSCs or later during any step of differentiation (Fig. 1B). AML is a heterogeneous disease and there may be multiple LSC clones found within the same patient (Horton and Huntly 2012). AML is usually classified by a morphological increase in BM blasts under microscope, and can be further characterized by cell surface markers analysed by flow cytometry and genetic changes by chromosomal karyotyping and mutational screening.

1.2.1 AML classification

AML is a heterogeneous disease. The World Health Organization (WHO) classification of AML was introduced in 2001, is based on morphology, genetic analysis by cytogenetics, and mutation screening as well as information on previous exposure to chemotherapy and radiation or previous MDS. This system classifies AML into four main categories; AML with recurrent genetic abnormalities, myelodysplasia-related AML, therapy-related AML and AML not otherwise specified. Recent advances in next-generation sequencing (NGS) technologies have made it possible to further subtype AML into sub-categories based on a more thorough genetic characterization. The last update of the WHO AML classification was published in 2016 (Arber, Orazi *et al.* 2016) (Table 1). Novelties in the last update include the establishment of two previously provisional entities to become new permanent entities: AML with *NPM1* mutation and AML with biallelic mutation of *CEBPA*. AML with mutated *RUNX1* is a new provisional entity. Although the primary diagnosis of AML is based on a bone marrow blast count above 20%, patients with translocations t(8:21), inv(16) or t(15:17) are classified as AML also with lower blast counts.

Table 1. WHO update on AML classifications

Acute myeloid leukaemia (AML) and related neoplasms	
AML with recurrent genetic abnormalities	Acute myelomonocytic leukemia
AML with t(8;21) (q22;q22.1);*RUNX1-RUNX1T1*	Acute monoblastic/monocytic leukemia
AML with inv(16)(p13.1q22) or t(16;16) (p13.1;q22);*CBFB-MYH11*	Pure erythroid leukemia
APL with *PML-RARA*	Acute megakaryoblastic leukemia
AML with t(9;11)(p21.3;q23.3);*MLLT3-KMT2A*	Acute basophilic leukemia
AML with t(6;9)(p23;q34.1);*DEK-NUP214*	Acute panmyelosis with myelofibrosis
AML with inv(3)(q21.3q26.2) or t(3;3) (q21.3;q26.2); *GATA2*, *MECOM*	Myeloid sarcoma
AML (megakaryoblastic) with t(1;22) (p13.3;q13.3);*RBM15-MKL1*	Myeloid proliferations related to Down syndrome
Provisional entity: AML with BCR-ABL1	Transient abnormal myelopoiesis (TAM)
AML with mutated *NPM1*	Myeloid leukemia associated with Down syndrome
AML with biallelic mutations of *CEBPA*	**Blastic plasmacytoid dendritic cell neoplasm**
Provisional entity: AML with mutated RUNX1	**Acute leukemias of ambiguous lineage**
AML with myelodysplasia-related changes	Acute undifferentiated leukemia
Therapy-related myeloid neoplasms	Mixed phenotype acute leukemia (MPAL) with t(9;22) (q34.1;q11.2); *BCR-ABL1*
AML, NOS	MPAL with t(v;11q23.3); *KMT2A* rearranged
AML with minimal differentiation	MPAL, B/myeloid, NOS
AML without maturation	MPAL, T/myeloid, NOS
AML with maturation	

Adapted from (Arber, Orazi *et al.* 2016)

1.2.2 AML prognostic factors

European LeukemiaNet (ELN) recommendations are based on chromosomal and molecular aberrations as main measures to predict prognosis and treatment outcome of AML (Dohner, Estey *et al.* 2017). Overall, age and pre-existing health issues have an adverse effect on treatment outcome and are often related to early death of AML patients (De Kouchkovsky and Abdul-Hay 2016). Standard risk stratification is based on cytogenetics and molecular abnormalities that classify AML patients into three risk groups with favourable, intermediate and adverse outcome, respectively. Although chromosomal abnormalities constitute the basis for the primary risk groups, commonly mutated genes such as *NPM1*, *FLT3-ITD*, *CEBPA*, *RUNX1*, *TP53* and *ASXL1* are part of the revised 2017 ELN classification (Table 2) (Dohner, Estey *et al.* 2017). Interestingly, most of these mutations are associated with normal karyotype which helps to give a more detailed classification of cytogenetically normal AML (CN-AML) patients. Still, the prognostic impact of genetic markers is dependent on a co-existence of other genetic lesions in context-dependent manner. Further understanding of the role of co-occurring mutations is required in order to achieve better prognostication.

Table 2. 2017 ELN risk stratification by genetics

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*#

Frequencies, response rates, and outcome measures should be reported by risk category, and, if sufficient numbers are available, by specific genetic lesions indicated.

*Prognostic impact of a marker is treatment-dependent and may change with new therapies.

†Low, low allelic ratio (,0.5); high, high allelic ratio ($0.5); semiquantitative assessment of *FLT3*-ITD allelic ratio (using DNA fragment analysis) is determined as ratio of the area under the curve "*FLT3*-ITD" divided by area under the curve "*FLT3*- wild type"; recent studies indicate that AML with *NPM1* mutation and *FLT3*-ITD low allelic ratio may also have a more favorable prognosis and patients should not routinely be assigned to allogeneic HCT.

‡The presence of t(9;11)(p21.3;q23.3) takes precedence over rare, concurrent adverse-risk gene mutations.

§Three or more unrelated chromosome abnormalities in the absence of 1 of the WHO-designated recurring translocations or inversions, that is, t(8;21), inv(16) or t(16;16), t(9;11), t(v;11)(v;q23.3), t(6;9), inv(3) or t(3;3); AML with *BCR-ABL1*.

‖Defined by the presence of 1 single monosomy (excluding loss of X or Y) in association with at least 1 additional monosomy or structural chromosome abnormality (excluding core-binding factor AML).

¶These markers should not be used as an adverse prognostic marker if they co-occur with favorable-risk AML subtypes.

#*TP53* mutations are significantly associated with AML with complex and monosomal karyotype

Adapted from (Dohner, Estey *et al.* 2017)

1.2.3 Genetics of AML

NGS techniques have significantly increased our knowledge about the genetic landscape of AML. In addition to the WHO classification, there are now additional suggestions on how to sub-classify AML based on the occurrence of different mutations. In 2013, the first thorough genetic characterization of AML using NGS on a large scale in AML was published. This study suggests gene mutations in AML to be divided into nine groups, based on the normal function of the mutated gene (Fig. 2) (Ley 2013).

FMS-like tyrosine kinase 3 (FLT3) receptor is expressed in hematopoietic progenitor cells. Mutations in *FLT3* intracellular tyrosine-kinase domain (*FLT3*-TKD) leads to constitutive proliferation activation signal through RAS-RAF, JAK-STAT or PI3K-AKT pathways (Fig. 2). Another mutation affecting the *FLT3* gene is the *FLT3*-internal tandem duplication (*FLT3*-ITD) in exon 14 and 15, which occurs frequently in AML. *FLT3*-ITD results from duplications and insertion in the juxtamembrane domain of FLT3 receptor. The insertion varies between 3 bp to up to 400 bp; this causes an auto-phosphorylation of FLT3 receptor and a constant activation of the tyrosine kinase. AML patients with *FLT3*-ITD mutation usually have poor prognosis (Stirewalt and Radich 2003).

Runt-related transcription factor 1 (RUNX1) is a transcription factor whose function has been implicated during early haematopoiesis (de Bruijn and Dzierzak 2017). Around 6-18% of *de novo* AML patients carry a mutation in the *RUNX1* gene. Most commonly, this consists of the chromosomal rearrangement t(8;21) which results in a *RUNX1-RUNX1T1* fusion. Double knockout of *RUNX1* gene in adult HSCs affects the transactivation domain of *RUNX1* and causes an increase in its DNA binding affinity, which leads to aberrant gene expression of downstream genes and results in multi-lineage differentiation blockage (Fig. 2). Further, biallelic missense and nonsense point mutations in *RUNX1* are reported to be associated with adverse prognosis in patients with CN-AML (Mangan and Speck 2011, Greif, Konstandin *et al.* 2012).

The most common mutation in AML affects **nucleophosmin (*NPM1*)**. The NPM1 protein has been implicated in critical cell functions through interacting with multiple proteins and shuttling between the cytoplasm and nucleus. NPM1 has been shown to be involved in stabilization of the Arf protein and ribosome biogenesis and export (Grisendi, Mecucci *et al.* 2006). An insertion mutation in the last exon of *NPM1* causes loss of the nuclear localization signal and aberrant cytoplasmic cellular location (Fig. 2) (Falini, Mecucci *et al.* 2005, Falini, Bolli *et al.* 2006). This type of mutation is referred to as NPM1c. However, the molecular mechanism of how *NPM1* mutations induces leukaemia is still not clear. Eviction of NPM1 from the nucleus along with their partner proteins potentially results in

disturbance of biological processes. A proposed mechanism of action of NPM1c in promoting leukemogenesis is by indirectly activating the onco-protein c-MYC through entrapping its suppressor Arf in the cytoplasm (Falini, Gionfriddo *et al.* 2011). Other mutations in genes like *DNMT3A* and *FLT3*-ITD are often associated with *NPM1* mutations in CN-AML (Papaemmanuil, Gerstung *et al.* 2016). While *NPM1* mutations are associated with a good prognosis, when they co-occur with *FLT*-ITD, the prognosis worsens (Dohner, Schlenk *et al.* 2005, Schnittger, Bacher *et al.* 2011).

DNA methyltransferase 3 alpha (DNMT3A) enzyme catalyses *de novo* DNA methylation. The *DNMT3A* gene is mutated in around 20-22% of all AML patients, preferentially in CN-AML (Ley, Ding *et al.* 2010, Gaidzik, Schlenk *et al.* 2013). Various types of mutations have been reported for *DNMT3A*, but heterozygous point mutations at arginine position 882 (R882) accounts for 58% of all *DNMT3A* mutations in AML patients. Functional studies on $DNMT3A^{R882}$ mutation have revealed that the mutant enzyme has less DNA-binding affinity compared to the wild type. This results in reduced enzymatic activity, which in turn leads to DNA hypomethylation (Holz-Schietinger, Matje *et al.* 2012, Russler-Germain, Spencer *et al.* 2014). The prognosis of *DNMT3A* mutations is context-dependent and affected by other recurrent mutations such as *NPM1* and *FLT*-ITD (Papaemmanuil, Gerstung *et al.* 2016). Moreover, $DNMT3A^{R882H}$ mutations have been found to cause global DNA hypomethylation in CN-AML patients (Qu, Lennartsson *et al.* 2014).

Mutations in spliceosome related genes are recurrent in AML and mainly affect **splicing factor 3b subunit 1 (*SF3B1)***, *U2AF1*, *SRSF2* and *ZRSR2*. Mutations in these genes result in an impaired spliceosome machinery (Fig. 2) (Lindsley, Mar *et al.* 2015). Spliceosome mutations are more common in refractory anaemia with ring sideroblasts (RARS) and MDS compared to *de novo* AML and are seen more commonly in AML secondary to MDS. *SF3B1* is the most investigated of the mutated spliceosome genes and a missense mutation that affects the core domain of SF3B1 protein causes aberrant RNA splicing. Targets of SF3B1 include genes such as *EZH*, *RUNX* and *ASLX1* (Dolatshad, Pellagatti *et al.* 2015).

