Linköping University Medical Dissertation No. 1900

DNA methylation in T cell leukaemia

Maike Bensberg

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Sammanfattning

T-cells akut lymfatisk leukemi (T-ALL) är en blodcancer som främst drabbar barn. Trots att sjukdomen har en överlevnadsgrad på 80% orsakar den intensiva behandlingen med kemoterapi allvarlig toxiska effekter och långsiktiga biverkningar. Dessutom är överlevnadsgrad för patienter i återfall mindre än 25%. Följaktligen finns det ett behov av att utveckla bättre alternativ för patienterna för att minska behandlingsrelaterade biverkningar och förbättra överlevnadschans vid återfall. Att rikta sig mot avvikande modifikationer på DNAt som inte medför ändring i genetiska koden, även kallade DNA-metylering, med hypometylerande läkemedel (HMAs), som minskar antalet av dessa modifikationer på DNAt, har varit framgångsrikt vid behandling av andra blodcancerformer, men har inte rutinmässigt använts vid behandling av T-ALL.

DNA-metylering är en kemisk modifiering av DNA som stänger av gener i dess närhet. Tidigare studier har visat att DNA-metyleringsprofiler förändras i alla cancerformer och särskilt gener som förhindrar utvecklingen av cancer, så kallade tumörsuppressorgener, stängs av genom DNA-metylering. I denna doktorsavhandling använde jag en omfattande uppsättning av laboratorie- och beräkningsmetoder för att genomföra en djupgående studie av DNA-metyleringens funktion i friska T-celler och T-ALL. Jag utforskade också möjligheten att korrigera DNA-metylering i cancerceller med hjälp av HMAs som ett behandlingsalternativ för T-ALL.

Vi gjorde upptäckten att den kända tumörsuppressorgenen *TET2* är mindre vanlig eller helt förlorad i T-ALL celler jämfört med friska celler. Vi såg också att *TET2*-genen var metylerad hos en grupp T-ALL-patienter, vilket tyder på att *TET2* självt kan stängs av genom DNA-metylering i T-ALL. Genen *TET2* förlorade sin DNA-metylering och aktiverades efter behandlingen av celler från T-ALL.

Vi såg även att traditionella HMAs, som redan används kliniskt, endast har en begränsad förmåga att ta bort DNA-metylering och att de främst dödar cancerceller genom att skada deras DNA. I jämförelse med detta kunde det nya läkemedlet GSK-3685032 minska DNA-metylering betydligt mer kraftfullt och gav oss möjligheten att studera konsekvenserna av den omfattande förlusten av DNA-metylering i T-ALL-celler.

Den tredje studien i denna avhandling fokuserade på det faktum att T-ALL är mer än två gånger vanligare hos pojkar än hos flickor. En stor skillnad mellan pojkar och flickor är att kvinnliga celler har två X-kromosomer medan manliga celler har en Y- och en X-kromosom. De flesta gener på en av X-kromosomerna hos kvinnor är inaktiverade, men cirka 15% av X-länkade gener undkommer denna avstängning. Detta medför att det är två kopior av dessa gener som är aktiva i kvinnliga XX-celler och endast en är aktiv i manliga XY-celler. Dessa ytterligare kopior av aktiva tumörsuppressorgener från X-kromosomen har föreslagits skydda flickor från att utveckla T-ALL och kan förklara den högre risken för att utveckla sjukdomen hos pojkar. Vi profilerade DNA-metylering under T-cellsutveckling och skapade en karta över aktivitet på gener från X-kromosomen i manliga och kvinnliga celler. Vi fann att vissa, men inte alla, föreslagna tumörsuppressorgener faktiskt undkom X-inaktivering och är aktiva från båda X-kromosomer. Dessa resultat understryker vikten av att profilera de friska celler som T-ALL uppstår från för att korrekt bedöma de funktionella konsekvenserna av förändringar som ses vid cancer.

I vår sista studie fokuserade vi på en specifik typ av DNA-metylering (kallad 6mdA) som är vanlig hos bakterier men mycket ovanligare hos människor. 6mdA har associerats med flera processer i friska celler och cancerceller. Vår studie ifrågasätter om 6mdA finns hos människor. Vi visade begränsningarna i de tekniker som används för analys av 6mdA. Förorening med bakteriellt DNA eller 6mA-innehållande RNA, ospecifik bindning av antikroppar och låg precision hos sekvenseringstekniker förhindrar upptäckt och undersökning av sällsynta DNA-modifikationer, såsom 6mdA.

Sammanfattningsvis är detta arbete en djupgående studie av DNA-metyleringens funktion och potential i biologin hos friska T-celler och T-cells cancer.

Abstract

T cell acute lymphoblastic leukaemia (T-ALL) is a predominantly paediatric cancer that stems from malignant transformation of developing T cells. While the disease has an overall survival rate of 80%, the intense chemotherapy treatment causes severe toxicity and long-term side effects. Furthermore, the survival rate for patients in relapse is less than 25%. Consequently, there is a need for improved therapy options to reduce treatment-related side effects and improve the survival rate of relapsed patients. Targeting aberrant DNA methylation with hypomethylating agents (HMAs) has been successful in the treatment of myelodysplastic syndromes and acute myeloid leukaemia but has not been routinely used in the treatment of T-ALL, despite DNA hypomethylation being observed in T-ALL patients. In this work, we employed a comprehensive set of molecular and sequencing-based techniques to explore the possibilities of HMAs as a treatment option for T-ALL.

We made the discovery that the DNA demethylating enzyme ten-eleven translocation 2, *TET2*, is downregulated or completely silenced in primary T-ALL. Moreover, the *TET2* promoter was highly methylated in a group of patients, suggesting that *TET2* itself can be silenced through DNA methylation in T-ALL. By treatment with HMAs, *TET2* was demethylated in T-ALL cell lines and was one of few genes that was activated upon loss of DNA methylation, indicating that *TET2* expression is regulated by DNA methylation in T-ALL cell lines. The development of a novel HMA, the DNMT1-specific inhibitor GSK-3685032, offers a tool to reveal the mechanism of action of the traditional HMAs, 5-azacytidine and decitabine, and to study the effects of acute loss of DNA methylation on cancer cells. We found that 5-azacytidine and decitabine are cytotoxic to T-ALL cells primarily by creating DNA double strand breaks. In contrast, GSK did not prompt a DNA damage response and instead reduced global DNA methylation to as little as 18% with limited

cytotoxicity only occurring after levels of DNA methylation had dropped below 30%, a level of demethylation not achieved with DEC or AZA.

T-ALL is more than two times more common in boys than girls and mutations in X-linked tumour suppressor genes that escape X inactivation, have been suggested as an underlying cause for the observed sex-bias. In theory, these aberrations would be more detrimental in XY-male cells than in XX-female cells due to the presence of an extra protective copy of the gene in females. We profiled DNA methylation during T cell development and created a map of sex-specific gene expression and expression from the inactive X chromosome, finding that some, but not all, suggested tumour suppressor genes in fact escape X inactivation. These results highlight the importance of profiling the healthy cells that T-ALL arises from to correctly judge the functional impact of gene dysregulation in cancer.

In the last study, we aimed to investigate the role of N6-adenine methylation (6mdA) during T cell differentiation. While 6mdA is common in bacteria it is much rarer in humans. Nevertheless, 6mdA has previously been associated with several cellular processes, including cancer progression. Our study calls the presence of 6mdA in mammals into question by exposing limitations of the techniques used in its analysis. We show that contamination with bacterial DNA or 6mA-containing RNA, nonspecific antibody binding, and low precision of third-generation sequencing techniques all hinder the detection and investigation of rare DNA modifications, such as 6mdA.

Together, this work is an in-depth study of the function and the potential of DNA methylation in the biology of healthy and malignant T cells.

Included Papers

This thesis is based on four published papers and manuscripts.

Paper I

Maike Bensberg*, Olof Rundquist*, Aida Selimović, Cathrine Lagerwall, Mikael Benson, Mika Gustafsson, Hartmut Vogt, Antonio Lentini, Colm E. Nestor

* Authors contributed equally.

TET2 as a tumor suppressor and therapeutic target in T-cell acute lymphoblastic leukemia

Proceedings of the National Academy of Sciences (PNAS), 2021, vol 118 (34)

Paper II

Maike Bensberg*, Aida Selimović*, Lisa Hedin Haglund, Júlia Goldmann, Sandra Hellberg, Colm E. Nestor

* Authors contributed equally.

No evidence for an epigenetic role in the cytotoxicity of traditional DNA de-methylating agents in cancer therapy

Manuscript

Paper III

Björn Gylemo*, Antonio Lentini*, Christina Lundqvist*, **Maike Bensberg**, Alessandro Camponeschi, Dóra Goldmann, Aida Selimović, Olov Ekwall, Colm E. Nestor

* Authors contributed equally.

A landscape of X-inactivation during human T-cell development

Manuscript

Paper IV

Karolos Douvlataniotis*, **Maike Bensberg***, Antonio Lentini*, Björn Gylemo, Colm E. Nestor

* Authors contributed equally.

No evidence for DNA N6-methyladenine in mammal

Science Advances, 2020, Volume 6, Issue 12

Abbreviations

4mdC	4-methylcytosine
5caC	5-carboxylcytosine
5fC	5-formylcytosine
5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
6mdA	6-methyladenine
AML	acute myeloid leukaemia
AZA	5- azacytidine
CGI	CpG island
CIMP	CpG island methylator phenotype
CpG	cytosine followed by guanine
DEC	5-aza-2′deoxycytidine, decitabine
DN	double negative
DNMT	DNA methyltransferase
DP	double positive
EM-seq	enzymatic methyl-conversion followed by sequencing
ERV	endogenous retrovirus
ESC	embryonic stem cell
ETP	early thymic progenitor
GSK	DNMT inhibitor GSK-3685032
H3K9me3	trimethylation of lysine 9 at histone 3

H3K27me3	trimethylation of lysine 27 at histone 3
HMA	hypomethylating agent
ICR	imprinting control region
IDH	isocitrate dehydrogenase
LINE	long interspersed repeat sequence element
MDS	myelodysplastic syndromes
MeDIP	methylated DNA immunoprecipitation
MRD	minimal residual disease
PCR	polymerase chain reaction
PRC	polycombe repressive complex
RNA-seq	RNA sequencing
ROS	reactive oxygen species
SMRT-seq	single molecule real-time sequencing
SP	single positive
T-ALL	T cell acute lymphoblastic leukaemia
TCR	T cell receptor
TE	transposable element
TET	ten-eleven translocation
Th	T helper cells
vitC	vitamin C, ascorbic acid
Xa	active X chromosome
XCI	X chromosome inactivation
Xi	inactive X chromosome

Introduction

The fine print of who we are: epigenetic regulation of gene expression

The term epigenetics as we know it, or ‘epigenotype’, was first devised by Conrad Hal Waddington in 1942 [1]. In his later publication, Waddington further described epigenetics as the mechanism of how a genotype becomes a phenotype. More specifically, Waddington’s famous epigenetic landscape suggests that during development an immature cell makes certain decisions (i.e. transcriptional regulation) which move it toward a specific differentiated state [2]. The basic question of how different cells with the same genome can perform vastly different tasks and how the environment can affect gene expression and thereby cell function is still the central question underlying most epigenetic research. Over the years, researchers have focused on three epigenetic mechanisms, which can dynamically regulate gene expression: DNA methylation, histone modifications, and non-coding RNAs. In this section, I will summarize the fundamentals of DNA and histone modifications.

DNA methylation

5-methylcytosine (5mC), the methylation of the 5th carbon of a cytosine, was first discovered in *Mycobacterium tuberculosis* in 1925 [3]. Since then, DNA methylation has been extensively researched with discoveries ranging from the identification of 5mC in mammals [4] and mapping of the interaction between DNA methylation and gene expression [5] to characterizing the dynamics of DNA methylation in development and disease [6] (beautifully summarized by Mattei *et al.* [7]).

5mC is the most abundant DNA methylation mark in mammals and occurs predominantly at CpG dinucleotides (cytosine followed by guanine). DNA methylation is generally symmetric, occurring on both DNA strands in the palindromic CpG sequence. An initial study in nine different tissues reported 1% of cytosines (estimated 70 – 80% of CpGs) in the human genome to be methylated [8]. Interestingly, while 5mC has almost exclusively been found at CpGs, regions of the genome that are enriched for CpG sites, so-called CpG islands (CGIs), are often devoid of any DNA methylation [9]. CGIs can frequently be found in promoter regions and the methylation of promoters is associated with silenced gene expression [10, 11]. A lack of 5mC at promoters facilitates gene expression but does not automatically result in active transcription [12, 13], indicating that other factors are necessary to induce gene expression or that only a selected set of genes are directly regulated by promoter DNA methylation [14].

Due to its direct and well-characterized functional impact, DNA methylation at promoters has been studied intensely, whereas the role of DNA methylation in other regions of the genome is less well understood. The presence of 5mC in gene bodies has been suggested to promote transcription [15] and at intergenic regions, DNA methylation silences retroviruses and retrotransposons preventing their activation and transposition [16]. There is some indication that loss of 5mC is connected to enhancer activation, but others have failed to replicate these results calling into question the role of DNA methylation at distal regulatory elements [17].

The clearest examples showing the regulation of gene expression by DNA methylation can be found at imprinted regions and across the inactive X chromosome (discussed further below). Imprinted genes are methylated early during development and silenced allele-specifically; on the same singular parental chromosome in all cells [18]. Apart from imprinting, the direct connection between promoter DNA methylation and loss of gene expression has been shown for some, often germline-specific, genes [19, 20]. Nevertheless, how this effect is mediated remains to be defined. Steric hindrance of transcription factor binding [21, 22], the activity of DNA methylation-binding proteins [23, 24], and recruitment of histone modifiers and chromatin remodelers [25-27] have all been suggested to play a role [28].

The dynamics of DNA methylation

After fertilization, DNA methylation is almost completely eliminated from both the paternal and maternal genome. During development, DNA methylation patterns are re-established and are a key factor of cellular commitment and differentiation into diverse cell lineages [29, 30]. For the dynamic remodelling of DNA methylation patterns seen during development, enzymes need to be in place that facilitate the addition and removal of methyl-groups from DNA (**Figure 1**).

A group of enzymes called DNA methyltransferases (DNMTs) have been shown to establish DNA methylation. DNMT3A and DNMT3B are *de novo* DNMTs, which establish novel DNA methylation at previously unmethylated cytosines. Loss of DNMT3A and DNMT3B in mouse embryonic stem cells (ESC) is lethal [31, 32], likely due to their role in establishing imprinting [33]. In contrast, DNMT1 is a maintenance methyltransferase, which preferentially binds to hemi-methylated CpG sites and adds DNA methylation to the unmethylated strand. As part of the DNA replication complex [34], DNMT1 is mainly active during S-phase, establishing DNA methylation patterns on the newly synthesized DNA strand [35]. Thus, DNMT1 is essential for the inheritance of DNA methylation patterns by ensuring maintenance of 5mC during mitotic cell division.

For many years it was assumed that no active removal of DNA methylation occurred. However, in 2009 it was discovered that ten-eleven translocation enzyme 1 (TET1) can oxidize 5mC to 5-hydroxymethylcytosine (5hmC) [36]. Shortly after, it became clear that all three enzymes of the TET family, TET1, TET2, and TET3, not only have the ability to oxidize 5mC to 5hmC, but further to 5-formylcytosine (5fC) and finally 5-carboxycytosine (5caC) (**Figure 1**) [37, 38]. As DNMT1 only maintains 5mC but neither of its derivatives, a passive demethylation pathway was proposed, whereby DNA methylation is lost during cell proliferation after oxidation of 5mC by the TETs. He *et al.* suggested there to also be an active demethylating process where 5fC and 5caC are replaced by unmethylated cytosine through base excision repair triggered by the thymine DNA glycosylase (TDG) [39] (**Figure 1**).

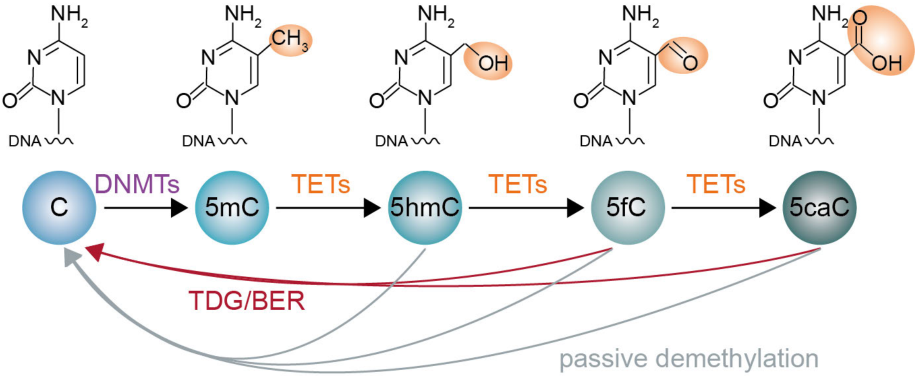


Figure 1: Dynamic DNA methylation. Methyl-groups are added to the fifth carbon of unmethylated cytosines (C) by DNA methyltransferases (DNMTs). 5-methylcytosine (5mC) can be oxidized by ten-eleven translocation (TET) enzymes to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and finally 5-carboxylcytosine (5caC). During active DNA demethylation, 5fC and 5caC are converted to unmethylated cytosine by thymine DNA glycosylase (TDG)-dependent base excision repair (BER). 5hmC, 5fC, and 5caC are not maintained during cell division and their existence can thus result in loss of DNA methylation during replication (passive demethylation).

Both passive and active demethylation has been shown to be responsible for loss of DNA methylation after initiation of the process by the TET enzymes [40, 41]. While 5hmC is relatively common in the mammalian genome, 5fC, and 5caC are significantly rarer. The discrepancy in the amount of 5mC derivatives is most likely caused by a reduced reaction efficiency of the oxidization reaction catalysed by TETs for the conversions of 5hmC to 5fC and of 5fC to 5caC [37]. As all three TET enzymes can perform the same DNA demethylating function, knockouts of individual TET enzymes in mice do not impair embryogenesis. Triple knockouts of all three *Tets*, however, are embryonically lethal [42], highlighting the vital role that remodelling of DNA methylation plays during embryogenesis.

Histone modifications

Apart from DNA methylation, chromatin organization is an essential epigenetic mechanism guiding gene expression. DNA is tightly wound around histones, forming a structure called the nucleosome. Each nucleosome is formed of eight globular histones, two of each of histones 2A, 2B, 3 and 4 (H2A, H2B, H3 and H4), around which 147 bases of double stranded DNA are wrapped twice [43, 44]. How closely together nucleosomes are packed affects the accessibility of the DNA and thus the possibility of active gene expression. Heterochromatin is characterized by condensed chromatin and transcriptional inactivity whereas euchromatin allows for active transcription as nucleosomes are less tightly packed (**Figure 2**). Modifications on histone tails, amino acid chains protruding from each histone in the nucleosome, can affect chromatin conformation and transcription. Since the first discovery of acetyl- and methyl-groups on histone tails and their effect on expression [45], a multitude of further histone modifications has been identified including acetylation, methylation, ubiquitylation, phosphorylation, sumoylation, ADP ribosylation, deamination, β-N-acetylglucosamine, and proline isomerization [46, 47]. However, lysine acetylation and lysine or arginine methylation remain the best described histone modifications (**Figure 2**).

Lysine acetylation is added by histone acetyltransferases (HATs), removed by histone deacetylases (HDACs), and directly affects chromatin packaging. The acetyl-group neutralizes the positive charge of lysine, weakening histone interaction with the negatively charged DNA and thereby promoting a more open chromatin structure and gene transcription [48]. Histone methylation can occur at lysine or arginine, where lysine are either mono-, di-, or trimethylated, while arginine show mono-, or dimethylation. Various lysine or arginine methylases and histone demethylases add and remove methylation groups [49].

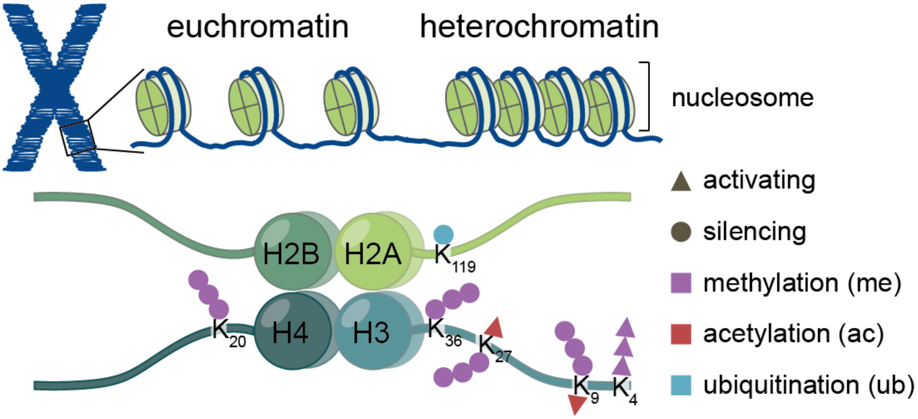


Figure 2: Chromatin. Chromatin can be divided into transcriptionally active euchromatin and silenced heterochromatin. Nucleosomes are formed of DNA wrapped around eight histones (H2A, H2B, H3 and H4). Many amino acids in histone tails that protrude from all four types of histones can be modified by various modifications. A selection of lysine (K) modifications are shown here.

The two repressive histone marks, trimethylation of lysine 9 and lysine 27 of histone 3 (H3K9me3 and H3K27me3) define constitutive and facultative heterochromatin, respectively. Constitutive heterochromatin, enriched for H3K9me3, can be found at centromeres, telomeres, repetitive elements, and other genomic regions that are permanently silenced [50]. Facultative heterochromatin on the other hand is located at genomic locations that are active in certain developmental contexts but are silenced in most differentiated tissues [47]. Proteins with methyl-binding domains are known to bind to methylated histones and in turn recruit effector proteins such as transcription factors or other chromatin modifiers [51]. Conversely, methylation at different positions of the histone tail can have opposing effects on gene expression. While

H3K9me3 and H3K27me3 are found in inactive genomic locations, H3K4me3 is associated with active transcription [52].

Histone modifications can interact with each other. For example, the inactivating mark H3K27me3 and the activating modification H3K27ac are mutually exclusive as they are found at the same amino acid and cannot co-occur, while H3K27me3 and ubiquitylation of lysine 119 of histone H2A (H2AK119Ub) promote establishment of each other [53]. Furthermore, patterns of DNA methylation and histone modifications are highly correlated. DNA methylation only rarely co-occurs with H3K27me3 but has been seen to overlap heterochromatic domains marked by H3K9me3 [54]. Indeed, to promote the establishment and maintenance of constitutive heterochromatin, DNA methylation-binding proteins can interact with histone modifiers [55, 56]. Methylation at H3K4 on the other hand has been suggested to prevent establishment of DNA methylation during development and DNA methylation is not found to overlap with H3K4me3 [57].

Non-canonical DNA methylation

Whereas the role of 5mC in transcriptional silencing, imprinting, and silencing of transposable elements (TEs) is well described, the function of non-canonical forms of DNA methylation is less clear.

5hmC has been found to not only be a transient mark during DNA demethylation but also a stable DNA modification in its own right [58]. 5hmC was first discovered to be present in cells of the central nervous system by Kriaucionis and Heintz who estimated 5hmC to represent up to 0.6% of all nucleotides (40% of 5mC) in Purkinje cells [59]. Further studies across several tissues found that 5hmC is present in all tissues at varying quantities [60-62]. 5hmC has been found primarily at gene bodies of tissue-specific genes where it is associated with active expression [63, 64]. If and how 5hmC promotes gene expression is still debated, but binding of transcription factors or chromatin modifiers to 5hmC has been suggested as a mechanism as well as the possibility that 5hmC is involved in mRNA processing by facilitating alternative splicing [65]. Another open question that remains is how 5hmC profiles at tissue-specific genes are established and maintained throughout cell division, considering that 5hmC modifications are not known to be retained during DNA replication [66]. Generally, 5hmC is considered to be vital during neuronal development and disruption of 5hmC has been associated with neurodevelopmental and neurodegenerative diseases [67].

While the majority of 5mC can be found at CpG dinucleotides, there have been an increasing number of reports of non-CpG DNA methylation. Initially, non-CpG methylation was found in ESCs and induced pluripotent cells and was thus suggested to be associated with pluripotency [68, 69]. The Meissner group reported up to 15% of cytosine methylation to be localized at non-CpG sites (12% CpA, 2.6% CpT and 1.2% CpC) [69]. More recent research identified non-CpG methylation in the brain of vertebrates, but not other species, at developmental genes conserved across vertebrates [70]. The exact function of non-CpG methylation is not yet clear but its presence in gene bodies has been associated with transcriptional silencing and disruption of non-CpG methylation has been seen in various cancers [71].

A different cytosine modification is known to be present in bacteria, namely methylation of the amine group at C4 of cytosines (4mdC). In bacteria, 4mdC is a part of the restriction-modification system, which protects bacteria against foreign DNA, and might be involved in bacterial virulence [72]. Although 4mdC is not a known epigenetic mark in eukaryotes, it has been identified in the invertebrates *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Bdelloid rotifers* in single studies that have not yet been reproduced [73, 74]. O'Brown and colleagues have revealed systemic overestimation of 4mdC in analyses using mass spectrometry and single molecule real-time sequencing [75]. Not surprisingly, the study of 4mdC currently focuses on the development and improvement of genome-wide analysis approaches of this modification [74, 76-79].

The methylation of adenine (6mdA) is the principal DNA modification in bacteria with well described roles in virulence and the restriction-modification system [80]. Methylation of adenine in RNA is common in mammals [81, 82] and in the last decade the DNA modification 6mdA has been described in various mammalian cell types, including ESCs and brain tissue [83-87]. 6mdA has been proposed to be involved in several cellular functions such as DNA damage control, cell cycle regulation, chromatin regulation, and activation of gene expression [88]. Stress response and cancer have also been associated with dysregulation of 6mdA [88]. However, others have questioned the existence of 6mdA in mammals [75, 89] and further studies are required to reveal the potential involvement of 6mdA in human biology.

DNA methylation and cancer

While chromatin remodelling and non-canonical DNA methylation have been associated with malignancies [90], this work will focus solely on the involvement of CpG methylation in cancer biology.

DNMTs and TETs, the enzymes involved in addition and removal of 5mC (**Figure 1**), are frequently mutated in cancers. Furthermore, mutations in the isocitrate dehydrogenases *IDH1* and *IDH2* result in reduced TET activity [91]. Mutations in genes coding for these eight enzymes are especially common in haematopoietic malignancies [38, 92-95], but have been frequently found in solid malignancies as well [96-100]. In particular, *TET2* is a commonly described tumour suppressor gene [101-103], which is dysregulated in 10-30% of myeloid cancers (myelodysplastic syndromes, chronic myelomonocytic leukaemia, myeloproliferative neoplasm) and 40-70% of patients with lymphoid malignancies (peripheral T-cell lymphoma, angioimmunoblastic T-cell lymphoma) [104]. The abundance of mutations in genes regulating DNA methylation underlines the significance of DNA methylation in cancer.

It has long been described that cancers undergo widespread changes in their DNA methylation landscape compared to healthy cells. Tumours are generally characterized by global loss of 5mC with locus-specific gains of DNA methylation [105-107] (**Figure 3**). Intergenic regions, which tend to be highly methylated in healthy cells, become hypomethylated, causing activation of TEs, demethylation of repeats and increased genome instability [105, 108-112]. Expression of imprinted genes has also been shown to be dysregulated in connection with gain of DNA methylation at the unmethylated allele of imprinting control regions (ICR) [113, 114]. Promoters of tumour suppressor genes are hypermethylated in malignant cells resulting in aberrant silencing [115, 116]. In contrast to tumour suppressor genes, oncogenes have been shown to be upregulated in response to promoter hypomethylation [117, 118] and gain of DNA methylation at their gene bodies [119] (**Figure 3**).

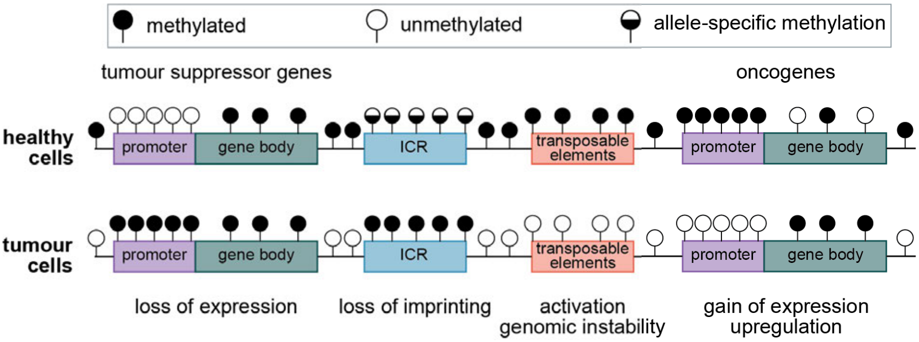

methylated
unmethylated
allele-specific methylation
tumour suppressor genes
oncogenes
healthy cells
tumour cells
promoter
gene body
ICR
transposable elements
promoter
gene body
loss of expression
loss of imprinting
activation
genomic instability
gain of expression
upregulation

Figure 3: DNA methylation in cancer. Tumour cells undergo extensive DNA methylation changes. Promoters of tumour suppressor genes, imprinting control regions (ICR), and gene bodies of oncogenes gain DNA methylation, while DNA methylation is lost at intergenic regions, such as transposable elements, and at promoters that regulate oncogene expression.

Considering that with histone modifications and DNA methylation several layers of epigenetic marks can often be found to work in tandem to maintain control of gene expression, many changes seen in DNA methylation in malignancies are most likely non-functional [13, 120, 121]. Nevertheless, extensive research has been conducted to identify specific CpGs or differentially methylated regions, which can identify tumour type, predict prognosis, or aid treatment decisions for cancer patients. The 'Heidelberg classifier' from the German Cancer Consortium, which uses DNA methylation array data to aid diagnosis of brain tumours [122], might be the most successful instance to date of integrating the analysis of DNA methylation into clinical use. Furthermore, the CpG island methylator phenotype (CIMP) has been suggested to predict prognosis in colorectal cancer and T cell acute lymphoblastic leukaemia. Based on hyper- or hypomethylation at specific CGIs, mainly in promoter regions, patients are classed as either CIMP$^+$ (high methylation) or CIMP$^-$ (low methylation). The CIMP profile has been shown to be indicative of prognosis and risk for relapse [123-127]. This valuable work, providing proof-of-principle for the use of DNA methylation patterns in the characterization of malignancies, encourages further study of 5mC in cancer, aiming for a better understanding of the underlying mechanisms and possibilities for patient benefit.

Silencing a whole chromosome: the special case of the X

Biologically male cells (46,XY) have two copies of each autosome and one copy each of the X and Y chromosome, while female cells (46,XX) have two X chromosomes. To account for differences in gene dosage between the X chromosome and autosomes and between male and female cells, expression from one X chromosome is upregulated in all cells and the second X in females is inactivated [128-130]. The process of X chromosome inactivation (XCI) is one of the most complex and well-studied epigenetic processes in mammals.

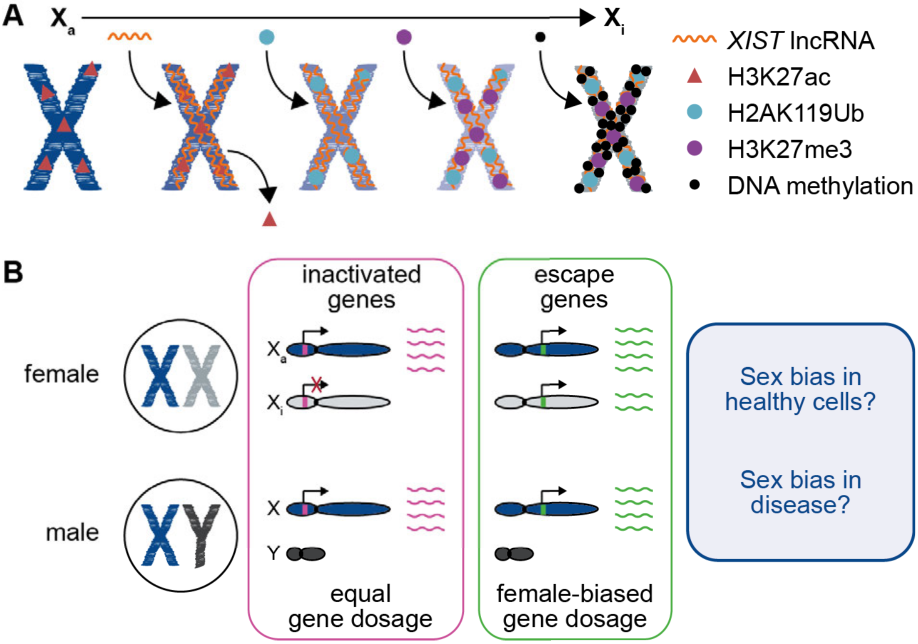
In 1949, the Canadian physician Murray Barr discovered a distinct difference between male and female cells. He described that a compact heterochromatic chromosome, which was later called the Barr body, could be seen at the edge of the nucleus in female but not male cells [131]. Twelve years later, in 1961, Mary Lyon suggested that this Barr body was the inactive version of one of the two X chromosomes in female cells [132]. The Lyon hypothesis proposes that all cells in humans and mice, except gametes, inactivate one X chromosome to compensate for the excess genetic material from the two X chromosomes found in females XX-cells compared to male XY-cells. She further hypothesized that the choice of which X chromosome becomes inactivated is random, but once XCI is established, the same X chromosome will be inactivated throughout all future cell divisions [133]. In the six decades since Lyon's pivotal hypothesis, the study of XCI has progressed significantly and the molecular mechanisms involved in this complex process have been extensively studied.

Establishing X chromosome inactivation

Inactivation of one X chromosome occurs rapidly during the early stages of embryogenesis in the blastocyst before implantation [134]. XCI is initiated by expression of the long non-coding RNA (lncRNA) *XIST* from the X inactivation centre (XIC) on one X chromosome. *XIST* binds in *cis* to the chromosome it is expressed from and marks it for inactivation [135]. Following coverage of the whole X chromosome by *XIST*, a complex of proteins is recruited, either by direct or indirect binding to *XIST* [136]. Several chromatin modifiers are part of the protein complex that bind to the future inactive X chromosome and establish a heterochromatin environment. HDAC3 removes the activating H3K27ac marks, followed by introduction of the repressive chromatin marks H2AK119Ub and H3K27me3 by polycomb repressive complex 1 (PRC1) and PRC2, respectively. Lastly, the inactive X chromosome (Xi) is covered by DNA methylation, resulting in the stable establishment of heterochromatin and silencing of gene expression [137] (**Figure 4A**). The process of XCI is best described in females but all cells with multiple X chromosomes, including Klinefelter syndrome (47,XXY) and other X chromosome aneuploidies, inactivate all X chromosomes except for one, suggesting that a mechanism exists that can 'count' the number of active X chromosomes in each cell [138].

Escape from X chromosome inactivation

The establishment of condensed heterochromatin on the Xi and its formation into a Barr body results in inactivation of most genes. However, not all genes on the Xi are completely silenced. Genes located in the pseudoautosomal regions (PAR) at each end of the X chromosome, which have complementary regions on the Y chromosome, are expressed from both the active X chromosome (Xa) and Xi. Apart from PAR genes, it is known that about 15% of genes on Xi remain expressed (**Figure 4B**).



A
X_a
X_i
XIST lncRNA
H3K27ac
H2AK119Ub
H3K27me3
DNA methylation
B
female
male
XX
XY
inactivated genes
X_a
X_i
X
Y
equal gene dosage
escape genes
female-biased gene dosage
Sex bias in healthy cells?
Sex bias in disease?

Figure 4: Establishment and escape from X chromosome inactivation. (**A**) Expression and binding of the long non-coding RNA *XIST* initiates the process of epigenetic remodelling, which forms an active X chromosome (Xa) into an inactive X chromosome (Xi). (**B**) Expression of escape genes from the inactive X chromosome results in higher expression of these genes in females compared to males. This female-biased gene dosage can have consequences for female health and disease.

These genes that escape XCI are known as ‘escape’ genes. The expression of escape genes from Xi varies, but is generally assumed to be roughly 50% of the expression from Xa [139]. A subset of escape genes has homologues on chromosome Y (e.g. KDM6A, DDX3X) and might be expressed from both X chromosomes in females to equalize gene expression dosage compared to male cells [140]. However, most escape genes are X-specific and are expressed more highly in female cells.

Attempts have been made to create consensus lists of genes that escape XCI in humans, but previous studies identified variability between individuals and across different tissues [141, 142]. While analysis of XCI escape is challenging when assaying several cells at the same time (e.g. bulk sequencing), the development of single cell sequencing is likely to advance our understanding of escape from XCI in human cells significantly [143].

Escape genes are characterised by the absence of repressive chromatin marks and a lack of DNA methylation at their promoters [139]. Nevertheless, how some genes are expressed from Xi remains to be clearly defined. The absence of long interspersed repeat sequence elements (LINE) in close proximity to escape genes and the physical structure of the Xi have both been suggested to facilitate escape. The Xi can be divided into two distinct topologically associating domains (TADs) that form the 3D structure of Xi, with escape genes preferentially located on the outside of the compact Barr body, suggesting easier access of for example transcription factors to escape genes [144].

T cells: the orchestrators of our immune system

Humans have developed complex mechanisms to fight against bacteria, viruses, and other pathogens, which we are constantly exposed to. Our immune system can be divided into two branches: the innate and the adaptive immune system. The innate immune system is responsible for a first quick response to an invading pathogen aimed at preventing the fast spread of an infection. Adaptive immunity offers a more targeted response to an antigen and retains a memory of the pathogen, which allows a faster clearance of the infection should the pathogen be re-encountered. T lymphocytes are a central part of the adaptive immune system involved in the detection, clearance, and memory of an infection. T cells also regulate our immune system and prevent targeting of self-antigens, thereby creating a balance between immunity and tolerance [145].

T cell development in the thymus

T cells develop in the thymus, where they undergo several selection steps to ensure proper T cell function (**Figure 5A**). Early thymic progenitor cells (ETPs) migrate from the bone marrow to the thymus. ETPs are pluripotent cells that have the ability to develop into multiple immune cell types apart from T cells, such as B cells or natural killer cells. Once ETPs have entered the cortex of the thymus, NOTCH signalling promotes their survival but also drives the progress of ETPs towards CD4 and CD8 negative cells (double negative, DN). DNs are committed to the T cell lineage and undergo T cell receptor (TCR)-β rearrangement. Pre-TCR signalling (β-selection) and NOTCH allow DN cells to develop into double positive cells (CD4$^+$ and CD8$^+$, DP), which undergo further TCR rearrangements (TCRα,γ,δ) [146]. Thymocyte development is closely regulated by the surrounding thymic epithelial cells (TECs) and the location of each precursor T cell in the thymus. Only those DP cells that are able to bind to the major histocompatibility complex (MHC), also called human leukocyte antigen (HLA), presented by cortical TECs, develop into either CD4$^+$ or CD8$^+$ single positive (SP) cells, while all other cells undergo apoptosis. Whether cells develop into CD4$^+$ or CD8$^+$ SP cells depends on which HLA class they bind to. CD4$^+$ SP T cells bind to HLA class II and CD8$^+$ cells to HLA class I. After this positive selection, travel SP cells into the medulla of the thymus to undergo negative selection, which entails presentation of self-antigens by medulla TECs to SP cells. Cells reacting to self-antigens are either undergo apoptosis or are induced to become regulatory T cells (Treg) (**Figure 5A**) [145-147]. Naïve CD4$^+$ and CD8$^+$ T cells that have passed both positive and negative selection then move out of the thymus into the blood and lymph nodes to become an active part of our immune system. T cell development occurs primarily during childhood, as the thymus shrinks with age and output of naïve T cells decreases in a process called thymic involution. Studies have suggested that the immunosuppression observed with older age could, in part, be caused by a lack of T cell diversity due to reduced thymic output [148, 149].

T cell function in the blood

CD4$^+$ and CD8$^+$ T cells have distinct functions to aid in the clearance of invading pathogens. Naïve CD4$^+$ T cells become activated in response to a combination of three signals, which are (i) recognition of an antigen by the TCR, (ii) an activation signal from an antigen presenting cell, and (iii) the presence of distinct cytokines. Depending on the type of pathogen, naïve CD4$^+$ T cells can differentiate into various subsets of T helper cells (Th) guided by stimulation by different cytokines, distinct signal cascades, and transcription factors (**Figure 5B**). Th1, Th2, and Th17 are the most well described T helper cells, but in recent years more subtypes have been described, such as Th9, Th22, and Tfh [145]. Th1 cells respond to bacteria, viruses, and other intracellular infections. Differentiation into Th2 cells occurs in response to multicellular extracellular pathogens, such as helminths, and Th17 cells are important for the defence against fungi and extracellular bacteria by promoting a local immune response in the mucosa often in cooperation with Th1 cells [145, 150]. Through the production of specific cytokines (**Figure 5B**), these T helper cells are vital for the immune response by promoting and enhancing the function of cytotoxic CD8$^+$ T cells and cells of the innate immune system, such as macrophages, mast cells, eosinophils, and neutrophils [151, 152]. In contrast, Tregs exercise a dampening effect on the immune response to prevent the overreaction of the immune system. Tregs also create tolerance to self-antigens to avoid autoimmunity [145, 150].

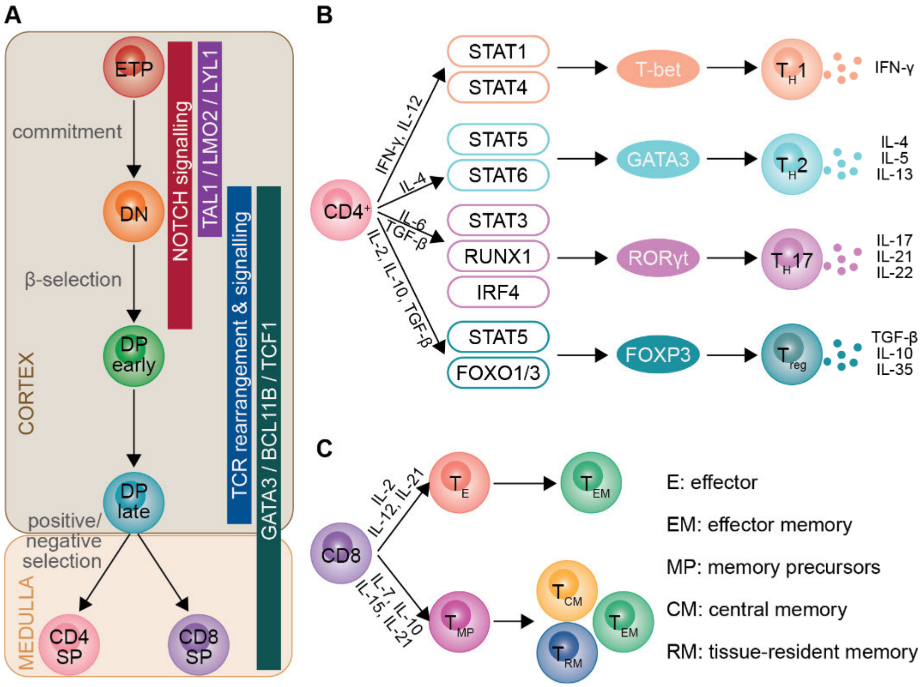

A
ETP
commitment
DN
β-selection
DP early
DP late
positive/ negative selection
CD4 SP
CD8 SP
CORTEX
MEDULLA
NOTCH signalling
TAL1 / LMO2 / LYL1
TCR rearrangement & signalling
GATA3 / BCL11B / TCF1
B
CD4+
IFN-γ, IL-12
IL-4
IL-6, TGF-β
IL-2, IL-10, TGF-β
STAT1
STAT4
STAT5
STAT6
STAT3
RUNX1
IRF4
STAT5
FOXO1/3
T-bet
GATA3
RORγt
FOXP3
TH1
TH2
TH17
Treg
IFN-γ
IL-4
IL-5
IL-13
IL-17
IL-21
IL-22
TGF-β
IL-10
IL-35
C
CD8
IL-2, IL-12, IL-21
IL-7, IL-10, IL-15, IL-21
TE
TEM
TMP
TCM
TEM
TRM
E: effector
EM: effector memory
MP: memory precursors
CM: central memory
RM: tissue-resident memory

Figure 5: T cell development and differentiation. (**A**) During their development in the thymus, T cell progenitors undergo multiple developmental and selection steps driven by various signalling and transcription factors. ETP, early thymic progenitors (CD34$^+$); DN, double negative (CD4$^-$, CD8$^-$); DP, double positive (CD4$^+$, CD8$^+$); SP, single positive (CD4$^+$ or CD8$^+$); TCR, T cell receptor. (**B**) Differentiation of naïve CD4$^+$ T cells into different T helper cell subtypes (T_H) driven by cytokine stimulation and the activation of key transcription factors. T_{reg}, regulatory T cells. (**C**) Differentiation of cytotoxic CD8$^+$ T lymphocytes into effector and memory T cells.

In contrast to CD4$^+$ T helper cells, have cytotoxic CD8$^+$ cells a direct role in the removal of infected cells. Upon antigen recognition, naïve CD8$^+$ T cells become effector CD8$^+$ T cells, which directly instigate pathogen clearance through their cytolytic capacity and the production of cytokines. After an initial expansion of CD4$^+$ and CD8$^+$ T cells by activation and proliferation at the beginning of an infection, the number of T cells decreases once the infection has been cleared [145]. However, both CD4 and CD8 T cells have the capacity to form memory cells (**Figure 5C**), which can persist for years and become re-activated when an antigen is re-encountered, thereby allowing for an immediate directed immune response [151, 153, 154]. Effector memory T cells (T_{EM}) can revert back to effector T cells (T_E) when exposed to an acute infection with a known pathogen while central memory T cells (T_{CM}) have been hypothesized to protect from chronic infections [155].

Epigenetic regulation in T cells

Dynamic changes of the epigenome are essential for both T cell development and T cell differentiation and have been extensively summarized before (for example: [156-158]). Knockouts of individual histone modifiers in mice have revealed the consequences of faulty chromatin remodelling during thymocyte development. For example, silencing of *Pax9* and suppression of the B cell lineage by PRC1-established H2AK119Ub is essential for the progression of DN cells [156]. Loss of EZH2, a member of PRC2, which introduces H3K27me3, stalls development of immature thymocytes and can result in progression of ETPs towards T cell acute lymphoblastic leukaemia in p53 deficient cells [159]. Histone modifications are further involved in T helper cell differentiation by establishing the necessary transcriptional profiles. Genes encoding lineage-specific interleukins and the key transcription factors, *TBET*, *GATA3*, *RORγt*, and *FOXP3*, have been shown to lose repressive histone marks (H3K27me3) and gain activating histone modifications (H3 acetylation and H3K4me3) [160-163].

Similarly, thymocytes and differentiating T cells undergo widespread alterations of their DNA methylation profile [158, 164]. Not only can remodelling of 5mC be seen in T cells, the presence of 5hmC at gene bodies of highly expressed genes that drive T cell development and differentiation has also been reported [165, 166]. While knockouts of individual TET enzymes in mice only slightly impair T cell development and activation [158, 167], this could be attributed to the impossibility of creating viable TET triple knockout mice. As DNMT1 primarily maintains DNA methylation profiles on the newly synthesized DNA strand during DNA replication, it can be expected that DNMT1 is highly active during stages including bursts of cell division. Indeed, Dnmt1 knockouts in mice show a lack of DP cells caused by lower cell proliferation after β-selection [168]. Inducing loss of DNMT3A in mouse models results in accumulation of immature thymocytes (ETP, DN) and can lead to the development of T cell acute lymphoblastic leukaemia (T-ALL) if combined with increased NOTCH signalling [169].

T cell acute lymphoblastic leukaemia: when thymocytes transform into cancer

The process of thymocyte development encompasses survival signals to immature ETP and DN cells through NOTCH but also rapid cell growth during the DP stages and TCR gene rearrangement. This combination of DNA rearrangement, cell proliferation, and survival needs to be strictly regulated by transcription factors and epigenetic processes because loss of control over thymic T cell development can lead to malignant transformation of thymocytes towards T-ALL (**Figure 6**) [170].

The biology of T cell acute lymphoblastic leukaemia

T-ALL is a haematopoietic cancer, which is typified by uncontrolled proliferation of immature T cells that infiltrate the bone marrow and blood. Patients often present with increased white blood cell count and haematopoietic failure (neutropenia, anaemia, thrombocytopenia). Malignant blasts can frequently also be found in the central nervous system (CNS) [171]. T-ALL is mainly a paediatric disease with an overall incidence of 2.6 cases per 1 million in the US, of which only about one third are adults [172, 173]. In 1981, T-ALL was first classified as a separate subgroup of ALL, distinct from the more common B-ALL. About 15% of paediatric and 25% of adult ALL cases are diagnosed as T-

ALL [174, 175]. Furthermore, T-ALL shows a prominent sex-bias with males having a more than 2-fold higher incidence than females [172, 174]. T-ALL can arise from any stage during thymocyte development after accumulation of multiple incidences of chromosome rearrangements, chromatin remodelling, and mutations in cell cycle regulators and key drivers of T cell development [170, 171]. Currently, patients are routinely grouped into subtypes based on the expression of surface and cytosolic markers, which indicate the cell type of origin (**Figure 6**) [171, 176, 177]. Particularly T-ALL originating from ETP (ETP-ALL) has been studied as a distinct class which encompasses about 10% of paediatric and 40-50% of adult cases [171]. However, it can be questioned how well the separation by surface and cytosolic markers reflects the complexity of T-ALL, particularly considering the diversity seen in patients with immature T-ALL [178].

With the rise of next-generation sequencing technologies, an increasing effort has been made to uncover mutations involved in T-ALL tumorigenesis [171, 179, 180]. The most prominent driver of T-ALL is continuous activation of NOTCH in the absence of its ligands. Gain of function mutations of *NOTCH1* can be found in up to 60% of T-ALL cases [181]. Furthermore, inactivating mutations of *FBXW7*, which is mutated in 30% of patients, similarly results in aberrant NOTCH1 activation [182, 183]. Together, over 80% of patients show *NOTCH1* dysregulation through mutations in *NOTCH1* or *FBXW7* [179]. The second most common mutation in T-ALL is deletion of the cell cycle regulator *CDKN2A*/*CDKN2B* in more than 70% of patients, which results in loss of the tumour suppressors p16 and p14 [171]. Both *NOTCH1* and *CDKN2A/2B* aberrations are relatively rare in ETP-ALL, but almost universal in more mature T-ALL (**Figure 6**) [171]. As NOTCH signalling is essential during the early stages of thymocyte development, the scarcity of *NOTCH1* mutations in immature T-ALL might be anticipated. In turn failure to inactivate NOTCH after β-selection seems to contribute to development of T-ALL. Several additional genes, which are essential for T cell development, have been found to be dysregulated and appear to be enriched in particular subgroups of T-ALL. The most prominent dysregulated genes are *BL11B*, *GATA3*, *HOXA*, *LMO1*, *LMO2*, *LYL1*, *NKX2-1*, *PTEN*, *RAS*, *TAL1*, *TLX1*, and *TLX3* (**Figure 6**) [171, 180].

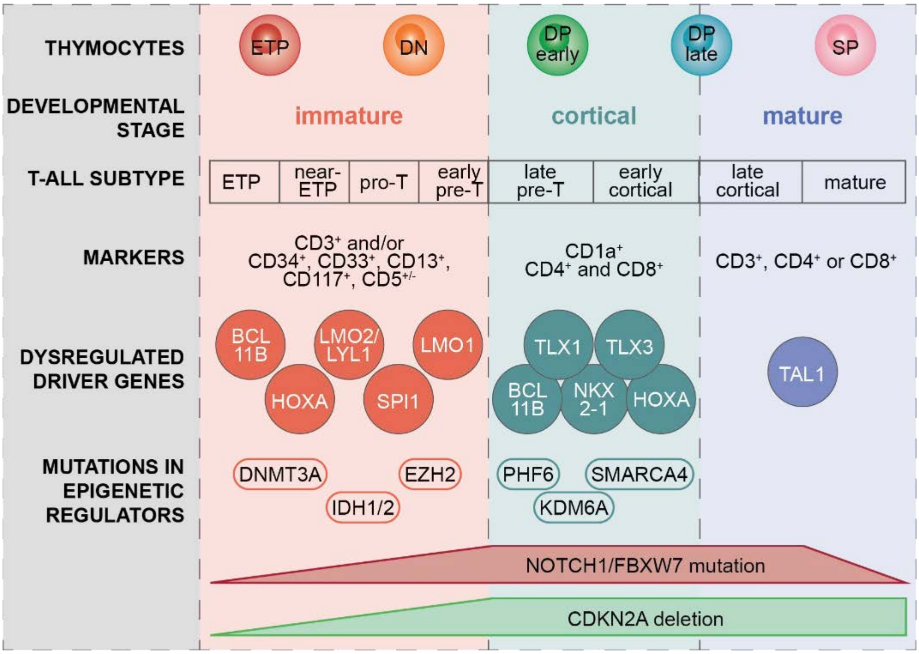

THYMOCYTES
ETP
DN
DP early
DP late
SP
DEVELOPMENTAL STAGE
immature
cortical
mature
T-ALL SUBTYPE
ETP
near-ETP
pro-T
early pre-T
late pre-T
early cortical
late cortical
mature
MARKERS
CD3⁺ and/or CD34⁺, CD33⁺, CD13⁺, CD117⁺, CD5⁺/⁻
CD1a⁺ CD4⁺ and CD8⁺
CD3⁺, CD4⁺ or CD8⁺
DYSREGULATED DRIVER GENES
BCL 11B
LMO2/ LYL1
LMO1
HOXA
SPI1
TLX1
TLX3
BCL 11B
NKX 2-1
HOXA
TAL1
MUTATIONS IN EPIGENETIC REGULATORS
DNMT3A
EZH2
IDH1/2
PHF6
SMARCA4
KDM6A
NOTCH1/FBXW7 mutation
CDKN2A deletion

Figure 6: T cell acute lymphoblastic leukaemia. Schematic representation of the different subgroups of T cell acute lymphoblastic leukaemia (T-ALL), including the stages of thymocyte development the cancer originates from. Markers used for classification, known drivers of T cell development that are commonly dysregulated, and mutations seen in epigenetic regulators are indicted for T-ALL from each developmental stage. ETP, early thymic progenitors; DN, double negative; DP, double positive; SP, single positive. Adapted from [184].

Prognosis, treatment, and relapse

Forty years ago, paediatric T-ALL was considered highly severe with a cure rate of only 10% [174]. Optimization of therapeutic regimes in the last decades has resulted in vast improvement of prognosis and represents one of the great success stories in cancer treatment. With current treatment protocols, over 80% of paediatric [185] and 60% of adult [186, 187] T-ALL patients survive. This improvement can be attributed to implementing a more intense treatment regime to both paediatric and adult T-ALL cases [188, 189]. Similarly, ETP-ALL was considered to have poorer prognosis and higher risk for failure of treatment induction [190]. However, therapy intensification has improved prognosis for both children and adults with ETP-ALL [178]. Despite these improvements in treatment regimes, patients experiencing relapse have only a 25% chance of long-term survival [191]. There are 12-15 different chemotherapeutic agents that have been used to treat T-ALL in different clinics with three to four drugs usually used in combination [192]. Methotrexate, dexamethasone, and asparaginase are often used for the treatment of T-ALL [176] and have shown favourable outcome in reports from several treatment consortia [192-194]. There is a markedly worse prognosis in cases of CNS involvement [195] and therefore radiation therapy has routinely been used to prevent the spread of malignant blasts to the CNS. Conversely, several retrospective studies have seen limited or no benefit of radiotherapy towards event free survival or reduction of risk for relapse [196, 197]. Minimum residual disease (MRD) after consolidation therapy is considered the most important predictor of prognosis in T-ALL [192, 198] despite the mentioned efforts to classify T-ALL patients into subgroups. More recently, researchers have suggested that combining MRD with white blood cell count and the presence of somatic or germline mutations in *NOTCH1*, *FBXW7*, *RAS*, and *PTEN* provides an even better indication for risk of relapse [199].

As relapsed T-ALL is often resistant to conventional chemotherapy agents and has a high rate of CNS infiltration, there is a need for better treatment options for patients in relapse. Nelarabine, which can target the

CNS, has been approved for treatment of relapsed and refractory T-ALL with an overall response of 40% and 33% in paediatric and adult patients, respectively [200-202]. Even transplantation of allogeneic haematopoietic stem cells has been considered for relapsed T-ALL [176]. Interestingly, there have been several case reports of successful treatment of relapse T-ALL with the DNMT inhibitors 5-azacytidine or decitabine in combination with the BCL2 inhibitor Venetoclax [203-205]. Further promising treatments are currently in clinical trials [192] and include the JAK-STAT inhibitor ruxolitinib [192] and CAR T cell therapy [198].

DNA methylation and T-ALL

The effort to characterize T-ALL subgroups by sequencing also offered the potential to investigate possible mutations in any of the genes involved in addition or removal of DNA methylation. However, while the majority of the 264 patients in a cohort from St Jude Children's Research Hospital had mutations in epigenetic regulators in general, only six (2.3%) presented with mutations in *DNMT1*, *DNMT3A*, *IDH2*, or *TET1* [179]. One reason why this study only found a limited number of mutations in genes connected to DNA methylation could be that they included almost exclusively paediatric patients. Other studies have shown that mutations in *DNMT3A* are rare in paediatric T-ALL [206] but occur in more than 16% of adult patients [207-209]. Genetic aberrations in *IDH2* have been similarly linked to older age [209, 210]. Mutations in both *DNMT3A* and *IDH2* were associated with the more immature ETP-ALL group (**Figure 6**) and were found to be a risk factor for shorter overall survival and relapse [171, 208, 210]. Interestingly, *TET1* and *TET2* seemed to have highly dissimilar roles in T-ALL. Poole *et al.* showed *TET1* to be overexpressed and *TET2* to be repressed in T-ALL compared to peripheral blood mononuclear cells [211, 212]. They further found that knockdown of *TET1* and overexpression of *TET2* in T-ALL cell lines resulted in distinct global profiles of 5mC and 5hmC, primarily linked to introns [211].

Studies using DNA methylation arrays have shown that T-ALL has a DNA methylation profile that is distinct from their thymic cells of origin [213] but DNA methylation can nevertheless be used as a classifier of thymocyte differentiation stage and T-ALL subtype [214, 215]. There has been an extensive effort to group patients based on the level of DNA methylation found at CGIs (CIMP). Studies by separate groups and in differing patient cohorts identified CpGs or promoters that were representative for CIMP status. These regions overlapped partially, but not completely. Nevertheless, all studies agreed that CIMP$^-$ (low DNA methylation) was associated with worse overall survival and an increased risk for relapse [215-219]. While there is strong evidence that CIMP can aid in determining prognosis, the functional consequences of the altered DNA methylation observed in T-ALL are more uncertain. Haider and colleagues, for example, observed that expression and DNA methylation correlated in only 30% of genes [219]. Furthermore, hypermethylated regions in T-ALL were enriched for H3K27me3 target genes that were not expressed in either T-ALL patient samples or primary thymocytes [125, 216]. These results suggest that changes in DNA methylation might be a representation of malignant transformation and replication instead of driving cancer progression by targeting of specific tumour suppressors or oncogenes [220]. Indeed, whole genome bisulfite sequencing of T-ALL and thymocytes revealed that gain of DNA methylation at promoters occurred randomly and no target genes could be distinguished that were consistently aberrantly methylated. The same study instead identified a group of hypermethylated T-ALL patients that showed an overall high level of DNA methylation with increased 5mC at promoters and no loss of DNA methylation at intergenic regions compared the healthy cells [221].

Epigenetic therapy against cancer: targeting DNA methylation

Widespread changes in the DNA methylation landscape are seen in all types of cancer. More importantly, in contrast to mutations in the DNA sequence, epigenetic aberrations are, in theory, reversible. Particularly the gain of DNA methylation and subsequent silencing of tumour suppressor genes represent a promising target for therapeutic intervention [222, 223].

Cytidine analogues as DNA hypomethylating agents

Currently, two drugs targeting DNA methylation are approved for clinical use in the treatment of the haematological malignancies myelodysplastic syndromes (MDS) and acute myeloid leukaemia (AML), namely 5-azacytidine (AZA) and 5-aza-2′deoxycytidine (decitabine, DEC). Both drugs are cytidine analogues (**Figure 7A**) that induce a loss of 5mC and are therefore summarized under the term hypomethylating agents (HMAs). AZA and DEC were first synthesized in 1964 and shown to inhibit cancer cell growth [224]. The epigenetic effects of these drugs were only discovered in 1980 by Taylor and Jones [225], who reported that the use of AZA and DEC resulted in DNA demethylation (for a timeline over HMA development and introduction into the clinic see **Figure 7B** [224-235]).

Incorporation of AZA and DEC into DNA causes trapping of the maintenance DNA methyltransferase DNMT1 on the newly synthesized DNA strand and ultimately results in degradation of DNMT1 and loss of DNA methylation [236-239]. After import into the cell, AZA and DEC undergo three phosphorylation steps to produce their active forms of 5-aza-CTP or 5-aza-dCTP, which are incorporated into RNA or DNA, respectively. Only 10-20% of AZA but 100% of DEC is incorporated into DNA [229]. Incorporation of AZA into RNA (80-90%) inhibits RNA formation, processing, and ultimately protein synthesis [240-242]. DEC has been shown to be more potent than AZA, prompting the same changes in DNA methylation at significantly lower doses. The increased potency of DEC over AZA is most likely associated with the considerably higher extent at which DEC in incorporated into DNA [243].

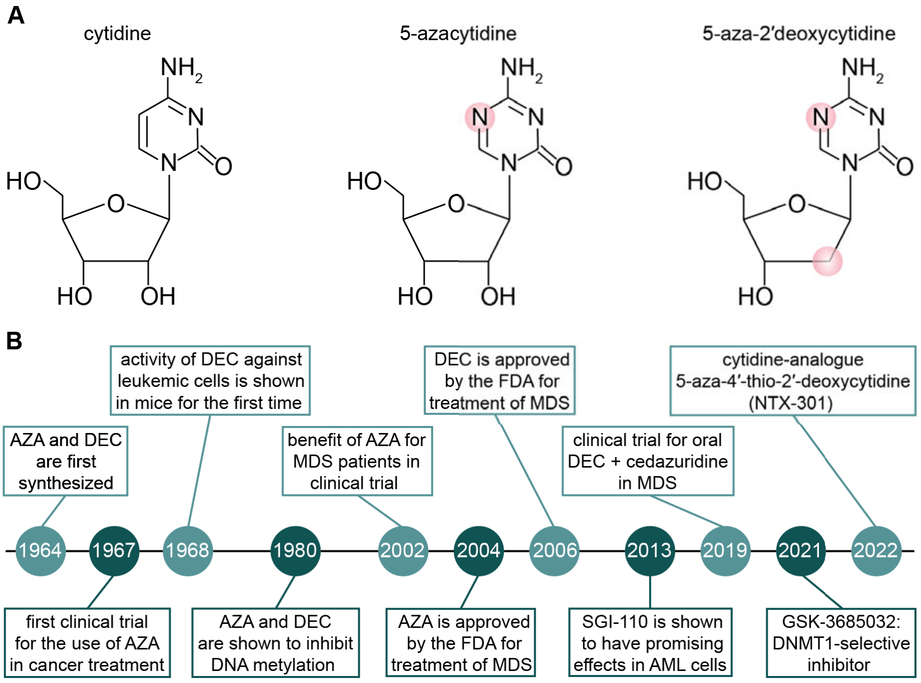

A
cytidine
5-azacytidine
5-aza-2′deoxycytidine
NH2
N
O
HO
HO
OH
B
AZA and DEC are first synthesized
activity of DEC against leukemic cells is shown in mice for the first time
benefit of AZA for MDS patients in clinical trial
DEC is approved by the FDA for treatment of MDS
clinical trial for oral DEC + cedazuridine in MDS
cytidine-analogue 5-aza-4′-thio-2′-deoxycytidine (NTX-301)
1964
1967
1968
1980
2002
2004
2006
2013
2019
2021
2022
first clinical trial for the use of AZA in cancer treatment
AZA and DEC are shown to inhibit DNA metylation
AZA is approved by the FDA for treatment of MDS
SGI-110 is shown to have promising effects in AML cells
GSK-3685032: DNMT1-selective inhibitor

Figure 7: Hypomethylating agents. (**A**) Chemical structure of the two traditional hypomethylating agents 5-azacytidine (AZA) and 5-aza-2′deoxycytidine (DEC). Differences compared to regular cytidine are highlighted. (**B**) A timeline of notable developments and clinical trials in the research on and clinical use of hypomethylating agents. MDS, myelodysplastic syndromes; AML, acute myeloid leukaemia

During initial clinical trials of AZA and DEC in the 1960s [226, 227], the use of high dosages led to major myelosuppression [229, 244] and their use in patients was discontinued for a time until interest in HMAs was renewed with the discovery of their activity as DNA demethylating agents [225]. More recent studies have shown that the epigenetic effect of HMAs can be observed after prolonged treatment with low drug concentrations[229, 245-247]. Indeed, MDS patients are treated for seven or five days with AZA or DEC, respectively [229]. The recent success of HMAs in the treatment of MDS and AML increased interest in their use in solid tumours and multiple clinical trials are currently under way [248].

However, it remains uncertain how DNA demethylation by HMAs can directly lead to cytotoxicity. A recent study in AML cells found no correlation between loss of DNA methylation in response to AZA treatment and gene expression or protein level [249]. Instead, it is well described that treatment with AZA and DEC causes a DNA damage response to double strand breaks, which results in growth inhibition and apoptosis [239, 250, 251]. Mutations in or downregulation of *DNMT1* have been associated with reduced response to HMAs both *in vitro* and in AML patients [252, 253], which could be connected to the involvement of DNMT1 in the DNA damage response described in mice [254].

Next-generation hypomethylating agents

In light of the success of AZA and DEC in MDS treatment researchers have aimed to develop novel HMAs and improve the existing drugs (**Figure 7B**). Due to the rapid deamination of AZA and DEC by cytidine deaminase, both currently used HMAs have a half-life of less than 30 minutes in adults [255-257]. One approach to circumvent drug clearance is to administer a pro-drug, such as SGI-110, which protects DEC from deamination and shows promising results in AML cells *in vitro* and *in vivo* [232]. Furthermore, oral administration of the traditional HMAs is evaluated in clinical trials [233] and additional cytidine analogues are being tested for their DNA demethylating activity, such as 5-aza-4′-thio-2′-deoxycytidine (NTX-301) [235]. The most promising advance in the field, however, was the publication of the DNMT1-selective inhibitors GSK-3685032 (GSK). GSK reversibly inhibits DNMT1 and induces widespread loss of DNA methylation without causing DNA damage like AZA and DEC do. The authors claim that GSK has similar or greater antitumour effects compared to the traditional HMAs while being less toxic *in vivo* [234].

Vitamin C

Another compound that has been investigated for its role in removing DNA methylation is vitamin V (vitC), also called ascorbic acid. For humans and other mammals that have lost the enzyme l-gulonolactone oxidase (GULO) and thereby the ability to synthesize vitC, vitC is an essential nutrient that needs to be taken up through the diet. Extreme deficiency of vitC, also called ascorbic acid, can lead to scurvy [258]. Healthy individuals that consume a balanced diet have a mean serum vitC concentration of between 42 µM and 75 µM with intracellular concentrations ranging from 0.5 mM to 10 mM due to the active import of vitC by sodium dependent transporters (SVCT1 and SVCT2) [259, 260]. Cancer patients, especially haematological cancer patients, are often deficient in vitC [261, 262].

The use of vitC in the treatment of malignancies has a long and controversial history. Cameron and Pauling proposed that cancer patients benefited from supplemental vitC in 1976 [263, 264]. However, a large clinical trial including 150 patients conducted at the Mayo clinic was not able to reproduce their promising results a few years later [265]. The most likely explanation for the discrepancy is the different ways at which vitC was administered in these studies. Oral administration of vitC, as used in the later clinical trial, results in 100-fold lower blood serum vitC concentration than intravenous injection, by which patients in the former study received vitC [260]. In the last 50 years there have been several clinical trials further studying whether vitC can enhance the effect of existing chemotherapy or reduce treatment related toxicities. Overall, there is little high quality clinical evidence that vitC supplementation benefits cancer patients [266]. However, such an overarching view might be too broad as vitC has varying function at different concentrations, which should be taken into consideration when judging the benefits of vitC treatment (**Figure 8**).

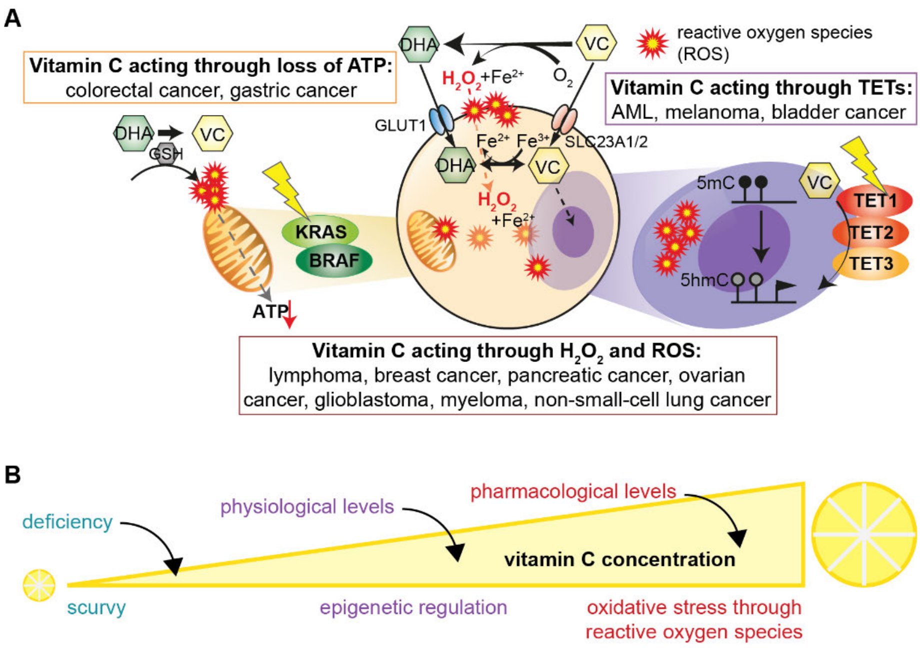

A
Vitamin C acting through loss of ATP:
colorectal cancer, gastric cancer
DHA
GSH
VC
ATP
KRAS
BRAF
GLUT1
DHA
VC
H2O2+Fe2+
O2
Fe2+ Fe3+
SLC23A1/2
DHA
VC
H2O2
+Fe2+
reactive oxygen species
(ROS)
Vitamin C acting through TETs:
AML, melanoma, bladder cancer
5mC
5hmC
VC
TET1
TET2
TET3
Vitamin C acting through H2O2 and ROS:
lymphoma, breast cancer, pancreatic cancer, ovarian cancer, glioblastoma, myeloma, non-small-cell lung cancer
B
deficiency
physiological levels
pharmacological levels
vitamin C concentration
scurvy
epigenetic regulation
oxidative stress through
reactive oxygen species

Figure 8: Vitamin C. (**A**) Different mechanisms of action of vitamin C (VC). VC (i) acts as a co-factor for the TET enzymes either by direct interaction or by facilitating cycling of iron (Fe), (ii) can be cytotoxic through production of hydrogen peroxide (H_2O_2) and reactive oxygen species (ROS), or (iii) by inducing loss of ATP in response to excessive intracellular ROS. Image reprinted from [267]. (**B**) Vitamin C has different modes of action dependent on its concentration.

As a reducing agent, vitC facilitates the cycling of iron by reducing Fe^{3+} to its active form Fe^{2+}. Thereby, vitC acts as a necessary co-factor for α-ketoglutarate-dependent dioxygenases, which are dependent on the presence of reduced iron for their catalytic activity [258]. Both the DNA demethylating TET enzymes and the Jumonji C-containing histone demethylases require vitC for their enzymatic function. As such, the addition of vitC to culture medium of ESCs and induced pluripotent stem cells (iPSCs) is required to facilitated remodelling of the epigenome [268, 269]. Blaschke *et al.* showed that supplementing cell culture medium with vitC promoted TET activity in mouse ESCs causing loss of 5mC and gain of 5hmC at genes that show dynamic DNA methylation throughout development, but not in regions that are stably silenced, such as ICR. This effect was TET-dependent as it was removed by double knockdown of *Tet1* and *Tet2* [269]. Only vitC, and no other reducing agent, affected the epigenetic profile of iPSCs [268], which could be due to a direct interaction of vitC with the C-terminal catalytic domain of TET2 as has been suggested by others [270]. There is evidence that vitC can promote DNA demethylation in a TET2 dependent manner in leukaemia cell lines, thereby driving differentiation and inducing cell death [271]. Additionally, vitC has been seen to promote active removal of DNA methylation by TET enzymes *in vivo* [272, 273] (**Figure 8A**).

Other studies, however, report that cancer cell death in response to vitC is dependent on the production of hydrogen peroxide (H_2O_2) and reactive oxygen species (ROS) during the extra- and intracellular oxidization of vitC to dehydroascorbic acid (DHA) [274]. This ROS dependent cytotoxicity might be intensified by the presence of mutations in *KRAS* or *BRAF* as seen in colorectal cancer cell lines [275] (**Figure 8A**).

Together, the current research on the activity of vitC suggests a model in which high, pharmacological, levels of vitC induce oxidative stress through the production of ROS, while lower, physiological concentrations, support epigenetic regulation, primarily through the TET enzymes (**Figure 8B**). Interestingly, the synergistic effects of HMAs and vitC have been reported *in vitro* and *in vivo* [262, 276]. In myeloid cancer patients that were vitC-deficient, oral administration of vitC was able to normalize blood vitC concentrations, which in turn resulted in an increase of 5hmC compared to 5mC in these patients [276], underlining the possibility of clinical use of vitC as an epigenetic regulator.

Activation of endogenous retroviruses and viral mimicry

A recent and intriguing mechanism of action that has been proposed for both HMAs and vitC is the activation of endogenous retroviruses (ERVs) in response to DNA demethylation and a subsequent activation of the adaptive and innate immune system in a process termed ‘viral mimicry’ [277]. ERVs are retroviral sequences that have integrated into our DNA throughout evolution and now make up about 8% of the human genome [278]. They are classified as TEs together with for example LINEs, as they theoretically have the ability to move to different places in the genome through retrotransposition. However, ERVs have lost their ability to form infectious particles or re-integrate into DNA in humans, due to extensive mutations accumulated over time [279, 280]. ERVs are, nevertheless, tightly regulated by both DNA methylation and histone modifications [278]. Extensive work in cancer cell lines has shown that ERVs are upregulated upon DNA demethylation by HMAs alone or in combination with vitC, which triggered an interferon (IFN) I response through recognition of intracellular double-stranded RNA by MDA5 and ultimately resulted in apoptosis [262, 281-284]. VitC might contribute to ERV activation not only by inducing DNA demethylation as a co-factor of TET but also by facilitating demethylation of H3K9me3, which is an additional epigenetic layer of ERV silencing [285]. While studies in immortalized cell lines have their advantages, they cannot replicate the complex interplay between the immune system and cancer cells in living organisms that is proposed by the model of viral. One of the few studies that have been conducted in mice found that a combination of HMAs and histone deacetylase inhibitors induced a type I IFN response and sensitized ovarian cancer to immune checkpoint therapy [286], showing that some of the results from *in vitro* studies are reproducible in a more complex *in vivo* context.

Aim of the Thesis

In light of (i) the dysregulation of DNA methylation observed in cancer in general and T-ALL in particular, (ii) the promising therapeutic benefits of targeting DNA methylation in other haematopoietic cancers, and (iii) the need for a deeper understanding of the consequences of changes in DNA methylation in T-ALL, I worked towards the following aim:

Revealing the function and possibilities of DNA methylation in T cell acute lymphoblastic leukaemia.

I will address four specific aims:

1. Dissecting the role of TET2 in T-ALL (**Paper I**)
2. Developing improved treatment options for T-ALL through pharmacological targeting of DNA methylation (**Paper I** and **Paper II**)
3. Characterizing gene expression and DNA methylation in primary human thymocytes (**Paper III**)
4. Illuminating the biology of DNA N6-methyladenine in human T cells (**Paper IV**)

Materials and Methods

Throughout this work a wide range of laboratory and computational techniques were utilised to study cellular properties, such as viability, gene expression and DNA methylation. Here, I will discuss advantages, challenges, and limitations of selected techniques. Technical details of experimental procedures and analysis can be found in the included papers.

Ethical considerations

Isolation and next generation sequencing of primary human thymocytes was approved by the Swedish Ethical Review Authority (Dnr 217-12, 2012-04-24 and Dnr 2022-07166-02). All T cells were obtained from anonymised buffy coats from Linköping University hospital and identification of the donors was not possible. Work on immortalized cell lines requires no ethical permission. All public data that was analysed did not include sensitive personal data and all next generation sequencing data that was produced in this work was handled in accordance with local and European data protection regulations.

Primary and immortalized cells

Studies on primary human thymocytes

The study of immature, developing T cells, so called thymocytes, has been conducted solely in model organisms until recently. Studies in mice have provided valuable insights, but T cell development and function differ substantially between mice and humans [287-289]. The publication of a single-cell atlas of human thymocytes in 2020 [147] therefore represented a significant advance in our understanding of human T cell biology. Thymocytes are isolated from thymic tissue that is removed during cardiac surgery, primarily in neonates suffering from cardiac heart defects. While thymectomies have been a standard procedure in the past, researchers have become increasingly aware of the long-term consequences on immune function and health in patients without a thymus [290-292]. Operating techniques have been called into question [293, 294] and surgeons are likely aiming to leave all, or the majority, of the thymus intact. Thus, studies on primary human thymocytes isolated from thymic tissue, as conducted in this work (**Paper III**) and by others [147, 295-298], provide a timely resource of data that might not be readily reproduced in future works.

T cell differentiation in vitro

The isolation of naïve T cells from buffy coats followed by *in vitro* activation and differentiation into T cell lineages is a widely used and well-established model to study T cell biology. Multiple aspects throughout *in vitro* T cell differentiation and expansion can affect the process and should be taken into consideration, such as isolation method (magnetic isolation or fluorescence-activated cell sorting), activating antibodies (bead or plate bound), and added cytokines [299, 300]. Nevertheless, investigating T cells *in vitro* is a powerful system that offers readily available study material, few confounding factors compared to *in vivo* studies, and numerous possibilities to apply interventions or manipulations of processes involved in T cell function. Differentiating T cells have been shown to undergo extensive remodelling of DNA methylation [166, 167], which makes them an ideal model to explore the dynamics of DNA methylation (**Paper IV**).

Immortalized T-ALL cell lines

In **Paper I**, **Paper II**, and **Paper IV** a range of immortalized T-ALL cell lines have been used, which have mutations in various enzymes involved in the regulation of DNA methylation (**Table 1**). Due to ease of access and handling, immortalized cell lines are commonly used in mechanistic studies, although it should be noted that introducing primary cells into culture and long-term culturing of cell lines causes profound molecular changes, including changes in the DNA methylation profile [301-303].

CELLS				**MUTATIONS**							
cell line	**age**	**sex**		***TET1***	***TET2***	***TET3***	***IDH1***	***IDH2***	***DNMT1***	***DNMT3A***	***DNMT3B***
ALL-SIL	17	m	relapse								
CCRF-CEM	3	f	relapse	m							
DND-41	13	m			s		m		m		f
HPB-ALL	14	m				m				m	
JURKAT	14	m	relapse		m				n,m	m	m
LOUCY	38	f	chemo-therapy resistant		s				m		
MOLT-3	19	m	relapse						m	f,m	
MOLT-4				m					m	f	n
PEER	4	f	relapse							m	
SUP-T1	8	m	relapse						m	n	
TALL-1	28	m	terminal lympho-sarcoma		s						

Table 1: T-ALL cell lines. List of T cell acute lymphoblastic leukaemia (T-ALL) cells used. Age and sex (m, male; f, female) of the patient is indicated. Information about mutations was collected from the Cancer Cell Line Encyclopedia (m, missense; f, frameshift; n, nonsense mutation). Information of silencing (s) of *TET2* is based on results from **Paper I**.

Analysis of cell viability

A multitude of *in vitro* assays have been developed to assess cell viability, cell proliferation, and drug cytotoxicity. They can be roughly divided into four groups, (i) dye exclusion, (ii) colorimetric and fluorometric, (iii) luminometric, and (iv) flow cytometry based. These assays have been summarized and reviewed in detail elsewhere [304-306]. I will focus on the two approaches used in this thesis, the colorimetric alamar blue assay and flow cytometry (**Paper I** and **Paper II**).

Alamar blue assay

The alamar blue assay [307, 308] is based on resazurin (7-hydroxy-10-oxidophenoxazin-10-ium-3-one), which is reduced by metabolically active cells. During the reduction of resazurin, a colour change occurs from blue to pink and the indicator dye becomes highly fluorescent. Therefore, the assay can be used as a colorimetric or fluorescent assay in 96-well plates with measurements of absorbance or fluorescence in a plate reader. Resazurin is cell permeable and not toxic, allowing its application in time course experiments with repeated measures. In this work, the colorimetric properties of the assay were leveraged for single, endpoint measurements of cell viability (**Paper I** and **Paper II**). The presence of vitC and other reducing agents reduces resazurin in the absence of any cells and confounds the alamar blue assay [309], therefore removal of vitC from the culture medium through careful washing was ensured (**Paper I**). A major limitation of the alamar blue assay is its use of metabolic activity as an indicator of cell viability, resulting in an inability to distinguish between cell death and treatment related metabolic changes. For instance, the presence of senescent cells or the inhibition of the mitochondrial reductase will confound results of the alamar blue assay when it is employed for cytotoxicity screening.

Flow cytometry: Annexin V and propidium iodide staining

To account for the limitations of the high throughput alamar blue assay, we complemented our cytotoxicity assay with Annexin V and propidium iodide (PI) staining followed by flow cytometry analysis. Staining of dead cells by PI is based on increased permeability of the cell membrane upon cell death. PI enters necrotic cells and intercalates into DNA, changing its fluorescent properties. The cell membrane of apoptotic cells, however, is still intact and PI staining on its own cannot identify apoptotic cells. Therefore, PI is often combined with Annexin V, a marker for apoptotic cells. Early during the initiation of apoptosis and necrosis the phospholipid phosphatidylserine is translocated from the inside to the outside of the cell membrane. Annexin V is a phosphatidylserine-binding protein that stains the outside of apoptotic cells when labelled with a fluorophore. By combining Annexin V and PI staining it is possible to distinguish between alive (Annexin V^-/PI^-), apoptotic (Annexin V^+/PI^-), and necrotic (Annexin V^+/PI^+) cells [310]. While Annexin V and PI staining is highly accurate in quantifying cell death, the complex handling of samples does not allow for high throughput screening of many samples.

Analysis of gene expression

There are many techniques to investigate gene expression. