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Leukaemia: Genetic or Epigenetic Disorder?

A Clarion Call for the Revision of the Two-Hit Hypothesis in Myeloid Leukaemogenesis

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Introduction

Over the past decades cancer research profoundly improved our understanding of tumour biology, which allowed for the development of efficient therapeutic strategies in several malignancies, thereby improving prognoses (Masters *et al.*, 2015). However, particularly in the elderly, long-term disease free survival in leukaemia still remains a problematic hurdle. Leukaemic cells prove to be very resistant to conventional chemotherapy that is (Stone, 2002; De Kouchkovsky & Abdul-Hay, 2016), and the majority of patients showing complete remission after revised intensive chemotherapeutic regimens eventually relapse despite receiving post-remission therapy (Kell, 2016). Yet, novel insights strongly convey the impression that a solution might be near at hand.

Leukaemia is defined as a "cancer that starts in blood-forming tissue such as the bone marrow and causes large numbers of blood cells to be produced and enter the bloodstream" (PubMed Health, n.d.). It represents the second most common blood cancer after lymphoma and includes several malignancies of which the four major syndromes comprise acute lymphocytic leukaemia (ALL), chronic lymphocytic leukaemia (CLL), acute myelogenous leukaemia (AML), and chronic myelogenous leukaemia (CML) (NIH, 2014). Accumulating evidence suggests leukaemia is caused by a combination of somatic mutations and epigenetic dysregulation rather than mere genetic events: a large number of mutations occur in genes which encode epigenetic regulators (Fathi & Abdel-Wahab, 2012; Sashida & Iwama, 2012; Shih *et al.*, 2012; TCGA, 2013). Disruption of these regulatory proteins – either through gain-of-function or loss-of-function mutations – clearly preludes epigenetic malfunction. A rationale for studying the epigenetic alterations in leukaemia lies in the very nature of epigenetic modifications. In contrast to somatic mutations that, for instance, irreversibly alter signal transduction pathways (Cooper, 1982), epigenetic modifications are theoretically reversible and thus hold the potential to be targeted therapeutically (Shih *et al.*, 2012).

The present thesis aims to investigate whether leukaemia truly is more than a mere genetic disorder, and if so, how this can potentially be put to use in the development of novel therapeutic strategies. Since mutations in epigenetic modifiers are most widely studied upon in myeloid malignancies, the current thesis will principally focus on AML[1] - a disease most often occurring in the older adult. The primary section provides an overview of the concept of epigenetics and how it contributes to the regulation of haematopoiesis in physiological context. Subsequently, by discussing two distinct recurrently mutated loci in AML, the way in which epigenetic dysregulation gives rise to the onset and maintenance of leukaemia is addressed in the second part. Exciting advances in the therapeutic strategies regarding the former genetic lesions will be reviewed in the third section. The final paragraph will provide some future perspectives.

Physiological haematopoiesis

The human blood comprises an enormous amount of blood cells. At least 14 different mature blood cell types exist in the normal adult, each type serving its own unique function (Sive & Göttgens, 2014) – for instance providing resistance to infections and toxins or transporting oxygen throughout the body. Since blood cells display remarkably high turnover rates (Orkin & Zon, 2008; Milo & Philips, n.d.), the body constantly needs to replenish the pool of circulating blood cells, such that the physiological requirements of the organism are met. Every hour an astonishing $1x10^9$ erythrocytes and $1x10^8$ leukocytes are generated from a single primary source, i.e. the haematopoietic stem cell (HSC), through a process called haematopoiesis (Wang & Wagers, 2014).

Haematopoiesis takes primarily place in the bone marrow (BM) where HSCs sit atop the apex of the complex hierarchical network of the haematopoietic system (**figure 1**) – 'hierarchical'

[1] **AML** is a clonal haematopoietic disorder characterised by the accumulation of immature myeloid cells coinciding with impeded normal haematopoiesis.

here implying that the potential to generate different cell types is progressively restricted with each branch point (Cedar & Bergman, 2011; Wang & Wagers, 2014). Two different types of HSCs reside in the BM: long-term HSCs (LT-HSCs) and short-term HSCs (ST-HSCs). LT-HSCs can either differentiate into ST-HSCs as an intermediate step or directly give rise to multipotent progenitors (MPPs), which form the initial step in haematopoiesis – the LT-HSC to ST-HSC transition not shown here. LT-HSCs are characterised by the ability to give rise to both lymphoid

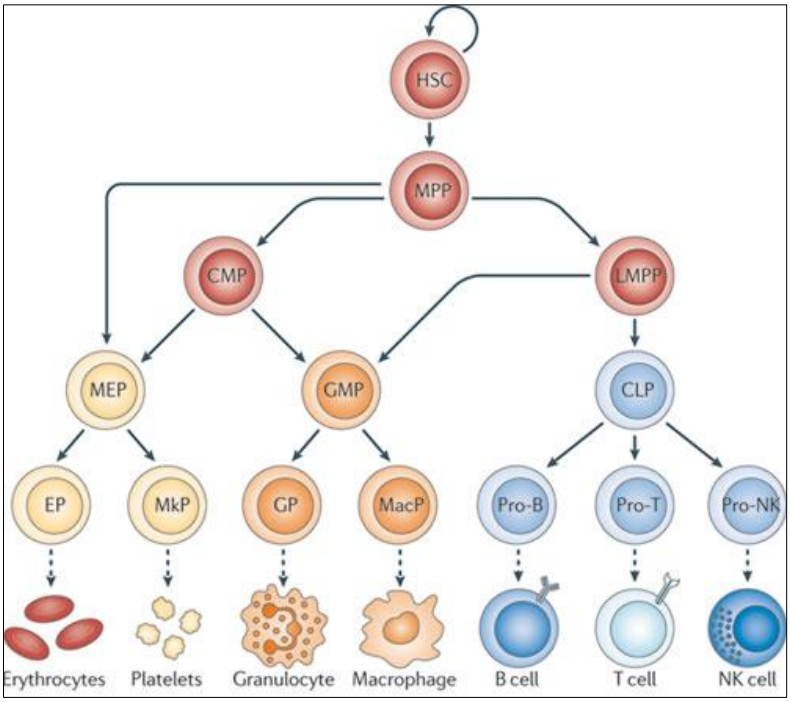


Figure 1 Hierarchy of differentiation in the haematopoietic system arising from a single primary source. The process of differentiation ultimately gives rise to all effector cells of the blood system, all of which are form a single progenitor i.e. the haematopoietic stem cell. Characteristically, the potential to generate different cell types is progressively restricted with each branch point. The differentiation of a committed progenitor to a mature effector cell is a multistage process, which is represented here as a dashed arrow. HSC, haematopoietic stem cell; MPP, multipotent progenitor; CMP, common myeloid progenitor; LMPP, lymphoid-primed multipotent progenitor; MEP, megakaryocyte-erythrocyte progenitor; GMP, granulocyte-macrophage progenitor; CLP, common lymphoid progenitor; EP, erythrocyte progenitor; MkP, megakaryocyte progenitor; GP, granulocyte progenitor; MacP, macrophage progenitor. Image obtained from Cedar & Bergman (2011).

and myeloerythroid lineages for life after transplantation into lethally irradiated recipients, whereas ST-HSCs have relatively limited self-renewal capacity and can only sustain aforementioned lineages for 8-12 weeks (Christensen & Weissman, 2001). Once MPPs are formed, the ability to undergo self-renewal is lost, although these cells still hold the potential to differentiate into every functional blood cell type. MPPs constitute the first branch point, wherein they can differentiate into either lymphoid-primed multipotent progenitors (LMPPs) or common myeloid progenitors (CMPs) – direct differentiation of MPPs into megakaryocyte-erythrocyte progenitors (MEPs) also constitutes a feasibility. The common lymphoid progenitors (CLPs), which stem from LMPPs, together with granulocyte-macrophage progenitors (GMPs) and megakaryocyte-erythrocyte progenitors (MEPs) form partially restricted progenitors responsible for the production of all lineage-committed effector cells of the blood (Kondo *et al.*, 1997; Akashi *et al.*, 2000; Cedar & Bergman, 2011).

The well-established concept that blood formation takes place in the BM stems from fundamental research in the 1950s. By making use of radiation-induced chromosomal markers, Ford *et al.* were first to demonstrate in mice that the BM contains cells capable of reconstituting the entire blood system of an irradiated recipient (1956). It remained unclear, however, whether it was a single stem cell that gave rise to all lineages or whether each lineage had its own specific stem cell. Answers came when Becker and colleagues proved the existence of "multipotent stem cells" in the spleen of irradiated mice that had been injected with BM (1961). To date, these putative multipotent stem cells are referred to as HSCs. Subsequent studies in animal models, such as the mouse and zebrafish, have greatly extended our knowledge of the complex haematopoietic network ever since.

Within this complex network, it is only the HSC that is capable of self-renewal – the production of additional HSCs to prevent stem cell pool exhaustion – and multi-lineage differentiation, indicating the ability to differentiate into all functional blood cell types (Orkin &

Zon, 2008; Cedar & Bergman, 2011; Manesso *et al.*, 2013; Nakamura-Ishizu *et al.*, 2014). Characteristically, HSCs divide very infrequently and are typically found in a quiescent state, which serves as a protective mechanism against oncogenic mutations (Wang & Dick, 2005; Van der Wath *et al.*, 2009) or becoming senescent once cells have reached their turnover maximum – often referred to as the 'Hayflick limit' (Hayflick & Moorhead, 1961; Nakamura-Ishizu *et al.*, 2014). The process of decision-making of HSCs as to whether or not exit quiescence and re-enter the cell cycle is driven by the interplay of external stimuli – both stochastic and deterministic – and intracellular regulators, including transcription factors (TFs), signal transduction pathways and niche factors (Sashida & Iwama, 2012; Nakamura-Ishizu *et al.*, 2014; Göttgens, 2015). Of note, such decisions have to be thoroughly kept in check, for it would well-poise HSCs to acquire a state of malignancy if dysregulated. Increasing evidence suggest that epigenetic regulators act in concert with these factors in that they are involved in decision-making, thus maintaining blood homeostasis (Álvarez-Errico *et al.*, 2015; Gore *et al.*, 2016). To understand how epigenetics are involved in the process of development and orchestrate choices of differentiation versus self-renewal in the HSC, it is essential to first obtain a clear overview of the principles of epigenetics and how epigenetic marks are established and maintained.

Principles of epigenetics

Prokaryotes and simples eukaryotes are characterised by relatively small genomes, which prove to be almost entirely and incessantly available to the transcriptional machinery (i.e. TFs, mediators and RNA polymerase). If genes are to be repressed, transcriptional-regulating factors are recruited and subsequently bind to highly specific sequences associated with the genes involved. Higher eukaryotes, however, often possess relatively large genomes in which as many as 50% of the genes can be transcriptionally silent or repressed in any particular cell type. Clearly, the mere binding of transcriptional-repressing factors to highly specific sequences would undoubtedly be insufficient. Ergo a different and more complex regulatory mechanism is required. Such a mechanism would be the heritable layer of 'epigenetic' information – a layer superimposed upon the DNA sequence which we have only just begun to read and appreciate (Bernstein *et al.*, 2007).

Epigenetics could well be defined as 'mechanisms that confer heritable, but potentially reversible, alterations in gene expression patterns, brought about via the modification of chromatin structure through covalent modifications of DNA and histones, though without actually changing the underlying DNA sequence' (Cedar & Bergman, 2011; Paluch *et al.*, 2016). Such modifications fall into two main categories: DNA methylation and histone modifications. Indeed, recent advances also indicate non-coding RNAs to be involved in epigenetic regulation (Bernstein & Allis, 2005; Sanchez-Elsner *et al.*, 2006; Rinn *et al.*, 2007).

The principle of epigenetics came into existence some 30 years ago when researchers demonstrated how gene regulation is dealt with in higher organisms. By making use of DNase I (Weintraub & Groudine, 1976) and RNA polymerases (Axel *et al.*, 1973), it was shown that the current enzymes were able to bind to actively transcribed genes, whereas the greater part of the genome remained untargeted. Herewith they proved that, although most of the DNA is packaged in a closed, tightly packed chromatin conformation, active genes are highly accessible to the transcription machinery. Chromatin structure is ever since thought to be important in regulating gene accessibility hence gene expression. Subsequent subsections will address the concepts of DNA methylation and histone modifications respectively, and illustrate how they contribute to remodelling of chromatin structure.

DNA methylation Genomic methylation serves as an epigenetic mark which is primarily established early in development and has a strong effect on decision-making during development (Cedar & Bergman, 2011). The process comprises enzymatic addition of a methyl group to the 5-carbon of cytosine, most often occurring at cytosine phospho-guanine dinucleotides (CpGs) in the genome (Gruenbaum *et al.*, 1981), after which a 5-methylcytosine (5mC) is formed. Interestingly, the presence of 5mC in DNA was first demonstrated in 1948, yet it took more than two decades until the concept of epigenetics came into existence (Hotchkiss,

1948). How are patterns of DNA methylation in the developing organism established and how are they propagated through cell division?
During early morula and blastula stages – which occur in the developing mammalian embryo following conception – DNA methylation patterns of both paternal and maternal genomes are eliminated and are later on re-established in a new bimodal methylation pattern upon implantation (Bernstein *et al.*, 2007; Cedar & Bergman, 2011; Paluch *et al.*, 2016). Enzymes that facilitate establishment and maintenance of genomic DNA methylation comprise DNA methyltransferases (DNMTs), including the *de novo* methyltransferases DNMT3A and DNMT3B, and the maintenance methyltransferase DNMT1 (Trowbridge *et al.*, 2009). These enzymes are thought to methylate the entire genome except for CpG islands[2], which are protected from methylation (Brandeis *et al.*, 1994). Before the G1-S transition of the cell cycle, the CpG sites of both DNA strands are methylated. During S-phase, DNA replication gives rise to hemi-methylated CpG dinucleotides in the newly-formed complementary strand, which are specifically recognised by DNMT1 (**figure 2**). The enzyme then methylates the second strand, whereas unmethylated CpG sites remain unmethylated (not shown). Thus, the original DNA methylation pattern is propagated through cell division (Bird, 2002; Cedar & Bergman, 2011) – hence explaining the terminology 'maintenance' in DNMT1 functioning.

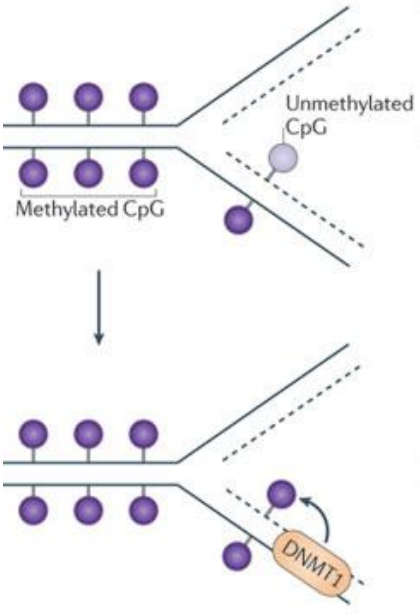


Figure 2 Mechanisms by which epigenetic methylation patterns are preserved through cell division. Before DNA replication occurs, the CpG sites of both DNA stands are methylated. During S-phase, the process of DNA replication gives rise to hemi-methylated CpG dinucleotides in the newly-formed complementary strand, which are specifically recognised by DNMT1. Subsequently, DNMT1 methylates the second DNA strand, whereas unmethylated CpG sites are left unmethylated (not shown). Thus, the original DNA methylation pattern is propagated through cell division. CpG, cytosine phospho-guanine; DNMT1, DNA methyltransferase-1. Image obtained from Cedar & Bergman (2011).

The effect DNA methylation elicits is readily complex to predict in that it can either promote or repress recruitment of regulatory proteins. Indeed, the precluding function of 5mC marks is best understood; 5mCs are recognised by a distinct family of methyl-CpG-binding proteins, which are thought to mediate transcriptional repression through interactions with histone deacetylases (Bird, 2002; Bernstein *et al.*, 2007). Moreover, cytosine methylation is also thought to repress transcription via the exclusion of DNA-binding proteins – which are to initiate transcription – from their target sites (Hark *et al.*, 2000). In addition, the presence of 5mC marks is associated with an altered chromatin structure, a tightly packed chromatin that is, which is considered to preclude gene transcription. For CpG islands do not contain 5mC marks, they automatically adopt an open conformation which is accessible to the transcriptional machinery, whereas many tissue specific genes become repressed due to global packaging into a closed chromatin structure (Keshet *et al.*, 1986; Cedar & Bergman, 2011). As cells become increasingly

[2] **CpG islands** are DNA sequences of 0.5-2 kilobases rich in CpG dinucleotides, which are often found upstream of housekeeping genes and some tissue-specific genes. They usually constitute unmethylated regions (Cedar & Bergman, 2011).

differentiated, overall methylation states change, resulting in the methylation of tissue-specific genes, indicating the transcriptional machinery is sterically hindered at particular loci (Bird, 2002; Cedar & Bergman, 2011). Taken together, DNA methylation represents a stable epigenetic mark that is crucial in the developmental decision-making process. Long-term lineage stability is propagated through every cell division via DNMT1 activity.

Histone modifications The entire two-meter-long DNA is packaged within the nucleus around histones into a repeating nucleosome pattern, sometimes referred to as 'beats on a sting'. Nucleosomes constitute the fundamental unit of chromatin and are composed of octamers of the four core histones – H2A, H2B, H3, H4 – around which approximately 147 DNA base pairs are wrapped (Kouzarides, 2007; Sashida & Iwama, 2012; Paluch *et al.*, 2016). Core histone proteins are chiefly globular except for their N-terminal domains or 'tails' (Kouzarides, 2007; Alberts *et al.*, 2008). Nucleosomes are thought to encompass two major functions: i. providing a means for the compaction of the basic DNA within the nucleus and ii. to alter gene expression via modulating gene structure and accessibility through nucleosome positioning on DNA and through modifications of histone tails (Cedar & Bergman, 2011). The following section will primarily focus upon modifications of N-terminal histone tails, in particular methylation and ubiquitination, as these are thought to contribute significantly to the induction and maintenance of leukaemia if dysregulated.

More than 60 different residues of the N-terminal portion of histone core proteins are thought to be subjected to post-translational modifications (PTMs) (Kouzarides, 2007), collectively referred to as 'histone modifications' (**figure 3**). At least eight distinct types of PTMs have been demonstrated thus far, four of which are shown (**table 1**). The current modifications are brought about by enzymes that: establish chemical groups ('writers'), interpret certain modifications ('readers') and eliminate chemical groups ('erasers') – and protein factors that recognise specific DNA sequence motifs facilitate their recruitment (Cedar & Bergman, 2011; Paluch *et al.*, 2016). These histone-modifying enzymes regulate indirectly, by means of histone modifications, transcription by influencing the degree to which chromatin (hence gene promoters) is accessible to the transcriptional machinery; PTMs promote transcription through induction of an open chromatin conformation, thus providing access to the transcriptional machinery, or suppress transcription by functioning as platforms for reader proteins to facilitate condensation of chromatin (Schones & Zhao, 2008; Paluch *et al.*, 2016).

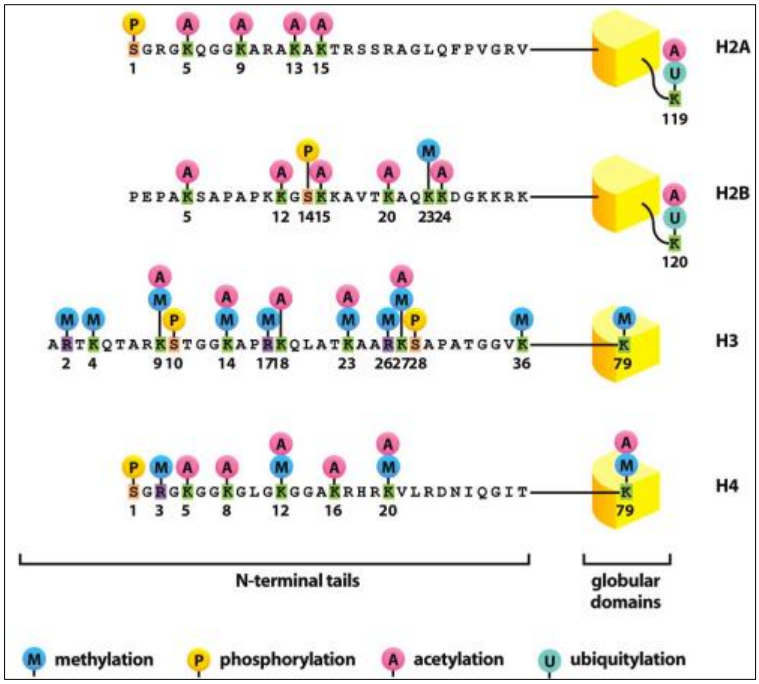
Profound progress has been made in our understanding of lysine acetylation and methylation: lysine acetylation is almost always correlated to an open chromatin configuration and transcriptional activity, whereas lysine methylation elicits different effects which are depending upon the specific residue being modified. Thus, some modifications are involved in transcriptional activation, whereas others prevent genes from being transcribed (**figure 4**) (Campos & Reinberg, 2009; Cedar & Bergman, 2011).

Table 1 Four different classes of modifications on histones

Histone modifications	Residues modified	Functions regulated	Enzymes involved ('writers')
Acetylation	**K**-Ac	Transcription, repair, replication, condensation	Histone acetyltransferases
Methylation	**K**-Me1, **K**-Me2, **K**-Me3 **R**-me1, **R**-Me2	Transcription, repair Transcription	Histone methyltransferases
Phosphorylation	**S**-P, **T**-P	Transcription, repair, condensation	Serine/threonine kinases
Ubiquitination	**K**-Ub	Transcription, repair	Ubiquitilases

Above-mentioned classes of histone modifications are major players in the onset and maintenance of leukaemia if dysregulated. The residues associated with a particular modification and the functions they have are shown. Note that lysines can undergo mono-, di-, or tri-methylation, whereas arginines are only found mono-, or di-methylated. Amino acid one-letter abbreviations: K, lysine; R, arginine; S, serine; T, threonine. Information obtained from Kouzarides (2007).

Modifications that are thought to promote transcription are collectively known as euchromatin modifications, including: acetylation of histone 3 and histone 4 (H3 and H4), or mono- (Me1), di- (Me2) or tri-methylation (Me3) of H3K4, and H3K79. Modifications that are associated with transcriptional repression are commonly referred to as heterochromatin modifications, comprising: mono-ubiquitination of H2AK119 as well as H3K9Me2, H3K9Me3, and H3K27Me3 (Kouzarides, 2007; Li *et al.*, 2007; Schones & Zhao, 2008). Importantly, some of the covalent modifications are mutually exclusive, for instance, H3K9 can be modified either by methylation or acetylation, though not simultaneously. The vast majority of histone modifications, however, remain to be elucidated. Indeed, major complexity arises from the fact that methylation at, for instance, lysines may be one of three different forms – mono-, di-, or tri-methyllysines may be found – of which the implications are poorly understood (Kouzarides, 2007). In conclusion, histone modifications comprise many PTMs that influence the direct interaction between DNA and histone octamers, thereby affecting gene expression. Some modifications induce transcriptional activity, whilst others are involved in gene silencing.



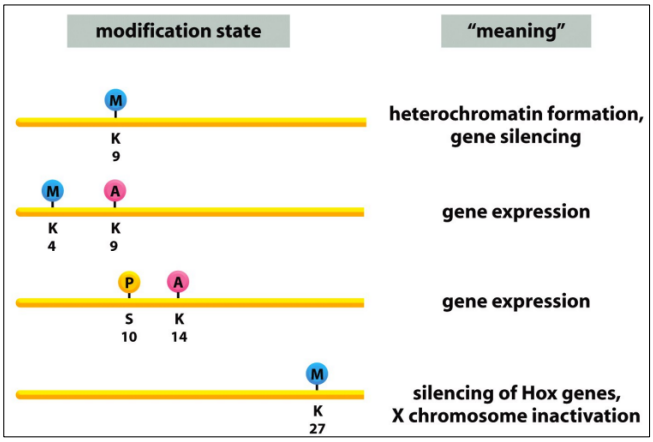


Figure 4 Some specific meanings of the histone modifications of histone tail-3. The N-terminal portion of H3 can be subjected to different combinations of modifications, which convey a specific meaning to the chromatin structure of particular loci involved. For instance, acetylation of H3 and H4 usually indicates actively transcribed chromatin, whereas H3K9 di-, or tri-methylation results in gene silencing. H3K9, lysine 9 of histone 3. Image obtained from Alberts *et al.* (2008).

Figure 3 The covalent modification of core histone N-terminal domains. Well-documented PTMs of the four core histones are shown. Importantly, methylation is indicated here as a single symbol, however, each lysine or arginine can be methylated in multiple ways. Also, note that some of the covalent modifications are mutually exclusive, e.g. H3K9 can be modified either by methylation or acetylation, though not simultaneously. H3K9, lysine 9 of H3. Image obtained from Alberts *et al.* (2008).

Epigenetic regulation of haematopoiesis

The molecular biology of cell lineage development in the complex process of haematopoiesis has been widely studied in the past, both by identifying the complete arsenal of cell surface markers at each stage of differentiation (Kiel *et al.*, 2005; Pronk *et al.*, 2007) and by executing transcriptional profiling for these distinct stages (Novershtern *et al.*, 2011). Indeed, the current studies provided striking insight into the very nature of these cells and the key TFs involved in the process of differentiation. Questions remained, however, of how developmental decisions in haematopoiesis or lineage commitment are mechanistically regulated. The answer lies in the epigenetic structure of the genome, as it forms the key player in regulating these decisions and commits cells to a specific lineage. The next paragraph will discuss the roles of DNA methylation status and histone modifications in the aforementioned features respectively.

DNA methylation status Ji *et al.* were first to actually show the role of DNA methylation in lineage determination in haematopoiesis downstream the HSC (2010). By means of genome-wide analysis of DNA methylation patterns using microarray technology, they uncovered some fascinating features of epigenetic modifications associated with developing blood cells. Many genes that were initially methylated in HSCs and MPPs were shown to undergo profound demethylation, which occurred in a lineage-specific manner. For instance, lymphoid-specific genes that underwent demethylation comprise both *Lck* and *Pou2af1* in T cells and B cells respectively, whilst *Mpo* and *Cxcr2* form myeloid-specific genes proved to undergo demethylation in GMPs (Borgel *et al.*, 2010; Ji *et al.*, 2010). Although the precise mechanisms by which these lineage-specific demethylation events occur are not completely understood, recent evidence demonstrates that the 5-methylcytosine dioxygenase TET enzymes could well be responsible for the demethylation of the former-mentioned genes (Cedar & Bergman, 2011) – owing to their ability to facilitate demethylation through a process that requires the base excision repair pathway as shown by Guo *et al.* (2011). Admittedly, these enzymes are often found mutated in AML, suggesting a potential role for these enzymes in maintaining physiological haematopoiesis and, in particular, myelopoiesis (discussed in the section 'aberrant haematopoiesis'). Importantly, removal of the 5-methyl mark on CpG alone does not fully explain the mechanism through which lineage determination is established.

As a matter of fact, some other subsets of lineage-specific genes are subjected to *de novo* methylation during the course of their development. For example, *Dach1* is considered as actively transcribed in MPPs and CMPs, in view of its packaging in an open chromatin structure. However, upon their transition to CLPs or double negative thymocytes, the latter gene becomes significantly methylated – thus facilitating differentiation towards myeloid lineage (Ji *et al.*, 2010). Moreover, genes involved in maintaining the feature of multipotency (e.g. *Meis1*) are found unmethylated in MPPs, though they become hypermethylated as differentiation progresses. Obtaining a status of hypermethylation is independent of myeloid or lymphoid cell fate. As indicated in the previous paragraph, DNA methylation constitutes a stable change in gene structure which is preserved through cell division (**figure 2**), therefore changes in DNA methylation status are thought to strengthen lineage determination (Bird, 2002; Cedar & Bergman, 2011).

In addition to its regulatory function in lineage commitment, and even prior to the microarray analysis in 2010, two research groups were independently able to assign a direct role for DNA methylation in the regulation of HSC self-renewal and its commitment to lymphoid versus myeloid cell fates (Bröske *et al.*, 2009; Trowbridge *et al.*, 2009). They created HSCs that contained a hypomorphic allele for *Dnmt1* resulting in low expression patterns of the former enzyme. Consequently, these cells demonstrated loss of self-renewal capacity and displayed aberrant differentiation, as they primarily developed into the myeloid direction. These results suggested premature demethylation of a specific set of genes, thereby gaining transcriptional activation, which pushed these cells into the myeloid direction. Following on from this, there might well be specific genes residing in the genome which either have to be demethylated in order to navigate into the myeloid direction or remain transcriptionally silent in the lymphoid lineage. Interestingly, Tadokoro *et al*. showed that *de novo* DNA methylation *per se* – a process facilitated by DNA methyltransferases 3A and 3B (DNMT3A/B) – is not necessary for HSC to adequately give rise to all blood cell lineages, for *Dnmt3a* and *Dnmt3b* knockout HSCs displayed normal differentiation patterns (2007). Importantly, *Dnmt3a* fulfils an important role in regulating the ability to switch from a self-renewal to a differentiation programme, as will be discussed in detail in the context of *DNMT3A* mutations in AML (see p. 12 'DNMT3A mutations'). In summary, DNA methylation status contributes in two distinct ways to lineage determination: a particular set of lineage-specific genes may be subjected to demethylation, whereas others undergo *de novo* methylation as the process of differentiation progresses. Moreover, genes required for multipotency become progressively hypermethylated as differentiation progresses towards either myeloid or lymphoid cell fates, thereby restricting the potential to generate different cell types.

Histone modifications

In addition to the well-defined role of DNA methylation in self-renewal and lineage commitment, accumulating evidence suggests that epigenetic regulators involved in the establishment of histone modifications also have critical roles in the maintenance of self-renewing HSCs (Iwama *et al.*, 2005; Cedar & Bergman, 2011; Sashida & Iwama, 2012). Among these epigenetic regulators, the polycomb group (PcG) repressive complexes are best characterised in HSCs (Bracken & Helin, 2009) and will therefore be the point of focus here.

Initially, Polycomb-group proteins were identified in *Drosophila* as regulators of body segmentation via the **repression** of putative homeotic genes (Lewis, 1978), and were subsequently identified in mammals as '*HOX*' genes wherein they fulfil an equally repressive function (Iwama *et al.*, 2005; Sashida & Iwama, 2012; van den Boom *et al.*, 2014). The mammalian genome contains 39 *HOX* TFs which are clustered on four chromosomal loci, termed *HOXA* through *HOXD*. The current TFs are crucial in the specification of the positional identities of cells (Rinn *et al.*, 2007). Interestingly, the temporal and spatial pattern in which *HOX* genes are to be expressed strongly correlates to their genomic location within each locus (Kmita & Duboule, 2003), and aberrant *HOX* expression patterns are often observed in myeloid leukemia (Abdel-Wahab *et al.*, 2012).

In general, PcG proteins constitute two major complexes: Polycomb respressive complex (PRC) 1 and 2 (**figure 5**) (Cedar & Bergman, 2011; Sashida & Iwama, 2012; van den Boom *et al.*, 2014). Polycomb repressive complex 2 is composed of the three core subunits: suppressor of zeste 12 (SUZ12), embryonic ectoderm development (EED), and enhancer of zeste homolog 2 (EZH2). It mediates – or rather **initiates** – transcriptional repression through tri-methylation of H3K27. Within this complex EZH2 shows methyltransferase activity, thus brings about the H3K27Me3 mark. Subsequently, canonical[3] PRC1 is recruited following recognition of H3K27Me3 by the chromodomain of the CBX core subunit of cPRC1 (Bernstein *et al.*, 2006; Sashida & Iwama, 2012). The RING1 subunit then mono-ubiquitinates H2A at lysine 119 (H2AK119Ub) via its ubiquitin ligase activity, which is to **maintain** transcriptional silencing (Iwama *et al.*, 2005). Indeed, multiple studies support the concept of H2AK119Ub-mediated transcriptional silencing in that it would inhibit RNA polymerase II-dependent transcriptional elongation (Zhou *et al.*, 2008) followed by local heterochromatin formation (Francis *et al.*, 2004; Pietersen & van Lohuizen, 2008).

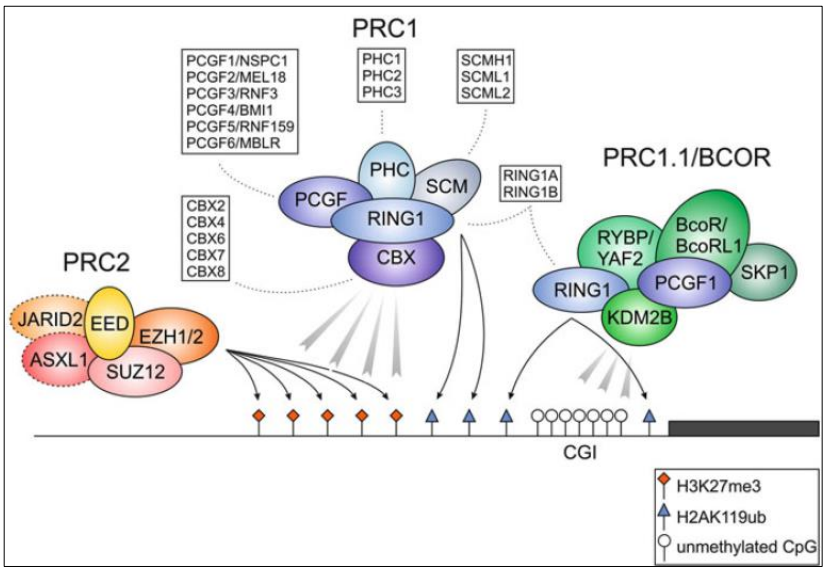


Figure 5 Graphical representation of PRC signalling. The PRC2 tri-methylates H3K27 conferred by its EZH2 subunit that specifically exhibits H3K27 methyltransferase activity. Subsequently, the PRC1 is recruited to chromatin due to the affinity of chromodomains in CBX proteins for H3K27 tri-methylation marks. Recruitment of PRC1 is followed by RING1-mediated mono-ubiquitination of H2AK119, which is thought to represent to entail transcriptional silencing. Image obtained from van den Boom *et al.* (2014).

[3] **Canonical** implying the current histone modification events occur through a generalised and commonly accepted pathway, whereas 'non-canonical' implies the present pathway deviates from the canonical paradigm.

Human PRC1 is composed of five subunit proteins: polycomb group ring finger (PCGF), polyhomeotic-like protein (PHC), chromobox homolog (CBX), sex combs on midleg (SCM), and really interesting new gene (RING). The former PRC1 components are known to have multiple paralog family members: six PCGF members (PCGF1/NSPC1, PCGF2/MEL18, PCGF3, PCGF4/BMI1, PCGF5, and PCGF6/MBLR), three PHC members (PHC1, PHC2, and PHC3), five CBX members (CBX2, CBX4, CBX6, CBX7, and CBX8), three SCM members (SCML1, SCML2, and SMLH1), and two RING1 members (RING1A and RING1B) – as indicated in **figure 5** (van den Boom *et al.*, 2014). Of note, the presence of one paralog of a specific family mutually excludes presence of the other family members, e.g. if CBX2 is present in a particular PRC1 then CBX4, 6, 7, 8, cannot reside in the same complex. The following paragraph will concisely discuss the roles of PRC1 and PRC2 in normal haematopoiesis respectively. For the sake of concision, the function of only one member per complex will be addressed, although the other complex members are equally important in the regulation of normal haematopoiesis.

Role of PRC1 Self-renewal of HSCs is thought to be under tight epigenetic regulation. Well-established findings demonstrate components of the PRC1 to be very important in regulation of HSC self-renewal. Indeed, Osawa *et al.* conducted a detailed expression analysis of PcG genes in mice in 1996, which revealed that members of the PRC1 are highly expressed in BM HSCs, whereas these cells display profound downregulation of the former genes upon their differentiation. Among these PRC1 genes, the role *Bmi1* has best been characterised in haematopoiesis. This gene is known to have a central role in HSC self-renewal, for it mediates induction of symmetrical cell division, as shown by multiple independent analyses of the present gene. (Park *et al.*, 2003; Iwama *et al.*, 2005; Sashida & Iwama, 2012; van den Boom *et al.*, 2014). Knockout mouse models for *Bmi1* (*Bmi1*$^{-/-}$) revealed loss of HSC self-renewal capacity, leading to severe pancytopenia.

BMI1 is able to repress – via RING1 E3 ligase activity - two distinct loci by directly binding to the promoters of the cyclin-dependent kinase inhibitor gene *Ink4a* and the tumour suppressor gene *Arf*. Expression of these genes in HSCs preludes cell cycle arrest and subsequent p53-mediated apoptosis. *Bmi1*$^{-/-}$ is thought to significantly reduce H2AK119Ub marks at the current loci, hence inducing re-expression of the two genes. One could think of creating knockout mice for both *Bmi1* and *Ink4a/Arf*. Indeed, mice deficient for the former three loci demonstrated substantial restoration of defective self-renewal capacity of HSCs (Sashida & Iwama, 2012), albeit not a complete rescue (van den Boom *et al.*, 2014). In addition, BMI1 also fulfils a role in the regulation of mitochondrial functioning, as it induces expression of proteins that are to keep reactive oxygen species generation in check (Sashida & Iwama, 2012). Taken together, PRC1 regulates through its subunit BMI1 self-renewal of HSCs, at least partially by repressing *Ink4a* and *Arf*, and by the preservation through normal mitochondrial functioning.

Role of PRC2 Contrary to the well-studied role of PRC1 members in physiological haematopoiesis, relatively few studies are conducted regarding the role of PRC2 in HSCs. Considering the PRC2 members, the histone methyltransferase EZH2 has best been characterised. An EZH2 null background in mice is somewhat harder to create, for the *Ezh2* $^{-/-}$ phenotype is not compatible with life in murine embryos. Therefore, conditional knockout models were developed as described by O'Carroll *et al.* in 2001. Absence of EZH2 does not interfere with adult haematopoiesis, albeit severely impairing lymphoid differentiation (van den Boom *et al.*, 2014). In addition, no aberrancy in self-renewal capacity of *Ezh2* $^{-/-}$ HSCs is found. Interestingly, H3K27Me3 marks are significantly preserved in the EZH2 null background (Shen *et al.* in van den Boom *et al.*, 2014), suggesting EZH1 somehow partially compensates for the absence of EZH2-mediated H3K27 tri-methylation (Sashida & Iwama, 2012). In conclusion, EZH2 seems to be important in lymphopoiesis, albeit not primarily necessary for the preservation of normal HSC functioning.

Aberrant haematopoiesis

The first part of this thesis provided a clear overview of the basic principles of epigenetics, constituting a tool to better comprehend the complex regulation of the hierarchical network of haematopoiesis in physiological context. Both DNA methylation and posttranslational histone modifications inevitably form a means through which HSC fate is linked to the functional requirements of the organism. However, changes in cytogenetics as well as somatic mutations are well-poised to distort the former link, hence preluding haematopoietic malfunction and malignancy. Although many blood-associated malignancies are subject of intensive research to date, and exciting advancements in our understanding of these syndromes truly deserve attention, the current paragraph will for the sake of concision only focus upon acute myeloid leukaemia pathogenesis.

The two-hit hypothesis of myeloid leukaemogenesis

For years, the putative 'two-hit hypothesis' formed a widely accepted model in explaining myeloid leukaemogenesis[4]. According to this hypothesis at least two distinct types of genetic events are required for **acute** malignant transformation of myeloid precursor cells to occur (Kelly & Gilliland, 2002; Shen *et al.*, 2011; Shih *et al.*, 2012; O'Brien *et al.*, 2015). Class I mutations comprise genetic aberrations conferring constitutive activity of various signalling pathways, leading to uncontrolled cellular proliferation and evasion of apoptosis (e.g. BCR-ABL, Flt3, c-KIT, and RAS). Class II mutations were thought to alter expression of key TFs involved in the process of myelopoiesis, hence inhibiting cellular differentiation (e.g. CBF, RARα, and MLL) (Shen *et al.*, 2011; Shih *et al.*, 2012; O'Brien *et al.*, 2015). Importantly, these classes of mutations may cause myeloid pathologies when occurring isolated from each other. Yet both have to be present to give rise to AML (Kelly & Gilliland, 2002). Nevertheless, compelling evidence now suggests class I and II mutations to be only one part of a more complex picture (**figure 6**).

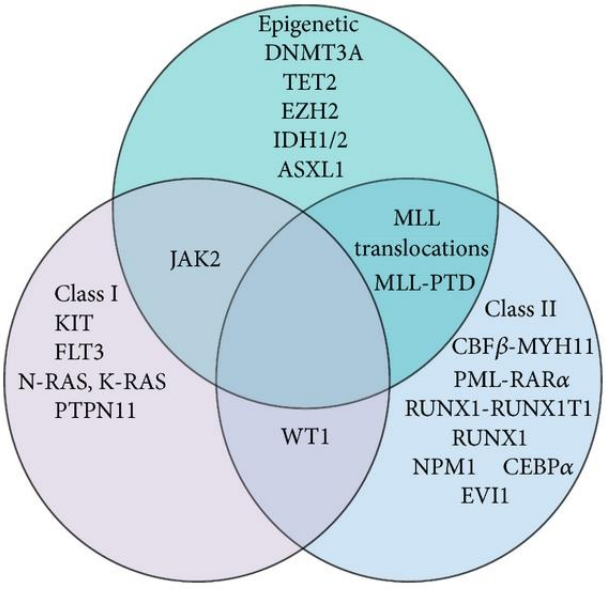


Figure 6 Venn diagram displaying commonly found recurrent driver genes in AML patients. Note that an additional class of genes has been added to the well-accepted class I and II mutations, comprising mutations occurring in genes encoding epigenetic modifiers. Image obtained from O'Brien *et al.* (2015).

[4] **Leukaemogenesis** is defined as the pathological process of induction and development of leukaemia in the bone marrow.

A third class of leukaemic disease alleles? Epigenetic modifiers

Nearly 50% of AML patients display a normal karyotype (normal karyotype-AML; NK-AML) and absence of copy number variations, suggesting some other concealed underlying cause for this disease. In the last years, the genomes of AML patients have therefore been subjected to next-generation sequencing techniques, including targeted sequencing, exome sequencing, and genome-wide sequencing, which remarkably revealed an additional class of mutations commonly occurring in AML. In 2013, the entire genome of 200 AML patients was sequenced and the results were published in The New England Journal of Medicine (TCGA, 2013). More than 20 recurrent driver mutations were identified (**figure 7**), strongly extending the known repertoire of genetic lesions found in AML. Many of these recurrently mutated genes are known to act as epigenetic regulators of transcription, including *TET2* (ten-eleven translocation 2), *IDH1/2* (isocitrate dehydrogenase 1/2), *ASXL1* (additional sex combs-like 1), *EZH2* (enhancer of zeste homologue 2), and *DNMT3A* (DNA methyltransferase 3A) (Shih *et al.*, 2012; Abdel-Wahab & Levine, 2013; TCGA, 2013; Falini et al., 2015). Shih and colleagues (2012) reported the year before that "functional studies have revealed that perturbations of such epigenetic modifiers alone can confer self-renewal and lead to myeloid transformation" [*sic*]. Interestingly, mutations in such epigenetic regulatory genes are now recognised as being the 'first hits'. Corces-Zimmerman and colleagues developed a model in which they powerfully demonstrated that the former mutations can promote haematopoietic stem and progenitor cells to acquire a putative "preleukaemic" state (**figure 8**). According to the present model, epigenetic-modifying genes always constitute founding mutations that are thought to induce AML pathology, as these actually are the only mutations present putative preleukaemic HSCs[5] (2014). In addition, studies of paired diagnosis and relapse samples suggested the current mutations to be stable during disease evolution, meaning preleukaemic HSCs carrying such mutations might survive initial therapeutic regimens and contribute to relapse (Chou *et al.*, 2010; Hou *et al.*, 2012). More importantly, as they indisputably hold the potential to be therapeutically targeted (introductory section), such founder mutations are of profound interest. Therefore, the following sections will review – based on two specific founder mutations in epigenetic modifiers – how these contribute to aberrant repatterning of the epigenome hence paving way for myeloid leukaemogenesis to thrive.

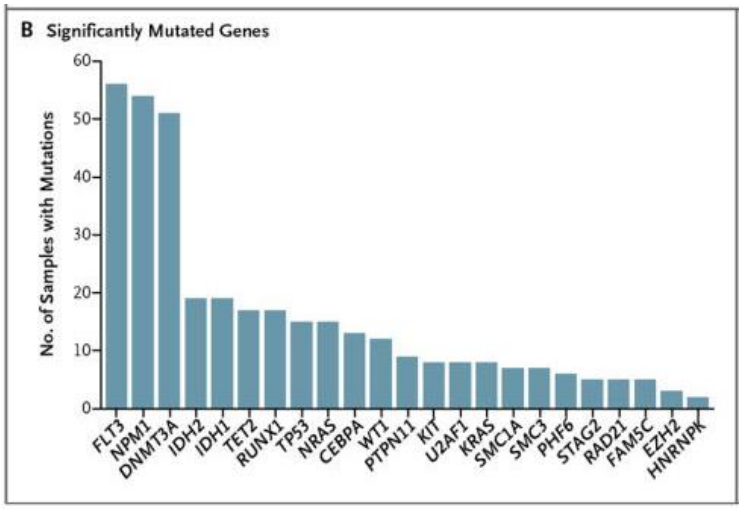


Figure 7 Depiction of significantly mutated genes in AML. The number of samples out of 200 carrying a particular mutation is displayed on the y-axis. Mutations were identified by the putative MuSiC analysis suite (Dees *et al.*, 2012 in: TCGA, 2013). Note that four genes encoding epigenetic modifiers (i.e. DNMT3A, IDH2, IDH1, and TET2) make up the top six based on frequency. DNMT3A, DNA methyltransferase 3A; IDH, isocitrate dehydrogenase; TET2, ten-eleven translocation 2. Image obtained from The Cancer Genome Atlas (2013).

[5] **Preleukaemic HSCs** are genetically distinct from normal HSCs in that they only harbour a subset of leukaemia-specific mutations, hence they are well-poised to become actually leukaemic when acquiring more mutations (Corces-Zimmerman *et al.*, 2014)

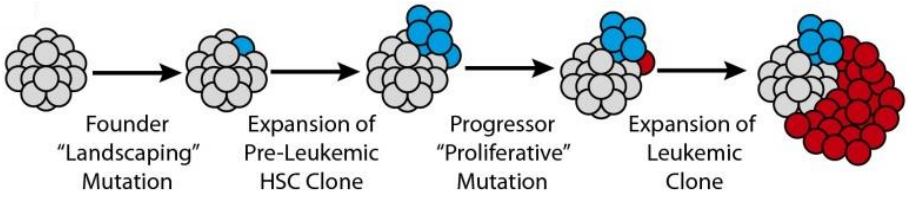


Figure 8 Model of mutation acquisition. The earliest acquired mutations in the process of AML evolution occur in epigenetic-modifying genes (e.g. *DNMT3A* or *IDH1/2*), whilst mutations in genes involved in signal transduction pathways and cellular proliferation (e.g. *RAS* or *FLT3*) are thought to occur late. Image obtained from Corces-Zimmerman *et al.* (2014).

<u>*DNMT3A* mutations</u>

DNA methyltransferase 3A is a member of the mammalian family of methyltransferases, an enzyme that functions by *de novo* adding a methyl group to the C5 position of the cytosine pyrimidine ring to form 5mC, usually in the context of CpG dinucleotides (see p. 4 'principles of epigenetics'). This subparagraph first gives a concise overview of normal DNMT3A functioning followed by genetic lesions in *DNMT3A* that contribute to leukaemogenesis.

Our very understanding of the physiological role of *DNMT3A* primarily stems from murine knockout models. Back in 1999, Okano and colleagues showed that mice carrying a $Dnmt3a^{-/-}$ died one month after birth, whilst $Dnmt3b^{-/-}$ mice already deceased halfway the gestation period. To put the current gene into haematological context, *DMNT3A* functions in HSCs by regulating "the ability to switch from a self-renewal to a differentiation programme" (Yang *et al.*, 2015). The latter followed from the observation in mice that $Dnmt3a^{-/-}$ HSCs exhibited increased self-renewal along with a markedly reduced differentiation (Challen *et al.*, 2012). A proposed mechanism for this would be the substantial hypomethylation of genes associated with stem cell self-renewal and cancer (e.g. *Hoxa9* and *Meis1*), resulting in their upregulation (Challen *et al.*, 2012; Jeong *et al.*, 2014). The $Dnmt3a^{-/-}$-induced reduction in differentiation is thought to be partially caused by an upregulation of β-catenin, for a knockdown of *Ctnnb1* (which encodes β-catenin) moderately restored the likelihood HSCs would favour differentiation over either self-renewal (Challen *et al.*, 2014 in: Yang *et al.*, 2015).

By means of whole-genome sequencing[6] efforts Ley *et al.* were first to expose *DNMT3A* as being strongly implicated in AML pathogenesis (2010); they demonstrated *DNMT3A* mutations in 22% of NK-AML cases. Since then, *DNMT3A* is perceived as one of the most frequently mutated genes in NK-AML, occurring up to 36% (Marcucci *et al.*, 2012). The prognostic impact of $DNMT3A^{mut}$ conditions has proven to be hard to interpret, for it generates much controversy between different studies. Several studies suggest $DNMT3A^{mut}$ to confer an adverse effect on overall survival (OS) (Shen *et al.*, 2011; Thol *et al.*, 2011; Marcucci *et al.*, 2012; Yuan *et al.*, 2016), whilst others report the opposite (Patel *et al.*, 2012). Thorough literature search provided more studies stating adverse effects on OS, which will therefore be the conclusion here, albeit with a grain of salt.

Mutations in *DNMT3A* are consistent heterozygous and – although many distinct types of mutations are reported for *DNMT3A* including frameshifts, nonsense, splice-site mutations, and deletions – a mutational hotspot is predicted to be at arginine 882 (R882) (**figure 9a-b**) (Ley *et al.*, 2010; Falini *et al.*, 2015). Missense mutations at R882 occur in nearly 60% of all *DNMT3A*-mutated samples (Ley *et al.*, 2010; Aumann & Abdel-Wahab, 2014) – usually generating R882H – and have been shown to abrogate catalytic activity and resulting in reduced DNA binding affinity (Gowher *et al.*, 2006; Yan *et al.*, 2011; Kim *et al.*, 2013; Russler-Germain *et al.*, 2014). To be functionally active, DNMT3A normally forms tetramers through protein-protein interactions,

[6] **Whole-genome sequencing** is an unbiased approach for discovering somatic variations in cancer genomes

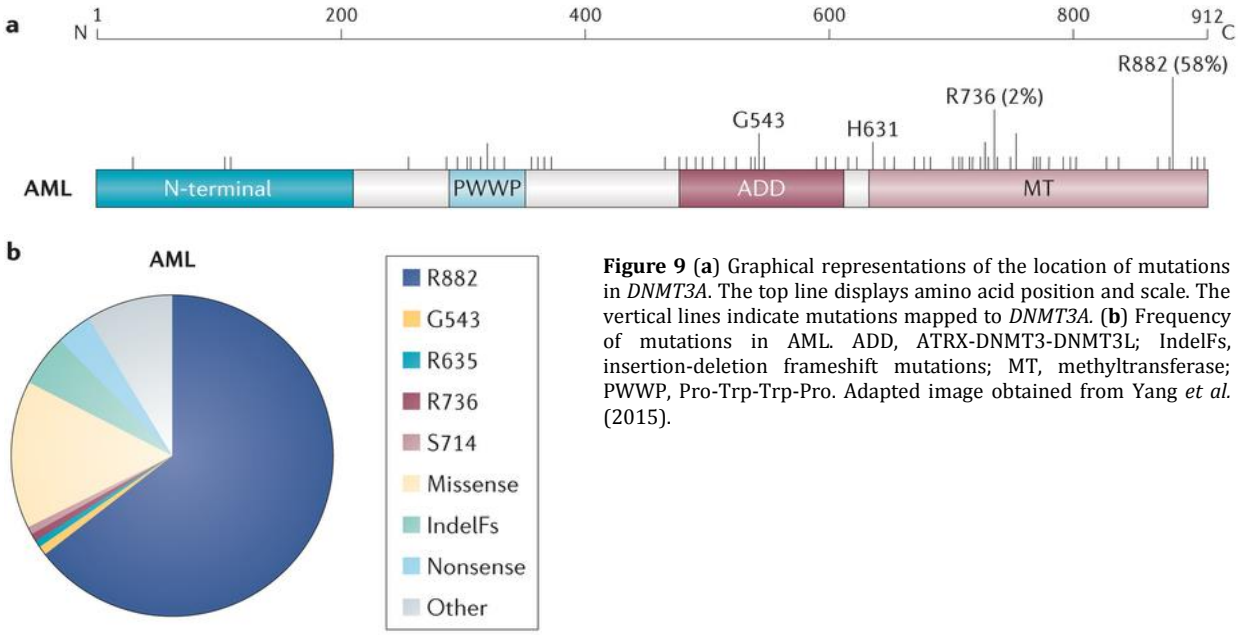


Figure 9 (**a**) Graphical representations of the location of mutations in *DNMT3A*. The top line displays amino acid position and scale. The vertical lines indicate mutations mapped to *DNMT3A.* (**b**) Frequency of mutations in AML. ADD, ATRX-DNMT3-DNMT3L; IndelFs, insertion-deletion frameshift mutations; MT, methyltransferase; PWWP, Pro-Trp-Trp-Pro. Adapted image obtained from Yang *et al.* (2015).

either by interacting with itself or by oligomerisation with DNMT3B or DNMT3L. $DNMT3A^{R882H}$ has dominant-negative activity in that it interferes with appropriate tetramer functioning, thereby inhibiting methyltransferase activity of $DNMT3A^{WT}$, thus creating a global signature of hypomethylation (Kim *et al.*, 2013; Jeong *et al.*, 2014; Russler-Germain *et al.*, 2014). Importantly, regardless of its high prevalence, the R882 variant possesses another feature which makes it of special interest. Holz-Schietinger and colleagues showed that in a state of heterozygous $DNMT3A^{R882}$, about 20% of $DNMT3A^{WT}$ functions remain, likely caused by either homodimeric oligomerisation of $DNMT3A^{WT}$ or through other concealed protein-protein interactions. If $DNMT3A^{R882}$ could somehow be therapeutically inhibited, we would probably substantially restore $DNMT3A^{WT}$ functions (2012).

To date, it is well-established that *DNMT3A* mutations are predominantly present in the founding clones of AML patients, indicating they might – at least partially – induce myeloid malignancy (TCGA, 2013; Corces-Zimmerman, 2014; Shlush *et al.*, 2014). Moreover, they represent stable mutations in disease evolution and are thought to survive chemotherapy, hence persisting in preleukaemic HSCs and may therefore be well-poised to contribute to relapse (Hou *et al.*, 2012). However, the exact mechanisms through which lesions in *DNMT3A* contribute to leukaemogenesis, unfortunately, remain elusive. Loss-of-function mutations (e.g. R882) in *DNMT3A* strongly correlate with genome-wide hypomethylation, principally at CpG islands and promoters (Yang *et al.*, 2015). Indeed, Jeong and colleagues created murine *Dnmt3a*-null HSCs in which they demonstrated a clear global hypomethylation signature. Moreover, The Cancer Genome Atlas (2013) conducted unsupervised analysis of changes in DNA methylation in a background of *DNMT3A*-mutated AML and equally showed whole-genome DNA hypomethylation. Of note, loss of DNMT3A functions *per se* do not cause leukaemia, more additionally acquired mutations are necessary (Tadokoro *et al.*, 2007). We still do not understand by far how hypomethylation patterns actually link mechanistically to leukaemia development.

Yang *et al.* report that multiple studies emphasis the relative absence of correlation between changes in DNA methylation and gene expression (Wu *et al.*, 2010; Raddatz *et al.*, 2012 in: Yang *et al.*, 2015), which additionally impedes mechanistic understanding. In addition to changes in promoter methylation state, enhancers might as well be important sites for hypomethylating events. Understanding of the latter, however, is still at a burgeoning state. Taken together, normal DNMT3A functioning is crucial for maintaining haematopoietic integrity, for it enables HSCs to opt for differentiation. Murine knockout models for *Dnmt3a* demonstrated that loss of

the present gene impeded HSCs to switch from self-renewal to differentiation, hence preluding leukaemia development. *DNMT3A*^{mut} signifies poor prognosis, establishes global hypomethylation patterns, though the underlying mechanisms remain unknown.

IDH1/2 mutations

In normal cells, IDH1 and IDH2 constitute homodimeric enzymes that are crucially implicated in the Krebs cycle, wherein they facilitate the conversion of isocitrate to α-ketoglutarate (α-KG) in an $NADP^+$-dependent manner (**figure 10**). IDH1 functions in the cytosol and peroxisomes, whereas IDH2 resides in the mitochondrial matrix (Falini *et al.*, 2015). Mardis and colleagues were first to identify *IDH1* as recurrently mutated in AML (2009) and subsequent candidate-gene studies also found its homologue *IDH2* often mutated in AML (Mardis *et al.*, 2009 in: Shih *et al.*, 2012). Mutations in *IDH1* and *IDH2* occur in approximately 20% of NK-AML patients (TCGA, 2013; Falini *et al.*, 2015; Coombs *et al.*, 2016). Corces-Zimmerman *et al.* (2014) and Shlush *et al.* (2014) independently showed *IDH1/2* mutations are acquired early in progression from normal HSCs to frank leukaemia and can persist in preleukaemic HSCs through chemotherapeutic interventions, suggesting they can ultimately re-establish disease conditions (Chou *et al.*, 2010). They constitute consistent heterozygous missense mutations occurring at one of three highly conserved argininyl moieties: IDH1-R132, IDH2-R140, and IDH2-R172 (Pastore & Levine, 2016). The current mutations confer a significant reduction in α-KG production, indicating loss-of-function mutations. However, subsequent studies that focused upon metabolic pathways demonstrated that corrupted IDH acquired neomorphic enzyme activity, wherein α-KG is converted to 2-hydroxyglutarate (2-HG) rather then not produced (Dang *et al.*, 2009; Ward *et al.*, 2010). They are now considered as aberrant gain-of-function mutations (Shih *et al.*, 2012). Normally, two enantiomers of 2-HG are present within cells at low concentrations: (*R*)-2-HG and (*S*)-2-HG. Corrupted IDH1/2 only produce (*R*)-2-HG (Pastore & Levine, 2016). One could therefore think of (*R*)-2-HG as a potential biomarker, indicative of *IDH1/2* mutations. Indeed, Ward *et al.* demonstrated elevated serum levels of (*R*)-2-HG in AML patients that harbour *IDH1/*2 mutations. Admittedly, those mutations do not seem to compromise epigenome integrity at first sight. Though when looked upon more closely they actually do, albeit in an indirect fashion.

Due to structural similarity, increased levels of (*R*)-2-HG can competitively antagonise α-KG in the active site of α-KG-dependent enzymes, thereby impairing enzyme functioning. α-ketoglutarate forms an essential substrate for two distinct clases of enzymes: i. the TET family of DNA demethylases and the Jumonji-C (JmjC) domain-containing family of histone lysine demethylases important in regulating chromatin structure, and ii. the propyl and lysyl hydroxylases that are required for adequate collagen folding and HIF signalling (Fathi & Abdel-Wahab, 2011; Prensner & Chinnaiyan, 2011; Abdel-Wahab & Levine, 2013). Recall the important roles of DNA and histone methylation in the normal regulation of haematopoiesis. Clearly, interfering with the first class of enzymes results in aberrant increases in DNA and histone methylation patters. Indeed, Figueroa *et al.* recently conducted DNA methylome analyses of 398 IDH mutation-positive AML patients and found that mutations in *IDH1/2* are associated with consistent and aberrant hypermethylation of various promoter sites involved in myeloid differentiation and leukaemogenesis (Figueroa *et al.*, 2010 in: Fathi & Abdel-Wahab, 2011). In addition, Xu *et al*. (2011) demonstrated a dose-dependent increase of various lysine methyl marks (H3K4Me3, H3K9Me2, H3K27Me2, and H3K79Me2) and upregulation of *HOXA* cluster genes, after (*R*)-2-HG treatment. Increased presence of those K-Me marks clearly suggests (*R*)-2-HG-induced JmjC malfunction. The actual effects of the former epigenetic alterations on haematopoiesis were shown by Sasaki and colleagues (2012), who expressed *IDH1* and *IDH2* mutant alleles in mouse bone marrow-derived cells and observed a block in myeloid differentiation and accumulation of immature haematopoietic progenitors (Sasaki *et al.*, 2012). Moreover, an *IDH*^{mut} phenotype may further promote leukaemogenesis by reducing hydoxycollagen formation through inhibition of propyl and lysyl hydroxylases. Hydroxylated collagen is critically implicated in HSCs proliferation and differentiation and its absence may

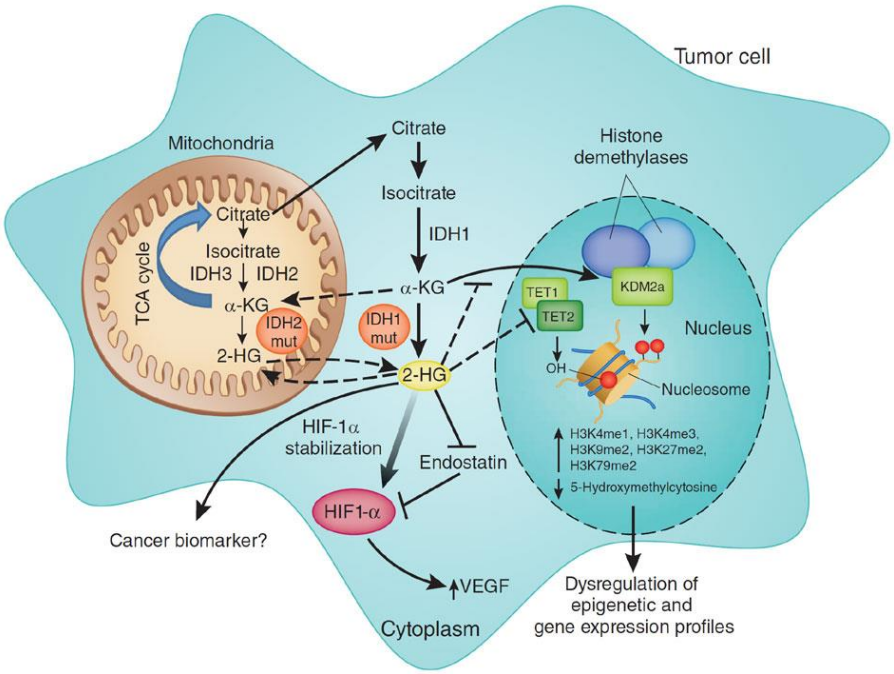


Figure 9 Wild-type *IDH1* and *IDH2* encode enzymes which are critically implicated in the Krebs cycle; they facilitate the cytosolic and mitochondrial matrix conversion of isocitrate into α-KG respectively. The latter being a crucial substrate for two distinct clases of enzymes: i. the TET family of DNA demethylases and the JmjC-containing family of histone lysine demethylases important in regulating chromatin structure, and ii. the propyl and lysyl hydroxylases that are required for adequate collagen folding and HIF signalling. Corrupted IDH1/2 convert α-KG into 2-HG, hence reducing α-KG levels, leading to impairment of the aforementioned enzymes. The final steps comprise epigenetic distortion, EMC alterations and response to anoxia, ultimately promoting leukaemogenesis. α-KG, α-ketogluterate; 2-HG, 2-hydroxygluterate; IDH, isocitrate dehydrogenase; JmjC, Jumonji-C domain. Image obtained from Prensner & Chinnaiyan (2011).

well predispose HSCs to transform (Falini *et al.*, 2015). *IDH*^{mut}-mediated upregulation of HIF factors activated multiple cancer-associated signalling pathways, ever more contributing to leukaemia developement (Cairns & Mak, 2013). A hindmost feature through which corrupted IDH1/2 promotes leukaemia evolution was recently exposed by Chan *et al.* (2015). They demonstrated that mutant IDH1/2 distorted mitochondrial functioning via the repression of cytochrome c oxidase. They surmised induction of apoptosis might well be a feasibility upon BLC-2 inhibition, strongly indicating their application in *IDH*^{mut}-AML. In conclusion, *IDH1/2* mutations confer aberrant DNA and histone methylation patterns via the (*R*)-2-HG-mediated repression of multiple epigenetic regulators, hence distorting gene expression and well-poise HSCs to undergo leukaemic transformation. Equal to *DNMT3A* mutations, they represent early onset mutational events that persist thought chemotherapeutic intervention in preleukaemic HSCs, which makes them interesting therapeutic targets.

Therapeutic intervention

Now that the two early onset genetic lesions have been discussed in detail, how is the former-presented knowledge put into practise when it comes to the development of therapeutic interventions? Given that AML principally is a disease of older adults – the median age at diagnosis is 67 years (Estey & Dohner, 2006) – and older patients (defined here as ≥60 years) showing little or no improvement over the past three decades (Thein *et al.*, 2013), this paragraph will focus upon advances in therapeutic strategies in this category of patients. The first subsection will concisely review AML first-line treatment, followed by exciting recent advances considering *DNMT3A*^{mut} and *IDH1/2*^{mut} targeting strategies.

Induction therapy

To date, the putative '7+3' regimen always forms the first-line treatment for AML (often referred to as 'induction therapy'). It constitutes combination chemotherapy with standard-dose cytarabine (100-200 mg/m^2) for seven days, followed by an anthracycline (either daunorubucin or idarubicin) for three days (Gallipoli et al., 2015; Coombs *et al.*, 2016; Khaled *et al.*, 2016). The current strategy aims to achieve patient remission. However, if persistence of AML is observed on the 14th day, the patient will be re-treated with the same compounds, albeit in a '5+2' schedule (Khaled *et al.*, 2016). Despite 20 years of efforts to optimise chemotherapeutic interventions, only a mere 40-60% of older patients are expected to go into complete remission. Furthermore, clinical outcome is very poor in that overall long-term survival is in the unfortunate range of 20-30% (Gallipoli et al., 2015). Indeed, many studies suggest that we have maximised the anti-leukaemic potential of chemotherapeutic regimens (Gallipoli *et al.*, 2015; Coombs *et al.*, 2016; Khaled *et al.*, 2016). Novel therapeutic strategies are therefore urgently needed if we want to enhance overall long-term survival.

DNMT3A mutations

Two distinct putative 'hypomethylating agents' (HPAs) are currently approved for clinical application in AML: 5-azacytidine (**azacytidine**) and 5-aza-2'-deoxycytidine (**decitabine**), both of which constitute pyrimidine analogue that function through the inhibition of DNMTs (Wouters & Delwel, 2016). A novel HPA and analogue of decitabine is **Guadecitabine** (**SGI-110**). Issa *et al.* are conducting a multicentre, open-label, phase I study in which they assess the safety and clinical activity of subcutaneously administered guadecitabine in AML patients (2015). They report that the current compound is well tolerated and is clinically and biologically active in AML patients. Interestingly, by means of combining docking-based virtual screening with biochemical analyses, Chen *et al*. recently identified a novel compound, termed **DC_05**, which is a non-nucleoside DNMT1 inhibiting agent (2014). Through further similarity-based analogue searching revealed two additional compounds were identified, **DC_501** and **DC_517**, which were shown to be more potent than DC_05. Strikingly, the present DNMT1-inhibiting agents significantly restricted cancer cell proliferation.

IDH1/2 mutations

The identification of the acquired neomorphic enzyme activity of corrupted IDH1/2 provided an exceptional target in leukaemia-specific therapeutic intervention. Many *IDH1* and *IDH2* metabolic enzyme inhibitors are currently being assessed in preclinical models. Discovered through computational compound screen using the ZINC library of Irwin *et al.* (2012), **HMS-101** specifically targets *IDH1^{mut}* and elicits its inhibition (Gallipoli *et al.*, 2015). Chaturvedi and colleagues assessed HMS-101 efficacy *in vitro* and demonstrated the present compound was able to actively target *IDH1* mutations both in murine BM cells and primary human AML (2013). Following on from this, HMS-101 exhibited *in vivo* suppression of (*R*)-2-HG production, conferred anti-proliferating effects and induced differentiation of leukaemic cells (Chaturvedi *et al.*, 2014). In addition, recent advances also provided IDH2^{mut}-inhibiting agents. In 2013, Wang *et al.* developed a small molecule termed **AGI-6780**, which showed selective targeting of IDH2^{R140Q}. *In vitro* treatment with AGI-6780 induced substantial differentiation in primary human AML cells.

These major improvements led to development of a multicentre, open-label, phase I dose-escalation study which examined **AG-221**, a highly selective and potent competitive antagonist of IDH2^{mut} (Coombs *et al.*, 2016). Coombs *et al.* reported in *Nature Reviews Clinical Oncology* last May that AG-221 is well tolerated, and that the maximum-tolerated dose has not yet been achieved (Stein *et al.*, 2014; DiNardo *et al.*, 2015 in: Coombs *et al.*, 2016). These trials, however, are still ongoing and more results have to be awaited for firm conclusions to be drawn on the actual future applicability of the present compound (Coombs *et al.*, 2016). Moreover, additional compounds are current being evaluated: **AG-120** and **AG-881**, specific IDH1 and dual IDH1-IDH2 inhibitors respectively, are in a precocious state and results are said to be underway (de

Botton *et al.*, 2015; US National Library of Medicine, 2015 in: Coombs *et al.*, 2016). The same applies to **IDH305**, IDH1-R132^{mut} specific inhibitor (ClinicalTrials.gov identifier: NCT02381886).

Experimental evidence also strikingly proposes alternative mode of actions that could target *IDH* mutations. Chan and colleagues showed induction of BCL-2 dependence in the context of corrupted IDH in AML, which paves way for BCL-2 inhibitors in future AML treatment. Even more interesting, Schumacher *et al.* reported in 2014 *IDH1*^{R132H} mutations to be a potential tumour-specific neo-antigen, which might well be implicated in the development of a vaccination regimen that ultimately acts as a provocation of T-cell responses against these mutations.

Future perspectives

The present thesis aimed to investigate whether acute myeloid leukaemia truly is more than a mere genetic disorder. The first indication which positively confirmed this question followed from the detailed description of how haematopoiesis is physiologically regulated. Epigenetics are critically implicated in the tight orchestration of HSC cell fates: DNA methylation guides progenitor cells in opting for the correct lineage and to persevere in their choice, whereas posttranslational histone modifications aid in preserving the functional HSC pool by regulating the process of self-renewal. This was further substantiated in the subsequent section 'aberrant haematopoiesis', which demonstrated genetic lesions in epigenetic-modifying genes to represent early onset events – already present in a preleukaemic state. A highly important observation lies in the very fact that such mutations are able to survive chemotherapeutic intervention. Haematopoietic stem and progenitor cells harbouring such mutations thus constitute sources of potential malignancy which are well-poised to give rise to disease re-establishment. These putative preleukaemic, epigenetic gene lesion-harbouring HSCs could well be compared with opportunistic pathogens; they are latently present and appear expectantly upon 'advantageous' conditions.

Despite its well-established importance, our understanding of the biological implications of epigenetic modifications is still at a burgeoning stage. Certainly, thirty years of extensive research have provided vast insight into the specific actions of isolated genes. In addition, we have created comprehensive models that show how transcription is affected by epigenetic modifications, and The Cancer Genome Atlas Research Network profoundly extended our knowledge regarding the genomic landscapes found in AML patients. Yet, the genome-wide consequences of such modifications are poorly understood – let alone in the context of disrupted epigenome integrity, as shown in the context of *DNMT3A* mutations. Despite efforts revealing, by and large, *DMNT3A* functions in the haematopoietic system (see p. 12 '*DNMT3A* mutations'), profound literature research in the context of current thesis did not show any ongoing studies addressing *DMNT3A* functioning in a more broader sense. For instance, the way in which DNMT3A is recruited to chromatin or whether it targets particular loci are topics that require to be elucidated to allow for the development of targeted therapies. Future research should therefore focus upon elucidating the aforementioned.

Remarkably and contrary to the well-established concept that *IDH1/2* mutations are mutually exclusive with *TET2* mutations (Figueroa *et al.*, 2010; Shih *et al.*, 2012; Pastore & Levine, 2016), the genomes of 2 out of 200 AML patients in the data set of The Cancer Genome Atlas Research Network contained both mutations in the *IDH1* and *TET2* loci (**Supplementary figure 1**). The former concept might therefore need revision.

For long, cytogenetic factors have extensively been put to use as a means for the prognostic stratification of patients. However, nearly 50% of AML patients display a normal karyotype (NK-AML) and show absence of structural abnormalities. Therefore, the conventional, well-established model of the 'two-hit hypothesis' in myeloid leukaemogenesis certainly needs modification.

The current thesis also demonstrated *IDH1/2* mutations to occur in a preleukaemic stage. *IDH1/2*^{mut} give rise to a readily identifiable cancer biomarker (i.e. (*R*)-2-HG), indicating that perhaps in the near future we might well be able to run diagnostic tests and screen for genetic lesions like the aforementioned. Moreover, considering the fact that the former mutations are

gain-of-function mutations, which can already be targeted – think of ongoing phase I trials regarding the *IDH1* and *IDH2* metabolic enzyme inhibitors – this might pave ways in which we are able to restore cellular metabolism hence genomic integrity and even prevent leukaemia from happening.

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Appendix

Supplementary figure 1 contrary to the well-established concept that *IDH1/2* mutations are mutually exclusive with *TET2* mutations (Figueroa *et al.*, 2010; Shih *et al.*, 2012; Pastore & Levine, 2016), the genomes of 2 out of 200 AML patients in the current data set contained both mutations in the *IDH1* and *TET2* loci. Adapted image obtained from TCGA (2013).