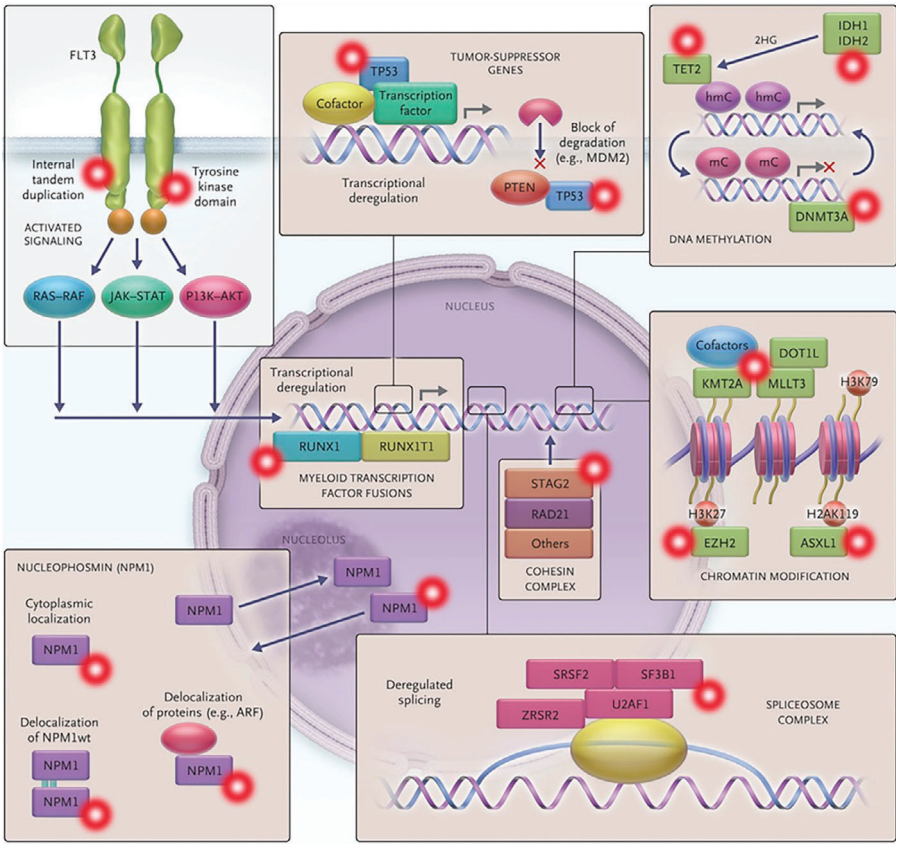

Figure 2. Most recurrent mutated genes in AML based on their function. Reproduced with permission from (Dohner, Weisdorf et al. 2015), Copyright Massachusetts Medical Society

TP53 is a tumour suppressor gene that functions as a transcription factor that becomes activated in response to DNA damage. While *TP53* mutations are found in 5-10% of AML patients, it is commonly associated with complex karyotype and an adverse prognosis. Mutations in *TP53* leads to impairment of its activity and often, as a consequence, overexpression of its negative regulators mouse double minute 2 homology (*MDM2*) and tensin homologue (*PTEN*) proteins (Fig. 2) (Kojima, Konopleva *et al.* 2005).

The cohesin complex along with CTCF maintains chromatin looping and interactions that regulate and facilitate gene regulation as well as chromosome condensation during cell divisions. Mutations in AML can affect cohesin complex subunits, mainly of two types, either truncating mutations in ***STAG2*** and ***RAD21*** or missense mutations in ***SMC3*** and ***SMC12A*** (Welch, Ley *et al.* 2012) (Fig. 2).

The exact mechanism by which different mutations impact the cohesin complex is to be further investigated. However, some mutations have been suggested to have dominant negative effect, while others induce transcript degradation. Interestingly, mutations in cohesin genes are mutually exclusive with *DNMT3A*, *FLT3*, *NPM1* and *PTPN11* mutations. While cohesin mutations cause chromosomal instability and aneuploidy it is often found in CN-AML (Ley 2013).

Ten-eleven translocation (*TET*) methylcytosine dioxygenase (*TET2*) plays a key role in DNA demethylation through catalysing the conversion of methylcytosine to 5-hydroxymethylcytosine (Ito, D'Alessio *et al.* 2010). Mutations in *TET2* are loss of function mutations that cause global DNA hypermethylation (Figueroa, Abdel-Wahab *et al.* 2010). *TET2* mutations are present in 23-27% of AML patient and commonly found in malignancies like MDS and myeloproliferative neoplasms (NPM) (Tefferi, Lim *et al.* 2009, Papaemmanuil, Gerstung *et al.* 2016). Detection of *TET2* mutations in early myeloid and lymphoid progenitors as well as in normal CD34+ cells, imply its role in clonal haematopoiesis (Smith, Mohamedali *et al.* 2010). In AML, *TET2* mutations often occur together with *NPM1*, *DNMT3A* and *FLT3* mutations (Papaemmanuil, Gerstung *et al.* 2016). It is still elusive how these mutations contribute to leukemogenesis. As mentioned, *TET2* mutations are associated to hypermethylation and one affected locus is the *GATA2* promoter causing gene downregulation leading to block in differentiation and leukaemia development (Shih, Jiang *et al.* 2015). The prognostic impact of the *TET2* mutations has been debated and is still unclear. A recent meta-analysis looked at 16 studies and found an adverse effect of *TET2* mutations on prognosis in general (Wang, Gao *et al.* 2019).

Isocitrate dehydrogenase 1 and 2 (*IDH1/2*) are frequently mutated genes in AML. Whereas mutations in *IDH1* are frequently affecting the arginine residue 132 ($IDH1^{R132}$), arginine 140 and 172 are frequently mutated in *IDH2* ($IDH2^{R140}$ and $IDH2^{R172}$ respectively). These are gain of function mutations that affect the catalytic domain of the IDH enzyme and consequently cause excessive conversion of alpha-ketoglutarate (α-KG) into the oncometabolite 2-hydroxyglutarate (2-HG) (Ward, Patel *et al.* 2010). $IDH2^{R140}$ is more common than IDH^{R172} despite that *IDH1* and *IDH2* mutations being mutually exclusive as with *TET2* mutations (Gaidzik, Paschka *et al.* 2012). Furthermore, *NPM1* mutations have been found to be mutually exclusive with $IDH2^{R140}$ but not $IDH2^{R172}$. The accumulation of the oncometabolite 2-HG in *IDH1/2* mutated AML has been shown to lead to DNA hypermethylation (Fig. 2) (Figueroa, Abdel-Wahab *et al.* 2010, Stein, DiNardo *et al.* 2019).

Mutations in chromatin remodelling genes like the histone modifiers **additional sex comb-like (*ASXL1*)** and **enhancer of zeste homolog 2 (*EZH2*)** leads to loss

of histone methylation of H2AK119 and H3K27, respectively, which results in more open chromatin and decreased histone occupancy (Fig. 2) (Simon and Lange 2008, Abdel-Wahab, Adli *et al.* 2012). *ASXL1* is a putative member of polycomb group (PcG) and is mutated in 5-10% of AML patients, although more frequent (16%) in older patients (> 60 years) (Metzeler, Becker *et al.* 2011). While EZH2 is a member PcG Repressive Complex 2 (PRC2), both ASXL1 and EZH2 interact to remove the repressive histone mark H3K27me3 (Gelsi-Boyer, Brecqueville *et al.* 2012).

CCAAT Enhancer Binding Protein α (CEBPA) is an important transcription factor that is involved in granulocyte differentiation (Ma, Hong *et al.* 2014). Loss of function mutations in *CEBPA* have been reported in 15-19% of CN-AML patients (Longo, Döhner *et al.* 2015). In particular, biallelic mutations are common in *CEBPA*, where one allele could harbour a frame shift mutation which results in a truncated protein at its N-terminal, while the other allele has an insertion/deletion at the bZIP domain. Moreover, biallelic mutations in CEBPA predict a favourable prognosis and a higher complete remission rate (Fasan, Haferlach *et al.* 2014).

More recently, Papaemmanuil *et al.,* have conducted a study with 1540 intensively treated AML patients characterizing driver mutations, cytogenetics and the clinical data. They proposed an additional genomic classification of AML, also based on its significance for clinical outcome. Three main genomic categories are proposed to be added to "AML with recurrent genetic abnormalities" within the WHO classification; namely 1) AML with mutations in chromatin-spliceosome genes, 2) AML with *TP53* aneuploidy, and 3) AML with IDH^{R172} mutations. In total eleven subgroups are suggested and they are summarized in Table 3 (Papaemmanuil, Gerstung *et al.* 2016).

Mutations within the chromatin-spliceosome group includes AML types with aberrant RNA splicing (*SRSF2*, *SF3B1*, *U2AF1* and *ZRSR2*), chromatin organization (*ASXL1*, *STAG2*, *BCOR*, MLL^{PTD}, *EZH2* and *PHF6*), or transcription (*RUNX1*). In their cohort, this group accounted for 18% of the patients. Furthermore, in the Papaemmanuil study, 13% of the AML patients had *TP53* aneuploidy which defined a separate group (Papaemmanuil, Gerstung *et al.* 2016). Interestingly, ~16 AML patients had an IDH^{R172} mutation, which represents 1% of the cohort. While IDH^{R172} mutation has a role in gene expression and DNA methylation, it was found to occur less frequently with *NPM1* mutations compared to IDH^{R140} mutations which affects metabolic activity.

Table 3. Suggested genomic classification of AML by Papaemmanuil *et al.* 2016.

Genomic Subgroup	Frequency in the Study Cohort (N = 1540) no. of patients (%)	Most Frequently Mutated Genes* gene (%)
AML with *NPM1* mutation	418 (27)	***NPM1*** (100), *DNMT3A* (54), *FLT3*ITD (39), *NRAS* (19),*TET2* (16), *PTPN11* (15)
AML with mutated chromatin, RNA-splicing genes, or both†	275 (18)	***RUNX1*** (39), ***MLL***PTD (25), ***SRSF2*** (22), *DNMT3A* (20),***ASXL1*** (17), ***STAG2*** (16), *NRAS* (16), *TET2* (15), *FLT3*ITD (15)
AML with *TP53* mutations, chromosomal aneuploidy, or both‡	199 (13)	**Complex karyotype** (68), **−5/5q** (47), **−7/7q** (44), ***TP53*** (44), **−17/17p** (31), **−12/12p** (17), +8/8q (16)
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB–MYH11*	81 (5)	**inv(16)** (100), *NRAS* (53), +8/8q (16), +22 (16), *KIT* (15), $FLT3^{TKD}$ (15)
AML with biallelic *CEBPA* mutations	66 (4)	$\textbf{\textit{CEBPA}}^{biallelic}$ (100), *NRAS* (30), *WT1* (21), *GATA2* (20)
AML with t(15;17)(q22;q12); *PML–RARA*	60 (4)	**t(15;17)** (100), $FLT3^{ITD}$ (35), *WT1* (17)
AML with t(8;21)(q22;q22); *RUNX1–RUNX1T1*	60 (4)	**t(8;21)** (100), *KIT* (38), −Y (33), −9q (18)
AML with MLL fusion genes; t(x;11)(x;q23)§	44 (3)	**t(x;11q23)** (100), *NRAS* (23)
AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *GATA2, MECOM(EVI1)*	20 (1)	**inv(3)** (100), −7 (85), *KRAS* (30), *NRAS* (30), *PTPN11* (30), *ETV6* (15), *PHF6* (15), *SF3B1* (15)
AML with $IDH2^{R172}$ mutations and no other class-defining lesions	18 (1)	***IDH2***R172 (100), *DNMT3A* (67), +8/8q (17)
AML with t(6;9)(p23;q34); *DEK–NUP214*	15 (1)	**t(6;9)** (100), *FLT3*ITD (80), *KRAS* (20)
AML with driver mutations but no detected class-defining lesions	166 (11)	*FLT3*ITD (39), *DNMT3A* (16)
AML with no detected driver mutations	62 (4)	
AML meeting criteria for ≥2 genomic subgroups	56 (4)	

* Genes with a frequency of 15% or higher are shown in descending order of frequency. Key contributing genes in each class are shown in boldface type.

† Classification in this subgroup requires one or more driver mutations in *RUNX1*, *ASXL1*, *BCOR*, *STAG2*, *EZH2*, *SRSF2*, *SF3B1*, *U2AF1*, *ZRSR2*, or *MLLPTD*. In the presence of other class-defining lesions — namely, inv(16), t(15;17), t(8;21), t(6;9), MLL fusion genes, or complex karyotype or driver mutations in *TP53*, *NPM1*, or *CEBPA* biallelic — two or more chromatin–spliceosome mutations are required.

‡ Classification in this subgroup requires *TP53* mutation, complex karyotype, or in the absence of other class-defining lesions, one or more of the following: −7/7q, −5/5q, −4/4q, −9q, −12/12p, −17/−17p, −18/18q, −20/20q, +11/11q, +13, +21, or +22.

§ Multiple fusion partners for MLL were found, with the clinical implications depending on the specific fusion partner.

1.2.4 Clonality and clonal evolution in AML

Clonal heterogeneity is common in AML where sub-clones originate from a founding clone (Ley 2013). Furthermore, pre-leukemic HSCs have shown to acquire early initiation mutations such as *DNMT3A* followed by mutations such as *NPM1*, *FLT*-ITD (Shlush, Zandi *et al.* 2014). Analysis of HSC from healthy donors have shown that some harbour $DNMT3A^{R882H}$ mutation as a result of aging, but in order to develop AML, it requires a second genetic hit (Welch, Ley *et al.* 2012).

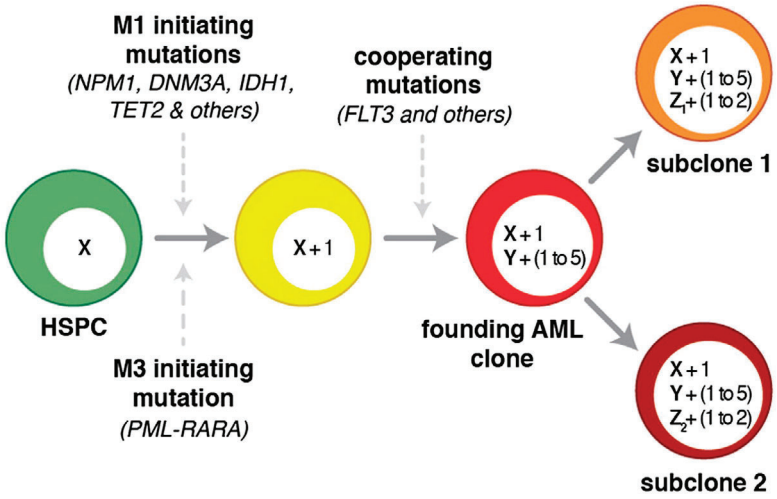

Figure 3. Graphical representation of the sequence of mutational events in HSCs. Adapted from (Welch, Ley et al. 2012)

Figure 3 shows a schematic representation of the sequence of mutational events from the time that the HSCs acquire an initiating mutation until AML develops and the generation of multiple sub-clones. The biological function of these mutations will help understanding disease development in AML.

1.2.5 Treatment of AML

During the last decades, there have been improvements in the treatment of AML with better survival following chemotherapy and hematopoietic stem cell transplantation HCST.

Induction therapy

The first line treatment in AML is named induction therapy and aims to eliminate leukemic blast cells to achieve a complete remission (CR). Classically, patients undergo daily cytarabine infusions for 7 days and anthracycline for 3 days, which known as the "7+3" treatment regimen. This is the preferred treatment for patients under the age of 70 and fit elderly patients (Dohner, Estey *et al.* 2017). CR is archived when BM blasts are <5%, platelets >100000/µl and neutrophil count >1000/µl (Cheson, Bennett *et al.* 2003). Younger patients have better CR rates (60-75%) compared to older patients (38-60%) (Longo, Döhner *et al.* 2015). Patient fitness is the main criteria for deciding treatment strategy and not age alone. Older AML patients with complex karyotype and *TP53* mutations may preferably be treated with the hypomethylating agents decitabine or azacitidine instead of intensive induction therapy, since these patients are highly resistant to chemotherapy (Quintas-Cardama, Ravandi *et al.* 2012, Klepin 2014).

Consolidation therapy

Consolidation therapy is a post-remission treatment to eliminate minimal residual disease (MDR) to prevent relapse. Usually it starts with chemotherapy and is followed by hematopoietic stem cell transplantation (HSCT). MRD is most commonly analysed by flow-cytometry for aberrant immune-phenotypes but genetically based MRD is increasingly utilized using conventional or next-generation sequencing techniques (Kohlmann, Nadarajah *et al.* 2014, Bill, Grimm *et al.* 2018). Patients within the favourable ELN genetic risk category have been suggested to benefit from repeated courses of high doses of cytarabine. Moreover, some studies have suggested combination therapy post-remission for adverse- risk cytogenetics group but have not shown better outcome compared to only cytarabine (Burnett, Russell *et al.* 2013). Importantly, intermediate and adverse risk group AML patients who are eligible for transplantation and achieve CR, are usually subjected to allogeneic HSCT. (Popat, de Lima *et al.* 2012).

New Therapies

Improving treatment outcome has always been a concern in AML and the development of new potent and less toxic drugs, resulting in novel therapeutic opportunities for AML, are examined continuously in clinical trials. Several of the new emerging drugs target specific recurrent mutations in AML. For instance, the first

generation of FLT3 inhibitors (sorafenib, sunitinib and midostaurin) have been shown to have a role in *FLT3* mutated AML but they also have off target effects as a result of their activity on other kinases (Weisberg, Roesel *et al.* 2010). More recently, second generation FLT3 inhibitors (crenolanib, gilteritinib and quizartinib) have shown higher specificity, potency and less toxicity due to less off target activity. First and second generation FLT3 inhibitors are also categorized based on their mechanisms of action; Type I (midostaurin, lestaurtinib, sunitinib, gilteritinib and crenolanib) and Type II (ponatinib, sorafenib and quizartinib). While Type I competes with ATP molecules and binds to the ATP-binding site of active tyrosine kinase domain (TKD), Type II blocks the activation of TKD when interacting with its hydrophobic region (Ke, Singh *et al.* 2015). To date, midostaurin results in a better survival in AML patients with *FLT3* mutation and been approved by FDA in combination with induction therapy. Meanwhile, gilteritinib is approved for relapsed or refractory AML patients with *FLT3* mutation (Dohner, Estey *et al.* 2017). Additionally, AML patients with *FLT3* mutations that are unfit for intensive induction treatment have been suggested to benefit from a combination of FLT3 inhibitors and hypomethylating agents (i.e. azacytidine and decitabine).

Other new promising treatment approaches include IDH inhibitors such as enasidenib, which blocks mutated IDH2 enzyme from excessively producing the 2-HG onco-metabolite and thereby promotes differentiation (Yen, Travins *et al.* 2017). Enasidenib has shown surprisingly good CR rates as monotherapy in relapsed or refractory AML patients and received FDA approval for clinical use (Stein, DiNardo *et al.* 2017). Likewise, ivosidenib inhibits mutated IDH1 enzyme and has similar results as enasidenib. As for *TET2* mutations, *IDH* mutation is associated with DNA hypermethylation and therefore, IDH inhibitors have been suggested to be combined with hypomethylating agents.

APR-246 is a small molecule that has been developed to specifically target mutated p53 protein by restoring the 3D structure of the mutated protein, and consequently to induce cell cycle arrest and apoptosis (Bykov and Wiman 2003). Since it has shown promising results in early clinical trials, APR-246 has now entered phase III clinical studies to prepare for registration in *TP53* mutated malignancies (Deneberg, Cherif *et al.* 2016). Although it has been developed to target mutant *TP53*, p53-independent effects have also been reported including the induction of oxidative and ER stress (Ali, Mohammad *et al.* 2016). *In vitro* experiments have shown that a combination APR-246 with other drugs improves cytotoxicity and can have synergistic effects in cancer cells. For instance, good combination effects have been shown in combination with cisplatin in ovarian cancer cells (Kobayashi, Abedini *et al.* 2013). Similarly, combination with wortmannin (PI3K inhibitor) or rapamycin (mTOR inhibitor) shows an increased cytotoxicity in primary AML cells (Ali, Mohammad *et al.* 2016).

1.3 Epigenetics of AML

AML differs from many other cancer types as it contains fewer genetic lesions compared to most other malignant diseases. On average, each patient harbours 13 potentially pathogenetic genetic mutations, of which five can be considered to be recurrent mutations (Ley 2013). Interestingly, many of these genetic mutations occur in pre-leukemic HSCs and commonly affect epigenetically regulating genes such as *DNMT3A*, *TET2*, *IDH1/2* and *EZH2* (Walter, Shen *et al.* 2012, Shlush, Zandi *et al.* 2014). Indeed, aberrant DNA methylation is significant in AML and studies have shown that different subtypes of AML exhibit distinguished DNA methylation profiles dependent on the type of genetic mutation, which could also be in genes such as *NPM1* and *CEBPA* (Figueroa, Lugthart *et al.* 2010, Ley 2013).

1.3.1 Aberrant DNA methylation in AML

DNA methyltransferases (DNMTs) are a family of enzymes that have the ability to add methyl groups to cytosine residues. This process maintains the DNA methylation profile during replication by DNMT1 or catalyses *de novo* DNA methylation by DNMT3A and DNMT3B (Goll and Bestor 2005). In contrast, TET enzymes mediate a step in the removal of DNA methylation. In normal hematopoietic cells, the IDH enzyme catalyses decarboxylation of isocitrate to α-KG, meanwhile TET2 catalyses hydroxylation of 5-Methylcytosine (5mC) nucleotide in a α-KG dependant manner, resulting in 5-hydroxymethylcytosine (5hmC) which leads to DNA demethylation (Yang, Ye *et al.* 2012).

AML cells with $DNMT3A^{R882H}$ mutation display a global hypomethylated pattern compared to patients with wild-type *DNMT3A* (Qu, Lennartsson *et al.* 2014). This can result in activation of enhancers mediated by histone modifications, which can lead to aberrant expression of the HOXA cluster (Lu, Wang *et al.* 2016). R882H mutations exert a dominant negative effect on DNMT3A reducing its catalytic methyltransferases activity. Although $DNMT3A^{R882H}$ is able to form dimers, it fails to methylate neighbouring CpGs once it binds to the target site due to lack of the more effective tetramers and this causes a scattered methylation pattern (Ley, Ding *et al.* 2010). $DNMT3A^{R882H}$ mutations are found in pre-leukemic HSCs that undergo subsequent clonal evolution in a process leading to AML development. Unfortunately, there are currently no drugs targeting DNMT3A mutations, which potentially could be a way to prevent pre-leukemic lesions to develop to AML. On the other hand and as described above, mutations in *TET* and *IDH* genes impair DNA demethylation causing global hypermethylation (Figueroa, Abdel-Wahab *et al.* 2010). Since TET2 is dependent on α-KG, it becomes inhibited by 2-HG which causes genome-wide hypermethylation (Figueroa, Abdel-Wahab *et al.* 2010). In a mouse model, overexpression of mutated IDH^{R132H} resulted in a block in differentiation within the myeloid lineage, however, AML was not developed solely

based on the introduction of an *IDH*^{R132H} mutation which shows that other lesions also are needed for AML development (Sasaki, Knobbe *et al.* 2012). Furthermore, *TET2* and *IDH* mutations are mutually exclusive and both these mutations result in a similar pattern of global DNA hypermethylation (Figueroa, Abdel-Wahab *et al.* 2010, Ley 2013). Functional studies have shown that *TET2* mutations abrogate the enzymatic activity of wild-type TET2 function (Rickman, Soong *et al.* 2012). Rasmussen *et al.* have reported *TET2* to be expressed in pre-leukemic HSCs in a murine model and they found that DNA hypermethylation targets active enhancer regions (Rasmussen, Jia *et al.* 2015). Moreover, *TET2* mutations in combination with *FLT3*-ITD cause differentiation block, leading to accumulation of GMP cells (Shih, Jiang *et al.* 2015). In addition, *TET2* mutations can alter gene expression through methylating the consensus binding site of chromatin remodelling protein CTCF (Marina, Sturgill *et al.* 2016).

1.3.2 Chromatin remodeling proteins and CTCF

There is an interplay between DNA methylation, chromatin interactions and chromosomal architecture. As mentioned above, aberrant methylation can affect the binding of the architectural protein CTCF, causing a change in chromatin looping and gene expression. CTCF is a key player in chromatin organization working together with cohesin in order to shape the chromatin architecture through regulating chromatin looping and formation of topologically associating domains (TADs) (Wendt, Yoshida *et al.* 2008, Merkenschlager and Odom 2013). Interestingly, and as described above, genes building up the cohesin complex subunits STAG2, SMC3, SMC12A and RAD21 are recurrently mutated in AML (Welch, Ley *et al.* 2012). Furthermore, cohesin and CTCF co-localize in the nucleus and they work together to facilitate chromatin interactions (Feinberg and Tycko 2004, Merkenschlager and Odom 2013). Also, CTCF recruits cohesin to exert its insulator function by looping out enhancers (Merkenschlager and Odom 2013, Losada 2014). The formation of DNA loops begins with extruding DNA through the cohesin ring complex and once the cohesin ring encounters CTCF, the loop becomes stabilized and forms a TAD (Fudenberg, Imakaev *et al.* 2016). Knockout studies of cohesin and CTCF have caused loss of chromatin interactions and altered chromatin looping as well as change in gene expression patterns (Zuin, Dixon *et al.* 2014).

DNA methylation has been suggested to shape the occupancy to TFs (Maurano, Wang *et al.* 2015). On the other hand, TFs can protect from DNA methylation. For example, SP1 binds to unmethylated CpGs and protects it from *de novo* DNA methylation (Brandeis, Frank *et al.* 1994). Also, CTCF maintains *Igf2-H19* region unmethylated (Schoenherr, Levorse *et al.* 2003). Despite the anti-correlation between CTCF binding and DNA methylation, some CTCF binding sites are insensitive for DNA methylation. This can be due to absence of CpGs in CTCF binding

motif (Maurano, Wang *et al.* 2015). Furthermore, some methylation insensitive TFs (for instance PU.1) bind to methylated DNA loci and induce DNA demethylation through recruiting TET enzymes (de la Rica, Rodriguez-Ubreva *et al.* 2013). This suggests how TFs could change chromatin structure through a dynamic alteration of DNA methylation. Interestingly, using CRISPR-dCas9 to modify sequence specific sites of DNA methylation, this can result in gains or losses of CTCF binding when combined with dCas9-TET2 or dCas9-DNMT3A respectively (Liu, Wu *et al.* 2016). Furthermore, knockout of *TET1* and *TET2* genes in mouse embryonic stem cells result in a change of CTCF occupancy and can lead to changes in gene express of neighbouring genes (Wiehle, Thorn *et al.* 2019). Hence, aberrant DNA methylation in AML can potentially influence the three dimensional structure of the chromatin through altering CTCF binding. Moreover, CTCF interacts with the NPM1 protein. Mutations affecting *NPM1* localization (*NPM1c*) can cause delocalisation of CTCF, consequently leading to aberrant gene expression (Wang, Han *et al.* 2019). CTCF is critical for maintaining chromatin boundaries of *HOXA* gene clusters and disruption of CTCF binding sites at these boundaries results in *HOXA9* upregulation in AML cells (Luo, Wang *et al.* 2018). Similar effects on *HOXA9* has been reported following CTCF delocalisation in *NPM1c* mutated cells (Wang, Han *et al.* 2019).

Histone modifications play a key role in chromatin remodelling and regulation of the chromatin status (Schubeler, MacAlpine *et al.* 2004). For example, mono-, di- and trimethylation of H3K79 by the histone methyltransferase *DOT1L* can lead to gene activation. High expression levels of *DOT1L* were found in AML with mixed-linkage leukaemia (MLL) (Liu, Deng *et al.* 2014). Overexpression of *DOT1L* causes H3K79 methylation in *HOXA9* promoter leading to upregulation of HOXA9, which has been shown to be critical for leukaemia development (Chen and Armstrong 2015).

Conversely, CTCF maintains gene repression by looping out and insulating genes through stabilization of polycomb domain boundaries. Thus, depletion of CTCF causes destabilization of polycomb domains and results in aberrant gene expression (Zhang, Niu *et al.* 2011, Dowen, Fan *et al.* 2014). Gain of function mutations in *EZH2* results in spread of H3K27me3 that leads to downregulation of tumour suppressor genes (Donaldson-Collier, Sungalee *et al.* 2019), which suggests this could be mediated by a gain of CTCF occupancy in order to stabilize repressive polycomb domain. Indeed, knockdown of *CTCF* in hepatocellular carcinoma causes loss of CTCF and EZH2 binding, decreased H3K27me3 marks and DNA hypomethylation at *SOCS3* promoter. *CTCF* depletion led to *SOCS3* upregulation which confirms the role of CTCF in gene silencing through recruitment of PcG subunits (Wei, Liu *et al.* 2020). In line with this, *ASXL1* is important for normal haematopoiesis (Abdel-Wahab, Gao *et al.* 2013) through interaction with cohesin

and stabilization of RAD21 and SMC1A subunits, mainly in promoter regions. Moreover, loss of *ASXL1* leads to genome wide decrease of cohesin occupancy and aberrant expression of genes that are critical for myeloid development (Li, Zhang *et al.* 2017). However, the direct impact of *ASXL1* mutation on chromatin structure has not yet been studied. Recently, cohesin was found to disrupt polycomb chromatin domain interactions in a CTCF independent manner. Furthermore, depletion of cohesin in ESCs led to stabilization of polycomb chromatin domain interactions and repression of polycomb target genes (Rhodes, Feldmann *et al.* 2020).

However, despite the key role of CTCF for gene expression, chromatin immunoprecipitation sequencing (ChIP-seq) data on CTCF occupancy in AML cells is still lacking. Such studies could reveal the impact of DNA methylation on CTCF binding and chromatin architecture in AML. Also, it could elucidate how different mutations in AML in genes such as *DNMT3A*, *TET2*, *IDH1*, *IDH2* and *NPM1* influence the 3D structure of the chromatin through changed CTCF binding in the nucleus.

2 AIM OF THE THESIS

With this thesis, we aim to increase the understanding of the genetic and epigenetic basis of AML.

Paper I

To examine the effect of APR-246 on AML cells and the role of oxidative stress in enhancing drug response through inhibiting the protective response of Nrf-2/HMOX1.

Paper II

To find a reliable source of germline DNA in bio-banked AML samples for genetic studies.

Paper III

To explore aberrant CTCF patterns in AML cells and the impact of certain mutations on CTCF occupancy.

3 METHODOLOGICAL APPROACHES

Detailed and comprehensive descriptions of experimental methodologies used to generate the data in this thesis are described in papers I-III. Key experiments are discussed below.

3.1 Cell culture and transfection

Throughout the projects various cell types and methods have been used to grow cells *in vitro.* Classically, immortalized cell lines were cultivated in suitable medium while primary cells required more optimized conditions to grow *in vitro*, as described below.

3.1.1 Bone marrow stroma culture

During BM aspiration from AML patients, stroma cells are also collected in the sample. To isolate stroma cells from BM samples, we used the ability of stroma cells to adhere the to plastic surface of culture flasks. In order to maximize cell recovery for culture, we cultured total mononuclear cells in culture flasks in MyeloCult™ H5100 (STEMCELL Technologies) supplemented with 10% FBS for the first two weeks. Thereafter, unattached cells (i.e., leukemic cells, lymphocytes etc.) were washed away. Then, new fresh DMEM-GlutaMax medium with 10% FBS was added to stroma cells for up to six weeks.

3.1.2 Primary AML cell culture

To assess the effect of different drugs on AML blast cells *in vitro*, cells were grown in duplicate in culture flasks. A modified protocol of long-term culture of AML cells without feeder cells was used (Griessinger, Anjos-Afonso *et al.* 2014). Total mononuclear cells from bone marrow aspirations were suspended in RPMI 1640 medium with 10% FBS supplemented with IL3, IL6, SCF (R&D Systems), GM-SCF, G-CSF and Flt- 3/flk2 ligand (STEMCELL Technologies). Cells were seeded onto 6-well plates and incubated at 37ºC and 5% CO_2.

3.1.3 RNA interference and transfection

RNA interference is a biological mechanism by which cells can control gene expression. Small interfering RNAs (siRNA) and mircoRNAs (miRNAs) are two of the main categories of non-coding RNA. siRNAs are derived from longer double-stranded RNAs that are produced by the cell. siRNAs are produced by an endonuclease protein called dicer, which cleaves along preRNA into short fragments (20-30 nucleotides). Then, double-stranded siRNA binds to the argonaute

protein whose antisense strand gets selected and stays bound to argonaute. Other proteins bind to siRNA-argonaute to form RNA-induced silencing complex (RISC). Antisense siRNA guides RISC to target mRNA. Once aligned to a perfect sequence match, catalytic RISC protein cleaves mRNA molecules that then will be degraded (Dana, Chalbatani *et al.* 2017). Scientists have used this approach by introducing synthetic siRNAs to manipulate and silence gene expression. In study I, we used siRNA to target Nrf2 in KBM3 and HG3 cells. While in study III, siRNA were targeting CTCF in K562 cells. NEON electroporation system was used to transfect the cells, which in principle uses electric current to create temporary pores in cell membranes allowing molecules to diffuse into cells.

3.2 Mutation detection by targeted sequencing

Pyrosequencing is a sequencing-by-synthesis method in which DNA polymerase complements single stand DNA and incorporates appropriate nucleosides. As a result, pyrophosphate is produced which then is converted to ATP by ATP sulfurylase. Finally, luciferase utilizes the ATP molecule to generate light signal as an indication of a successful addition of either an A, T, C or G base (Harrington, Lin *et al.* 2013). In study II we used pyrosequencing to detect somatic mutations in BMS cells by designing specific primers targeting the mutations of interest.

3.3 Fluorescence-activated cell sorting

Fluorescence-activated cell sorting (FACS) is a technique used to analyse and separate cell populations based on cell surface antigens (Cluster of differentiation (CD) markers). Cells are mixed with fluorophore-conjugated antibodies that recognize a specific CD marker, then passed through a beam of laser that excites the fluorophore that is bound to the antibody at a certain wave length where the emission is captured by a detector. A computer software analyses the signals to identify different cells types.

Since AML samples usually have a heterogeneous set of leukemic blast populations carrying different surface markers, we used a negative sorting strategy to sort out non-leukemic cells. CD45, CD3, CD19 and Nkp45 were used to mark T-cells, B-cells and NK cells, respectively, while CD33 was used to mark myoblasts and Aqua to determine and sort cells based on viability. We defined leukemic blast cells as CD45-, CD3-, CD19-, Nkp45-, Aqua negative and either CD33+ or CD33-. To minimize the impact of sorting on cells, samples were stained and formaldehyde fixed upon sorting only for ChIP-seq experiment. We used this strategy for the isolation of leukemic blast cells in paper III (Fig. 4).

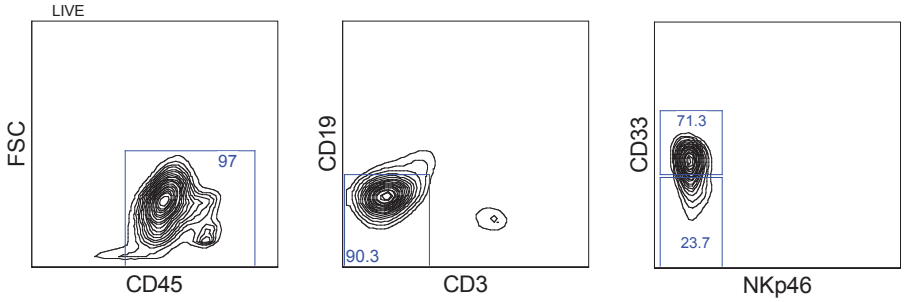

Figure 4. FACS sorting panel. FACS gating strategy for sorting leukemic blast cells.

Eukaryotic cells produce reactive oxygen species (ROS) as part of their normal metabolism. This results in the production of hydroxyl radical (OH) and hydrogen peroxide (H_2O_2) which contribute to intracellular oxidative stress. The development of fluorescent probes has made it possible to detect intercellular ROS using flow cytometry (Cossarizza, Ferraresi *et al.* 2009). A non-fluorescent H_2DCFH-DA molecule is used to detect ROS. It is highly sensitive to intracellular redox change and is a cell-membrane permeable dye. H_2DCFH-DA enters the cell, and then, esterase enzymes cleave it into 2′,7′-dichlorodihydrofluorescein (H_2DCF) which then utilize H_2O_2 to oxidize H_2DCF into the fluorescent molecule dichlorofluorescein (DCF). The signal emitted from DCF can be detected and quantified by flow cytometry or fluorescent microscopy. In study I, KBM3 cell cells were treated with different concentrations of APR-246 drug and cells were then stained with H_2DCFH-DA for 20 minutes and immediately analysed by flow cytometry. H_2O_2 was used as a positive control along with H_2DCFH-DA.

3.4 Immunocytochemistry

Immunocytochemistry is a method to detect intracellular proteins using a specific antibody that is linked to a fluorescent dye, which can be detected by microscope (Burry 2011). Cells are fixed and permeabilized with paraformaldehyde in order to allow antibodies to enter the cells. In study I, a primary mouse IgG antibody was used to detect human Nrf2 protein, while a FITC-labelled anti-IgG secondary antibody was used to visualize the detection of Nrf2. The signal was detected by confocal microscope.

3.5 Glutathione live detection

Thioltracker Violet is a thiol-reactive fluorescent dye used to detect intercellular reduced thiol (Mandavilli and Janes 2010). In study I, following the exposure of primary AML cells with APR-246, cells were stained with Thioltracher Violet dye and visualized under fluorescent microscope.

3.6 DNA methylation detection

The recent development of sequencing technologies and microarrays has made is possible to detect single nucleotide DNA methylation genome wide. In paper III, Infinium MethylationEPIC BeadChip was used.

3.6.1 Bisulfite conversion

Sequencing technologies are not able to directly detect 5mC and distinguish it from cytosine (C). However, chemical modification of C in a process called bisulfite conversion has made it possible to detect 5mC in whole genome (Hayatsu, Shiraishi *et al.* 2008). Treating genomic DNA with sodium bisulfite causes deamination of C into uracil (U), while 5mC remains protected from deamination by the methyl group. This allows detection methylation levels on single-nucleotide resolution by calculating the C/T ratio after PCR amplification. The main disadvantage of bisulfite conversion method is the fragmentation of genomic DNA caused by the harsh chemical treatment and also, its inability to distinguish between 5hmC and 5mC.

3.6.2 Illumina methylation arrays

The Infinium MethylationEPIC BeadChip (IlluminaEpic array) is a probe-based array that consists of the original HumanMethylation450 BeadChip with an additional 400,000 CpGs that mainly cover enhancer and other non-CpG island regions (Pidsley, Zotenko *et al.* 2016). IlluminaEpic array employs both Infimum type I and type II probes (Bibikova, Lin *et al.* 2006). Following bisulfite conversion and genomic DNA amplification and purification, BS converted DNA is applied to the BeadChip to hybridize with the probe on the chip. For Infinium type I, two probes are dedicated for same loci to detect either methylated or unmethylated CpG. However, Infinium type II, uses a single probe per loci where the 3' prime end hybridize directly upstream to the target CpG. Single nucleotide extension allows the incorporation of a fluorescently-labelled G or A to detect either methylated or unmethylated loci.

3.6.3 Whole genome bisulfite sequencing

Whole genome bisulfite sequencing (WGBS) or BS-Seq is the optimal method for genome-wild methylation profiling. The principle of WGBS is combining high-throughput NGS and bisulfite converted DNA (Mill, Yazdanpanah *et al.* 2006). Sequenced reads are mapped to the reference genome using specialized alignment algorithms that identifies C to T transitions and marks it as unmethylated cytosine. This powerful method can detect methylation status at single nucleotide resolution genome-wide. However, since it is dependent on BS DNA, it is not possible to differentiate between 5mC and 5-hydroxymethylcytosine (5hmC).

3.7 Chromatin immunoprecipitation and sequencing

DNA strands are wrapped around histone proteins to form nucleosomes, which is referred to as euchromatin (Hewish and Burgoyne 1973, Hyde and Walker 1975). Furthermore, other transcription factors and structural protein are also interacting with genomic DNA. To investigate these interactions between proteins and DNA, chromatin immunoprecipitation (ChIP) technique is used (Collas 2010). Molecules within the nucleus are in dynamic interaction so it is critical to fix the cells first where formaldehyde is used for the cross-linking of DNA to protein. To detect the specific loci of certain protein-DNA interactions, fixed chromatin must be fragmented using sonication. This is followed by immunoprecipitation by adding an antibody that recognizes the protein of interest to pull it down as DNA-bound protein complexes. While heat is used to reverse the cross-linking, proteases digest bound proteins so that DNA can be purified for downstream analysis (Fig. 5). Classically, PCR was used to amplify a genomic target loci where a protein of interest could possible bind. However, with the development of NGS, it is now possible to combine ChIP and sequencing (ChIP-seq) which makes it possibly to map proteins bound to DNA on a genome-wide level.

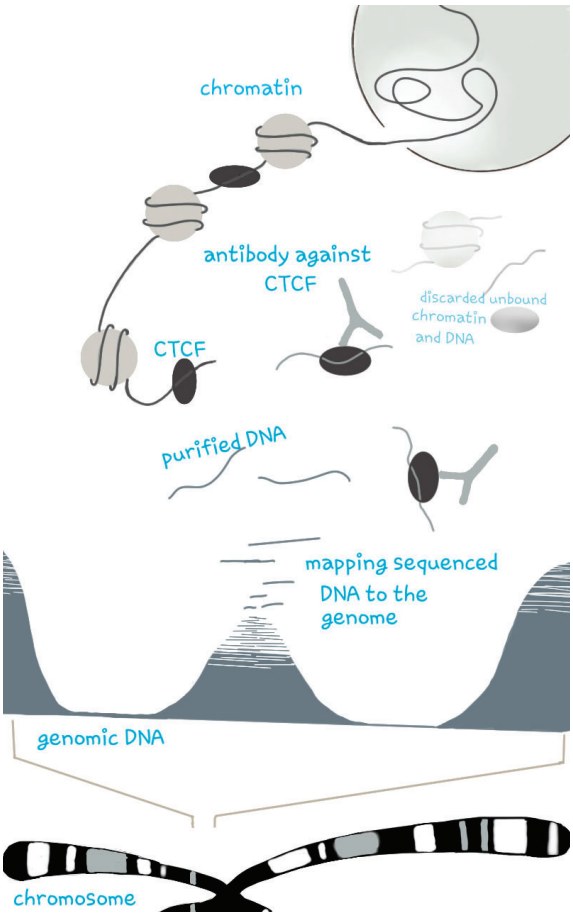

Figure 5. Schematic representation of ChIP-seq.

3.8 RNA sequencing

RNA sequencing is a method for whole transcriptome profiling using NGS technology. Briefly, total RNA is purified and transcribed to cDNA. The cDNA is then used to prepare sequencing libraries. In this case single-end sequencing was performed producing reads with an average length of 50 bp. Following alignment to the reference genome, this generates information of gene expression.

4 RESULTS AND DISCUSSION

4.1 Paper I

Mutations in the tumor suppressor *TP53* has been associated with resistance to chemotherapy in many cancers (Wattel, Preudhomme *et al.* 1994, Breen, Heenan *et al.* 2007). More than half of AML patients with a complex cytogenetic profile harbour mutant *TP53* (Rucker, Schlenk *et al.* 2012). APR-246 is a small molecule that has been developed to reactivate mutant TP53 protein (Bykov, Issaeva *et al.* 2002). However, off-target effects have previously been reported for APR-246 and such effects have also been found to induce apoptosis in primary AML cells regardless of *TP53* status (Ali, Jonsson-Videsater *et al.* 2011). Our goal was to further investigate the effects of APR-246 in AML. Expression profiling of KMB3 AML cell following exposure to APR-246 revealed that genes related to oxidative stress and heat shock were the most affected by APR-246 exposure. Among these genes were *HMOX1*, *RIT1* and *SLC7A11*, that had protective effects against ROS. Expression of *HMOX1* is inversely correlated with intercellular GSH and analysis of GSH in AML patient cells showed a dose-dependent reduction of intracellular GSH in response to APR-246 exposure. Furthermore, the combination of APR-246 with GSH inhibitor buthionine-[S,R]-sulfoximine (BSO) resulted in an extensive killing of KMB3 AML cells. In addition, we used the ROS scavenger N-acetylcysteine (NAC) to confirm that ROS induced by APR-246 exposure caused *HMOX1* up-regulation. NAC demolished the effect of APR-246 on *HMOX1* while exhibiting minimal cytotoxicity. On the other hand, we found that upregulation of *HMOX1* was independent from *TP53* mutational status in HCT116 colon cancer cell lines. Four different clones of HTC116 with either $TP53^{wt/wt}$, $TP53^{null/null}$, $TP53^{R243w/wt}$ or $TP53^{R248W/null}$ were treated with APR-246 and *HMOX1* expression was found to be upregulated in all four clones. Moreover, nuclear factor erythroid 2-related factor 2 (NRF2L) was activated upon exposure to APR-246. NRF2L is a transcription factor that binds to *HMOX1* promoter and induces its expression (Dhakshinamoorthy and Jaiswal 2001). Immunostaining of APR-246 treated KMB3 cells for NRF2L protein showed translocation for the protein from the cytoplasm and to the nucleus. NRF2L also had increased expression at the transcriptional level as measured by qPCR. To confirm that NRF2L mediates *HMOX1* activation following APR-246 exposure, we knocked down *NRF2L* in KMB3 cells and then incubated them with APR-246. As anticipated, *HMOX1* expression was suppressed. However, KMB3 cells with *NRF2L* knockdown were more sensitive to the cytotoxic effect of APR-246. To overcome the protective effect ROS, we combined the use of PI3K inhibitors wortmannin and the mTOR inhibitor rapamycin with APR-246. As a result, we could detect an improved anti-leukemic effect of APR-246.

4.2 Paper II

Advances in next generation sequencing (NGS) techniques have opened the doors for genome-wide characterization of genetic lesions in various cancers (Shao, Lin *et al.* 2016). In order to properly identify somatic genetic mutations in cancers, the use of reference germline DNA is crucial, and thus, it is important have access to a reliable source of germline DNA from non-malignant cells from same patient. There are studies using skin biopsies, buccal swabs and buccal washes from AML patients. However, these samples are often infiltrated by leukemic blast cells that makes the analysis more complicated (Ley, Mardis *et al.* 2008). In addition, they require separate invasive or non-invasive sampling which is not achieved retrospectively in deceased patients. T-cells are often also used as a source of germline DNA, however, evolutionary early somatic mutations such as *DNMT3A* mutations can be found in T-cells as well as in the leukemic clone (Shlush, Zandi *et al.* 2014).

In this study, we utilized bio-banked vitally frozen mononuclear cells from the diagnostic bone marrow sample as a source of germline DNA. Thus, we focus on non-hematopoietic cells in the diagnostic AML sample and hypothesized that bone marrow stroma (BMS) cells would be a reliable source. BMS cells were expanded in culture for up to six weeks to get enough genomic material and get rid of leukemic cells. Six AML patients, of which five harboured monosomy 7, were selected for the study. The presence of monosomy 7 facilitated the monitoring of malignant cells in the BMS population using Fluorescence *in-situ* hybridization (FISH). After six weeks of culture, BMS cells from all patients showed disomy of chromosome seven. The morphological appearance of BMS cells was consistent with a fibroblast-like shape with large nuclei. In immunophenotypical analysis of cell surface markers in AML and BMS cells by flow cytometry, AML blasts were positive for CD45, CD117, CD34, CD38, HLA-DR and CD13. However, one AML sample was also positive for CD7 and CD19, defining a biphenotypic AML. In contrast, expanded BMS cells did not express CD45 or CD34 but were positive for CD90, CD105 and CD73, a phenotype similar to that of mesenchymal stem cells (MSCs). Furthermore, differentiation assay showed that BMS cells could differentiate into either adipocyte or osteoblasts but not chondroblasts. This indicates that BMS cells are still able to differentiate to osteogenic cells after a long time in culture, which can be compared to BM MSCs that lost their differentiation potential following few passages (Halfon, Abramov *et al.* 2011). Targeted exome sequencing of the AML samples revealed the presence of recurrent AML mutations in the following genes: *DNMTT3A*, *EZH2*, *FLT3*, *IDH1*, *KRAS*, *NRAS*, *TET2*, *RUNX1*, *PTPN11*, *SF3B1*, *TP53* and *U2AF1*. Interestingly, none of these somatic mutations found in the AML cells were present in the BMS population after expansion. However, other studies have reported genetic aberrations of BMS (Huang, Basu *et al.* 2015,

Kim, Jekarl *et al.* 2015) but these mutations were distinct from the mutations in the malignant clone. The mechanisms of BMS specific mutations and their effect on AML development is still elusive and require further investigation. Regardless, BMS can serve as germline control to rule out the existence of germline genetic aberrations found in the AML clone.

4.3 Paper III

The chromatin modulator protein the CCTC-binding factor (CTCF) plays an important role in gene expression regulation by forming three-dimensional chromatin interactions (Fudenberg, Imakaev *et al.* 2016, Lu, Shan *et al.* 2016). CTCF binding can be affected by DNA methylation (Engel, West *et al.* 2004, Wang, Maurano *et al.* 2012), and thus, methylation could impact on chromatin structure through interfering with CTCF binding. CTCF is important for normal haematopoiesis (Torrano, Chernukhin *et al.* 2005, Splinter, Heath *et al.* 2006, Kim, Kim *et al.* 2017) but its impact on tumour development remains unclear (Kemp, Moore *et al.* 2014). In this study, we aimed to characterize CTCF occupancy in AML patient cells and its relation to DNA methylation, gene expression and chromatin structure. Our ChIP-seq data show that AML cells (AML^{all}) exhibit an aberrant gain in CTCF occupancy compared to normal CD34+ cells, especially in *TET2* mutated AML ($AML^{TET2mut}$). In contrast, AML with mutated *NPM1* ($AML^{NPM1mut}$) did not display strongly aberrant CTCF binding. The aberrantly bound CTCF binding sites (CBSs) in $AML^{TET2mut}$ were mainly in the promoters. In contrast, aberrantly bound CBSs in general (AML^{all}) were enriched for enhancer locations. Motif analysis of differentially bound CTCF sites (DBCs) in AML^{all} showed enrichment of motifs for CEBPA, PU.1, ETS1 and RUNX1 transcription factors (TFs), which are TFs that have been implicated in the development of AML. Besides, motifs for DBCs specific for $AML^{TET2mut}$ ($AML^{TET2mut}$ vs. AML^{TET2wt}) were also enriched for other transcription factors such as KLF7 (Schuettpelz, Gopalan *et al.* 2012), HIC1 (Britschgi, Jenal *et al.* 2008), SOX4 (Lu, Hsieh *et al.* 2017), BCL11A (Tao, Ma *et al.* 2016) and FOXH1 (Loizou, Banito *et al.* 2019) that have been proposed to play roles in haematopoiesis or leukaemia. To confirm the effect of CTCF on the binding to other TFs, we knockdown CTCF in K562 cells and performed ChIP-PCR for CTCF as well as RUNX1. ChIP-qPCR analysis of overlapping sites revealed a loss of binding of both CTCF and RUNX1 at several examined sites. To correlate changes in CTCF binding to changes chromatin organization, we also performed ChIP-Seq of selected histone marks (H3K18ac, H3Kt27ac, H3K4me1 and H2A.Z) in patients where data on CTCF binding was available. We found an increase in H3K27 acetylation, H3K18 acetylation and H3K4me1 at gained CTCF binding sites while some histone marks were decreased at lost DBCs in AML showing that increased CTCF binding is associated with increase in open chromatin and active transcription. DNA methylation and RNA-seq were performed on the same samples

to investigate the correlation between CTCF binding, DNA methylation and gene expression. The majority of CBSs were hypomethylated and CTCF binding anti-correlated with DNA methylation. There was a seemingly paradoxical finding in AML^{TET2mut} with increased DNA methylation in *TET2* mutated samples but still a gain of CTCF binding in the same samples. However, we found hypermethylation associated to *TET2* mutations to occur dominantly outside of CTCF binding sites both in general and in the promoters. Also, the gain of CTCF binding in AML^{TET2mut} occurred at sites that are hypomethylated in both AML^{TET2mut} and AML^{TET2wt}, and thus, these sites do not change methylation status. Moreover, by integrating CTCF and gene expression, we found that gained DBCs in general was associated with upregluation of gene expression while loss of CTCF binding was associated with down-regulation of genes. However, the relation was dependent on the location of the aberrant CTCF binding where changes in promoters showed a clearer positive correlation while regions distant from promoters showed more anti-correlation. In addition, upregulated genes that gained CBSs were hypomethylated, while downregulated genes that lost CBSs were hypermethylated. For instance, *DOT1L*, *ZBTB7A*, *PDCD1* and *FOSL2* were genes that showed both increase in CTCF binding and gene expression. To study the direct role of CTCF binding for gene expression, we knocked down CTCF in K562 cells and analysed CTCF binding and gene expression by ChIP-qPCR and qPCR, respectively. Following CTCF knockdown, *DOT1L*, *ZBTB7A* and *PDCD1* exhibited loss of CTCF binding which was paralleled by downregulation of *DOT1L* and *PDCD1*. Thus, a change of CTCF binding can affect gene expression of nearby genes but likely requires other factors as well.

We also treated AML^{TET2mut} with the demethylating drug azacytidine and investigated CTCF occupancy, DNA methylation and gene-expression in response to this drug. Azacytidine exposure induced DNA demethylation in AML cells and major changes in CTCF occupancy. Although sites were both gained and lost for CTCF, sites with a two-fold change were more common among gained sites. *KLF6*, a gene that has been shown to be dysregulated in AML (Matsumoto, Kubo *et al.* 2006, Adelman, Huang *et al.* 2019), was among the top genes that lost DNA methylation, gained CTCF binding, and was upregulated after azacytidine exposure. Finally, more than half of DBCs that were gained in AML^{TET2mut} in response to azacytidine were overlapping with CTCF binding sites occupied in normal CD34+ cells, implying a restoration CTCF occupancy to a status similar to normal cells.

5 CONCLUDING REMARKS

In study **I**, we found that leukemic cells protect themselves from APR-246 partly through expressing genes that counteract the oxidative stress caused by the drug, including activation of the NFE2L2/HMOX1 pathway. Indeed, knocking down NFE2L2 sensitized AML cells to the anti-leukaemic effects of APR-246. Additionally, disrupting NFE2L2/HMOX1 signalling by using PI3K and mTOR inhibitors significantly improved the cytotoxicity of APR-246. Thus, we suggest that such a combination therapy could increase efficacy of APR-246 and result in better treatment responses.

In study **II**, the most significant finding is the ability to obtain germline DNA from previously vitally frozen diagnostic AML material. In this case, bone marrow stroma cells derived from non-haematopoietic cells constitute the source of germline DNA. This can be an attractive approach to obtain germline DNA for reliable genetic characterization of AML cases in clinical routine as well as for research purposes.

In study **III,** we found that AML primary cells have an aberrant gain in CTCF occupancy mainly located in enhancers. AML with *TET2* mutation appears to have a greater impact on CTCF occupancy with a gain, mainly located in promoters. It is not clear how AML cells exhibit increased CTCF binding mechanistically. Apparently, consequences on gene expression profiles were related to CTCF gain of binding where genes that gained CTCT at promoters were mainly upregulated. In addition, active chromatin represented by active histone marks were found around gained CTCF sites. This suggests that aberrant CTCF binding could contribute to AML development by a change in chromatin organization that leads to aberrant gene expression patterns. However, to study the effect of AML specific changes in CTCF binding on the three-dimentional chromatin structure in more detail, studies using techniques such as HiC are warranted.

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و ها هنا فصل آخر قد أتم و لولا فضلكم الذي مهد الي الطريق لما وصلت الى هذا القدر من النجاح. والدتي الغاليةَ، يا أعظم الملكات كم سهرتي من الليالي و تحملتي الآم منذ نعومة أظافري لأجل راحتي و سعادتي. لازلتي منبع العطف و الحنان و الشمعة الي تنير لي الطريق ،إن كلمات الثناء لا تستطيع أن تفيك حقكي، إنني من كل تاريخي خجول فمهما قدمت لن أفيكي جزاً من مما تستحقين أيتها المعلمة الأصيلة. إخواني و أخواتي الأحباء، لطالما كنتم العون و السند على طول السنين فأنتم الأخلاء و الأوفياء. تعجز حروفي أن تكتب لكم كل ما حاولت ذلك، ولا أجد في قلبي ما أحمله لكم إلا الحب والعرفان والشكر على ما قدمتم لي و ان غبتم عن ناظري يوماً فأنتم دائما في القلب.

7 REFERENCES.

Abdel-Wahab, O., M. Adli, Lindsay M. LaFave, et al. (2012). "ASXL1 Mutations Promote Myeloid Transformation through Loss of PRC2-Mediated Gene Repression." Cancer Cell **22**(2): 180-193.

Abdel-Wahab, O., J. Gao, M. Adli, et al. (2013). "Deletion of Asxl1 results in myelodysplasia and severe developmental defects in vivo." J Exp Med **210**(12): 2641-2659.

Adelman, E. R., H. T. Huang, A. Roisman, et al. (2019). "Aging Human Hematopoietic Stem Cells Manifest Profound Epigenetic Reprogramming of Enhancers That May Predispose to Leukemia." Cancer Discov **9**(8): 1080-1101.

Ali, D., K. Jonsson-Videsater, S. Deneberg, et al. (2011). "APR-246 exhibits anti-leukemic activity and synergism with conventional chemotherapeutic drugs in acute myeloid leukemia cells." Eur J Haematol **86**(3): 206-215.

Ali, D., D. K. Mohammad, H. Mujahed, et al. (2016). "Anti-leukaemic effects induced by APR-246 are dependent on induction of oxidative stress and the NFE2L2/HMOX1 axis that can be targeted by PI3K and mTOR inhibitors in acute myeloid leukaemia cells." Br J Haematol **174**(1): 117-126.

Arber, D. A., A. Orazi, R. Hasserjian, et al. (2016). "The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia." Blood **127**(20): 2391-2405.

Arinobu, Y., S. Mizuno, Y. Chong, et al. (2007). "Reciprocal activation of GATA-1 and PU.1 marks initial specification of hematopoietic stem cells into myeloerythroid and myelolymphoid lineages." Cell Stem Cell **1**(4): 416-427.

Asada, N., Y. Kunisaki, H. Pierce, et al. (2017). "Differential cytokine contributions of perivascular haematopoietic stem cell niches." Nat Cell Biol **19**(3): 214-223.

Bao, E. L., A. N. Cheng and V. G. Sankaran (2019). "The genetics of human hematopoiesis and its disruption in disease." EMBO Mol Med **11**(8): e10316.

Bibikova, M., Z. Lin, L. Zhou, et al. (2006). "High-throughput DNA methylation profiling using universal bead arrays." Genome Res **16**(3): 383-393.

Bill, M., J. Grimm, M. Jentzsch, et al. (2018). "Digital droplet PCR-based absolute quantification of pre-transplant NPM1 mutation burden predicts relapse in acute myeloid leukemia patients." Ann Hematol **97**(10): 1757-1765.

Brandeis, M., D. Frank, I. Keshet, et al. (1994). "Sp1 elements protect a CpG island from de novo methylation." Nature **371**(6496): 435-438.

Breen, L., M. Heenan, V. Amberger-Murphy and M. Clynes (2007). "Investigation of the role of p53 in chemotherapy resistance of lung cancer cell lines." Anticancer Res **27**(3A): 1361-1364.

Britschgi, C., M. Jenal, M. Rizzi, et al. (2008). "HIC1 tumour suppressor gene is suppressed in acute myeloid leukaemia and induced during granulocytic differentiation." Br J Haematol **141**(2): 179-187.

Burnett, A. K., N. H. Russell, R. K. Hills, et al. (2013). "Optimization of chemotherapy for younger patients with acute myeloid leukemia: results of the medical research council AML15 trial." J Clin Oncol **31**(27): 3360-3368.

Burry, R. W. (2011). "Controls for immunocytochemistry: an update." J Histochem Cytochem **59**(1): 6-12.

Bykov, V. J., N. Issaeva, A. Shilov, et al. (2002). "Restoration of the tumor suppressor function to mutant p53 by a low-molecular-weight compound." Nat Med **8**(3): 282-288.

Bykov, V. J. and K. G. Wiman (2003). "Novel cancer therapy by reactivation of the p53 apoptosis pathway." Ann Med **35**(7): 458-465.

Calvi, L. M., G. B. Adams, K. W. Weibrecht, et al. (2003). "Osteoblastic cells regulate the haematopoietic stem cell niche." Nature **425**(6960): 841-846.

Chen, C. W. and S. A. Armstrong (2015). "Targeting DOT1L and HOX gene expression in MLL-rearranged leukemia and beyond." Exp Hematol **43**(8): 673-684.

Cheson, B. D., J. M. Bennett, K. J. Kopecky, et al. (2003). "Revised recommendations of the International Working Group for Diagnosis, Standardization of Response Criteria, Treatment Outcomes, and Reporting Standards for Therapeutic Trials in Acute Myeloid Leukemia." J Clin Oncol **21**(24): 4642-4649.

Collas, P. (2010). "The current state of chromatin immunoprecipitation." Mol Biotechnol **45**(1): 87-100.

Cossarizza, A., R. Ferraresi, L. Troiano, et al. (2009). "Simultaneous analysis of reactive oxygen species and reduced glutathione content in living cells by polychromatic flow cytometry." Nat Protoc **4**(12): 1790-1797.

Dana, H., G. M. Chalbatani, H. Mahmoodzadeh, et al. (2017). "Molecular Mechanisms and Biological Functions of siRNA." Int J Biomed Sci **13**(2): 48-57.

de Bruijn, M. and E. Dzierzak (2017). "Runx transcription factors in the development and function of the definitive hematopoietic system." Blood **129**(15): 2061-2069.

De Kouchkovsky, I. and M. Abdul-Hay (2016). "'Acute myeloid leukemia: a comprehensive review and 2016 update'." Blood Cancer J **6**(7): e441.

de la Rica, L., J. Rodriguez-Ubreva, M. Garcia, et al. (2013). "PU.1 target genes undergo Tet2-coupled demethylation and DNMT3b-mediated methylation in monocyte-to-osteoclast differentiation." Genome Biol **14**(9): R99.

Deneberg, S., H. Cherif, V. Lazarevic, et al. (2016). "An open-label phase I dose-finding study of APR-246 in hematological malignancies." Blood Cancer J **6**(7): e447.

Dhakshinamoorthy, S. and A. K. Jaiswal (2001). "Functional characterization and role of INrf2 in antioxidant response element-mediated expression and antioxidant induction of NAD(P)H:quinone oxidoreductase1 gene." Oncogene **20**(29): 3906-3917.

Dohner, H., E. Estey, D. Grimwade, et al. (2017). "Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel." Blood **129**(4): 424-447.

Dohner, H., D. J. Weisdorf and C. D. Bloomfield (2015). "Acute Myeloid Leukemia." N Engl J Med **373**(12): 1136-1152.

Dohner, K., R. F. Schlenk, M. Habdank, et al. (2005). "Mutant nucleophosmin (NPM1) predicts favorable prognosis in younger adults with acute myeloid leukemia and normal cytogenetics: interaction with other gene mutations." Blood **106**(12): 3740-3746.

Dolatshad, H., A. Pellagatti, M. Fernandez-Mercado, et al. (2015). "Disruption of SF3B1 results in deregulated expression and splicing of key genes and pathways in myelodysplastic syndrome hematopoietic stem and progenitor cells." Leukemia **29**(8): 1798.

Donaldson-Collier, M. C., S. Sungalee, M. Zufferey, et al. (2019). "EZH2 oncogenic mutations drive epigenetic, transcriptional, and structural changes within chromatin domains." Nat Genet **51**(3): 517-528.

Dowen, J. M., Z. P. Fan, D. Hnisz, et al. (2014). "Control of cell identity genes occurs in insulated neighborhoods in mammalian chromosomes." Cell **159**(2): 374-387.

Dutta, P., F. F. Hoyer, L. S. Grigoryeva, et al. (2015). "Macrophages retain hematopoietic stem cells in the spleen via VCAM-1." J Exp Med **212**(4): 497-512.

Engel, N., A. G. West, G. Felsenfeld and M. S. Bartolomei (2004). "Antagonism between DNA hypermethylation and enhancer-blocking activity at the H19 DMD is uncovered by CpG mutations." Nat Genet **36**(8): 883-888.

Falini, B., N. Bolli, J. Shan, et al. (2006). "Both carboxy-terminus NES motif and mutated tryptophan(s) are crucial for aberrant nuclear export of nucleophosmin leukemic mutants in NPMc+ AML." Blood **107**(11): 4514-4523.

Falini, B., I. Gionfriddo, F. Cecchetti, et al. (2011). "Acute myeloid leukemia with mutated nucleophosmin (NPM1): any hope for a targeted therapy?" Blood Rev **25**(6): 247-254.

Falini, B., C. Mecucci, E. Tiacci, et al. (2005). “Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype.” N Engl J Med **352**(3): 254-266.

Fasan, A., C. Haferlach, T. Alpermann, et al. (2014). “The role of different genetic subtypes of CEBPA mutated AML.” Leukemia **28**(4): 794-803.

Feinberg, A. P. and B. Tycko (2004). “The history of cancer epigenetics.” Nat Rev Cancer **4**(2): 143-153.

Figueroa, M. E., O. Abdel-Wahab, C. Lu, et al. (2010). “Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation.” Cancer Cell **18**(6): 553-567.

Figueroa, M. E., S. Lugthart, Y. Li, et al. (2010). “DNA methylation signatures identify biologically distinct subtypes in acute myeloid leukemia.” Cancer Cell **17**(1): 13-27.

Fudenberg, G., M. Imakaev, C. Lu, et al. (2016). “Formation of Chromosomal Domains by Loop Extrusion.” Cell Rep **15**(9): 2038-2049.

Gaidzik, V. I., P. Paschka, D. Spath, et al. (2012). “TET2 mutations in acute myeloid leukemia (AML): results from a comprehensive genetic and clinical analysis of the AML study group.” J Clin Oncol **30**(12): 1350-1357.

Gaidzik, V. I., R. F. Schlenk, P. Paschka, et al. (2013). “Clinical impact of DNMT3A mutations in younger adult patients with acute myeloid leukemia: results of the AML Study Group (AMLSG).” Blood **121**(23): 4769-4777.

Gelsi-Boyer, V., M. Brecqueville, R. Devillier, et al. (2012). “Mutations in ASXL1 are associated with poor prognosis across the spectrum of malignant myeloid diseases.” J Hematol Oncol **5**: 12.

Georgopoulos, K., M. Bigby, J. H. Wang, et al. (1994). “The Ikaros gene is required for the development of all lymphoid lineages.” Cell **79**(1): 143-156.

Goll, M. G. and T. H. Bestor (2005). “Eukaryotic cytosine methyltransferases.” Annu Rev Biochem **74**: 481-514.

Greif, P. A., N. P. Konstandin, K. H. Metzeler, et al. (2012). “RUNX1 mutations in cytogenetically normal acute myeloid leukemia are associated with a poor prognosis and up-regulation of lymphoid genes.” Haematologica **97**(12): 1909-1915.

Griessinger, E., F. Anjos-Afonso, I. Pizzitola, et al. (2014). “A niche-like culture system allowing the maintenance of primary human acute myeloid leukemia-initiating cells: a new tool to decipher their chemoresistance and self-renewal mechanisms.” Stem Cells Transl Med **3**(4): 520-529.

Grisendi, S., C. Mecucci, B. Falini and P. P. Pandolfi (2006). “Nucleophosmin and cancer.” Nat Rev Cancer **6**(7): 493-505.

Halfon, S., N. Abramov, B. Grinblat and I. Ginis (2011). “Markers distinguishing mesenchymal stem cells from fibroblasts are downregulated with passaging.” Stem Cells Dev **20**(1): 53-66.

Harrington, C. T., E. I. Lin, M. T. Olson and J. R. Eshleman (2013). “Fundamentals of pyrosequencing.” Arch Pathol Lab Med **137**(9): 1296-1303.

Hayatsu, H., M. Shiraishi and K. Negishi (2008). “Bisulfite modification for analysis of DNA methylation.” Curr Protoc Nucleic Acid Chem **Chapter 6**: Unit 6 10.

Hewish, D. R. and L. A. Burgoyne (1973). “Chromatin sub-structure. The digestion of chromatin DNA at regularly spaced sites by a nuclear deoxyribonuclease.” Biochem Biophys Res Commun **52**(2): 504-510.

Holz-Schietinger, C., D. M. Matje and N. O. Reich (2012). “Mutations in DNA methyltransferase (DNMT3A) observed in acute myeloid leukemia patients disrupt processive methylation.” J Biol Chem **287**(37): 30941-30951.

Horton, S. J. and B. J. P. Huntly (2012). “Recent advances in acute myeloid leukemia stem cell biology.” Haematologica **97**(7): 966-974.

Huang, J. C., S. K. Basu, X. Zhao, et al. (2015). “Mesenchymal stromal cells derived from acute myeloid leukemia bone marrow exhibit aberrant cytogenetics and cytokine elaboration.” Blood Cancer J **5**: e302.

Hyde, J. E. and I. O. Walker (1975). “A model for chromatin sub-structure incorporating symmetry considerations of histone oligomers.” Nucleic Acids Res **2**(3): 405-421.

Ito, S., A. C. D’Alessio, O. V. Taranova, et al. (2010). “Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification.” Nature **466**(7310): 1129-1133.

Ke, Y. Y., V. K. Singh, M. S. Coumar, et al. (2015). “Homology modeling of DFG-in FMS-like tyrosine kinase 3 (FLT3) and structure-based virtual screening for inhibitor identification.” Sci Rep **5**: 11702.

Kemp, C. J., J. M. Moore, R. Moser, et al. (2014). “CTCF haploinsufficiency destabilizes DNA methylation and predisposes to cancer.” Cell Rep **7**(4): 1020-1029.

Kfoury, Y. and D. T. Scadden (2015). “Mesenchymal cell contributions to the stem cell niche.” Cell Stem Cell **16**(3): 239-253.

Kim, T. G., S. Kim, S. Jung, et al. (2017). “CCCTC-binding factor is essential to the maintenance and quiescence of hematopoietic stem cells in mice.” Exp Mol Med **49**(8): e371.

Kim, Y., D. W. Jekarl, J. Kim, et al. (2015). “Genetic and epigenetic alterations of bone marrow stromal cells in myelodysplastic syndrome and acute myeloid leukemia patients.” Stem Cell Res **14**(2): 177-184.

Klepin, H. D. (2014). "Geriatric perspective: how to assess fitness for chemotherapy in acute myeloid leukemia." Hematology Am Soc Hematol Educ Program **2014**(1): 8-13.

Kobayashi, N., M. Abedini, N. Sakuragi and B. K. Tsang (2013). "PRIMA-1 increases cisplatin sensitivity in chemoresistant ovarian cancer cells with p53 mutation: a requirement for Akt down-regulation." J Ovarian Res **6**: 7.

Kohlmann, A., N. Nadarajah, T. Alpermann, et al. (2014). "Monitoring of residual disease by next-generation deep-sequencing of RUNX1 mutations can identify acute myeloid leukemia patients with resistant disease." Leukemia **28**(1): 129-137.

Kojima, K., M. Konopleva, I. J. Samudio, et al. (2005). "MDM2 antagonists induce p53-dependent apoptosis in AML: implications for leukemia therapy." Blood **106**(9): 3150-3159.

Ley, T. J. (2013). "Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia." N Engl J Med **368**(22): 2059-2074.

Ley, T. J., L. Ding, M. J. Walter, et al. (2010). "DNMT3A mutations in acute myeloid leukemia." N Engl J Med **363**(25): 2424-2433.

Ley, T. J., E. R. Mardis, L. Ding, et al. (2008). "DNA sequencing of a cytogenetically normal acute myeloid leukaemia genome." Nature **456**(7218): 66-72.

Li, Z., P. Zhang, A. Yan, et al. (2017). "ASXL1 interacts with the cohesin complex to maintain chromatid separation and gene expression for normal hematopoiesis." Sci Adv **3**(1): e1601602.

Lindsley, R. C., B. G. Mar, E. Mazzola, et al. (2015). "Acute myeloid leukemia ontogeny is defined by distinct somatic mutations." Blood **125**(9): 1367-1376.

Liu, W., L. Deng, Y. Song and M. Redell (2014). "DOT1L inhibition sensitizes MLL-rearranged AML to chemotherapy." PLoS One **9**(5): e98270.

Liu, X. S., H. Wu, X. Ji, et al. (2016). "Editing DNA Methylation in the Mammalian Genome." Cell **167**(1): 233-247 e217.

Loizou, E., A. Banito, G. Livshits, et al. (2019). "A Gain-of-Function p53-Mutant Oncogene Promotes Cell Fate Plasticity and Myeloid Leukemia through the Pluripotency Factor FOXH1." Cancer Discov **9**(7): 962-979.

Longo, D. L., H. Döhner, D. J. Weisdorf and C. D. Bloomfield (2015). "Acute Myeloid Leukemia." New England Journal of Medicine **373**(12): 1136-1152.

Losada, A. (2014). "Cohesin in cancer: chromosome segregation and beyond." Nat Rev Cancer **14**(6): 389-393.

Lu, J. W., M. S. Hsieh, H. A. Hou, et al. (2017). "Overexpression of SOX4 correlates with poor prognosis of acute myeloid leukemia and is leukemogenic in zebrafish." Blood Cancer J **7**(8): e593.

Lu, R., P. Wang, T. Parton, et al. (2016). "Epigenetic Perturbations by Arg882-Mutated DNMT3A Potentiate Aberrant Stem Cell Gene-Expression Program and Acute Leukemia Development." Cancer Cell **30**(1): 92-107.

Lu, Y., G. Shan, J. Xue, et al. (2016). "Defining the multivalent functions of CTCF from chromatin state and three-dimensional chromatin interactions." Nucleic Acids Res **44**(13): 6200-6212.

Luo, H., F. Wang, J. Zha, et al. (2018). "CTCF boundary remodels chromatin domain and drives aberrant HOX gene transcription in acute myeloid leukemia." Blood **132**(8): 837-848.

Ma, O., S. Hong, H. Guo, et al. (2014). "Granulopoiesis requires increased C/EBPalpha compared to monopoiesis, correlated with elevated Cebpa in immature G-CSF receptor versus M-CSF receptor expressing cells." PLoS One **9**(4): e95784.

Mandavilli, B. S. and M. S. Janes (2010). "Detection of intracellular glutathione using ThiolTracker violet stain and fluorescence microscopy." Curr Protoc Cytom **Chapter 9**: Unit 9 35.

Mangan, J. K. and N. A. Speck (2011). "RUNX1 mutations in clonal myeloid disorders: from conventional cytogenetics to next generation sequencing, a story 40 years in the making." Crit Rev Oncog **16**(1-2): 77-91.

Marina, R. J., D. Sturgill, M. A. Bailly, et al. (2016). "TET-catalyzed oxidation of intragenic 5-methylcytosine regulates CTCF-dependent alternative splicing." EMBO J **35**(3): 335-355.

Matsumoto, N., A. Kubo, H. Liu, et al. (2006). "Developmental regulation of yolk sac hematopoiesis by Kruppel-like factor 6." Blood **107**(4): 1357-1365.

Maurano, M. T., H. Wang, S. John, et al. (2015). "Role of DNA Methylation in Modulating Transcription Factor Occupancy." Cell Rep **12**(7): 1184-1195.

Merkenschlager, M. and D. T. Odom (2013). "CTCF and cohesin: linking gene regulatory elements with their targets." Cell **152**(6): 1285-1297.

Metzeler, K. H., H. Becker, K. Maharry, et al. (2011). "ASXL1 mutations identify a high-risk subgroup of older patients with primary cytogenetically normal AML within the ELN Favorable genetic category." Blood **118**(26): 6920-6929.

Mill, J., S. Yazdanpanah, E. Guckel, et al. (2006). "Whole genome amplification of sodium bisulfite-treated DNA allows the accurate estimate of methylated cytosine density in limited DNA resources." Biotechniques **41**(5): 603-607.

Nutt, S. L. and B. L. Kee (2007). "The transcriptional regulation of B cell lineage commitment." Immunity **26**(6): 715-725.

Papaemmanuil, E., M. Gerstung, L. Bullinger, et al. (2016). "Genomic Classification and Prognosis in Acute Myeloid Leukemia." New England Journal of Medicine **374**(23): 2209-2221.

Pidsley, R., E. Zotenko, T. J. Peters, et al. (2016). "Critical evaluation of the Illumina MethylationEPIC BeadChip microarray for whole-genome DNA methylation profiling." Genome Biol **17**(1): 208.

Pinho, S. and P. S. Frenette (2019). "Haematopoietic stem cell activity and interactions with the niche." Nat Rev Mol Cell Biol **20**(5): 303-320.

Popat, U., M. J. de Lima, R. M. Saliba, et al. (2012). "Long-term outcome of reduced-intensity allogeneic hematopoietic SCT in patients with AML in CR." Bone Marrow Transplant **47**(2): 212-216.

Qu, Y., A. Lennartsson, V. I. Gaidzik, et al. (2014). "Differential methylation in CN-AML preferentially targets non-CGI regions and is dictated by DNMT3A mutational status and associated with predominant hypomethylation of HOX genes." Epigenetics **9**(8): 1108-1119.

Quintas-Cardama, A., F. Ravandi, T. Liu-Dumlao, et al. (2012). "Epigenetic therapy is associated with similar survival compared with intensive chemotherapy in older patients with newly diagnosed acute myeloid leukemia." Blood **120**(24): 4840-4845.

Raaijmakers, M. H., S. Mukherjee, S. Guo, et al. (2010). "Bone progenitor dysfunction induces myelodysplasia and secondary leukaemia." Nature **464**(7290): 852-857.

Rasmussen, K. D., G. Jia, J. V. Johansen, et al. (2015). "Loss of TET2 in hematopoietic cells leads to DNA hypermethylation of active enhancers and induction of leukemogenesis." Genes Dev **29**(9): 910-922.

Rhodes, J. D. P., A. Feldmann, B. Hernandez-Rodriguez, et al. (2020). "Cohesin Disrupts Polycomb-Dependent Chromosome Interactions in Embryonic Stem Cells." Cell Rep **30**(3): 820-835 e810.

Rickman, D. S., T. D. Soong, B. Moss, et al. (2012). "Oncogene-mediated alterations in chromatin conformation." Proc Natl Acad Sci U S A **109**(23): 9083-9088.

Rucker, F. G., R. F. Schlenk, L. Bullinger, et al. (2012). "TP53 alterations in acute myeloid leukemia with complex karyotype correlate with specific copy number alterations, monosomal karyotype, and dismal outcome." Blood **119**(9): 2114-2121.

Russler-Germain, D. A., D. H. Spencer, M. A. Young, et al. (2014). "The R882H DNMT3A mutation associated with AML dominantly inhibits wild-type DNMT3A by blocking its ability to form active tetramers." Cancer Cell **25**(4): 442-454.

Sasaki, M., C. B. Knobbe, J. C. Munger, et al. (2012). "IDH1(R132H) mutation increases murine haematopoietic progenitors and alters epigenetics." Nature **488**(7413): 656-659.

Schnittger, S., U. Bacher, W. Kern, et al. (2011). "Prognostic impact of FLT3-ITD load in NPM1 mutated acute myeloid leukemia." Leukemia **25**(8): 1297-1304.

Schoenherr, C. J., J. M. Levorse and S. M. Tilghman (2003). "CTCF maintains differential methylation at the Igf2/H19 locus." Nat Genet **33**(1): 66-69.

Schubeler, D., D. M. MacAlpine, D. Scalzo, et al. (2004). "The histone modification pattern of active genes revealed through genome-wide chromatin analysis of a higher eukaryote." Genes Dev **18**(11): 1263-1271.

Schuettpelz, L. G., P. K. Gopalan, F. O. Giuste, et al. (2012). "Kruppel-like factor 7 overexpression suppresses hematopoietic stem and progenitor cell function." Blood **120**(15): 2981-2989.

Shao, D., Y. Lin, J. Liu, et al. (2016). "A targeted next-generation sequencing method for identifying clinically relevant mutation profiles in lung adenocarcinoma." Sci Rep **6**: 22338.

Shih, A. H., Y. Jiang, C. Meydan, et al. (2015). "Mutational cooperativity linked to combinatorial epigenetic gain of function in acute myeloid leukemia." Cancer Cell **27**(4): 502-515.

Shlush, L. I., S. Zandi, A. Mitchell, et al. (2014). "Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia." Nature **506**(7488): 328-333.

Simon, J. A. and C. A. Lange (2008). "Roles of the EZH2 histone methyltransferase in cancer epigenetics." Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis **647**(1-2): 21-29.

Smith, A. E., A. M. Mohamedali, A. Kulasekararaj, et al. (2010). "Next-generation sequencing of the TET2 gene in 355 MDS and CMML patients reveals low-abundance mutant clones with early origins, but indicates no definite prognostic value." Blood **116**(19): 3923-3932.

Splinter, E., H. Heath, J. Kooren, et al. (2006). "CTCF mediates long-range chromatin looping and local histone modification in the beta-globin locus." Genes Dev **20**(17): 2349-2354.

Stein, E. M., C. D. DiNardo, A. T. Fathi, et al. (2019). "Molecular remission and response patterns in patients with mutant-IDH2 acute myeloid leukemia treated with enasidenib." Blood **133**(7): 676-687.

Stein, E. M., C. D. DiNardo, D. A. Pollyea, et al. (2017). "Enasidenib in mutant IDH2 relapsed or refractory acute myeloid leukemia." Blood **130**(6): 722-731.

Stirewalt, D. L. and J. P. Radich (2003). "The role of FLT3 in haematopoietic malignancies." Nature Reviews Cancer **3**(9): 650-665.

Sugiyama, T., H. Kohara, M. Noda and T. Nagasawa (2006). "Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches." Immunity **25**(6): 977-988.

Suzuki, M., R. Shimizu and M. Yamamoto (2011). "Transcriptional regulation by GATA1 and GATA2 during erythropoiesis." Int J Hematol **93**(2): 150-155.

Tao, H., X. Ma, G. Su, et al. (2016). "BCL11A expression in acute myeloid leukemia." Leuk Res **41**: 71-75.

Tefferi, A., K. H. Lim, O. Abdel-Wahab, et al. (2009). "Detection of mutant TET2 in myeloid malignancies other than myeloproliferative neoplasms: CMML, MDS, MDS/MPN and AML." Leukemia **23**(7): 1343-1345.

Tikhonova, A. N., I. Dolgalev, H. Hu, et al. (2019). "The bone marrow microenvironment at single-cell resolution." Nature **569**(7755): 222-228.

Ting, C. N., M. C. Olson, K. P. Barton and J. M. Leiden (1996). "Transcription factor GATA-3 is required for development of the T-cell lineage." Nature **384**(6608): 474-478.

Torrano, V., I. Chernukhin, F. Docquier, et al. (2005). "CTCF regulates growth and erythroid differentiation of human myeloid leukemia cells." J Biol Chem **280**(30): 28152-28161.

Walkley, C. R., G. H. Olsen, S. Dworkin, et al. (2007). "A microenvironment-induced myeloproliferative syndrome caused by retinoic acid receptor gamma deficiency." Cell **129**(6): 1097-1110.

Walter, M. J., D. Shen, L. Ding, et al. (2012). "Clonal architecture of secondary acute myeloid leukemia." N Engl J Med **366**(12): 1090-1098.

Wang, A. J., Y. Han, N. Jia, et al. (2019). "NPM1c impedes CTCF functions through cytoplasmic mislocalization in acute myeloid leukemia." Leukemia.

Wang, H., M. T. Maurano, H. Qu, et al. (2012). "Widespread plasticity in CTCF occupancy linked to DNA methylation." Genome Res **22**(9): 1680-1688.

Wang, R., X. Gao and L. Yu (2019). "The prognostic impact of tet oncogene family member 2 mutations in patients with acute myeloid leukemia: a systematic-review and meta-analysis." BMC Cancer **19**(1): 389.

Ward, P. S., J. Patel, D. R. Wise, et al. (2010). "The common feature of leukemia-associated IDH1 and IDH2 mutations is a neomorphic enzyme activity converting alpha-ketoglutarate to 2-hydroxyglutarate." Cancer Cell **17**(3): 225-234.

Wattel, E., C. Preudhomme, B. Hecquet, et al. (1994). “p53 mutations are associated with resistance to chemotherapy and short survival in hematologic malignancies.” Blood **84**(9): 3148-3157.

Wei, L., Q. Liu, Y. Huang, et al. (2020). “Knockdown of CTCF reduces the binding of EZH2 and affects the methylation of the SOCS3 promoter in hepatocellular carcinoma.” Int J Biochem Cell Biol **120**: 105685.

Weisberg, E., J. Roesel, P. Furet, et al. (2010). “Antileukemic Effects of Novel First- and Second-Generation FLT3 Inhibitors: Structure-Affinity Comparison.” Genes Cancer **1**(10): 1021-1032.

Weissman, I. L. (2000). “Stem cells: units of development, units of regeneration, and units in evolution.” Cell **100**(1): 157-168.

Welch, John S., Timothy J. Ley, Daniel C. Link, et al. (2012). “The Origin and Evolution of Mutations in Acute Myeloid Leukemia.” Cell **150**(2): 264-278.

Wendt, K. S., K. Yoshida, T. Itoh, et al. (2008). “Cohesin mediates transcriptional insulation by CCCTC-binding factor.” Nature **451**(7180): 796-801.

Wiehle, L., G. J. Thorn, G. Raddatz, et al. (2019). “DNA (de)methylation in embryonic stem cells controls CTCF-dependent chromatin boundaries.” Genome Res **29**(5): 750-761.

Yang, H., D. Ye, K. L. Guan and Y. Xiong (2012). “IDH1 and IDH2 mutations in tumorigenesis: mechanistic insights and clinical perspectives.” Clin Cancer Res **18**(20): 5562-5571.

Yen, K., J. Travins, F. Wang, et al. (2017). “AG-221, a First-in-Class Therapy Targeting Acute Myeloid Leukemia Harboring Oncogenic IDH2 Mutations.” Cancer Discov **7**(5): 478-493.

Zhang, H., B. Niu, J. F. Hu, et al. (2011). “Interruption of intrachromosomal looping by CCCTC binding factor decoy proteins abrogates genomic imprinting of human insulin-like growth factor II.” J Cell Biol **193**(3): 475-487.

Zhao, M., J. M. Perry, H. Marshall, et al. (2014). “Megakaryocytes maintain homeostatic quiescence and promote post-injury regeneration of hematopoietic stem cells.” Nat Med **20**(11): 1321-1326.

Zuin, J., J. R. Dixon, M. I. van der Reijden, et al. (2014). “Cohesin and CTCF differentially affect chromatin architecture and gene expression in human cells.” Proc Natl Acad Sci U S A **111**(3): 996-1001.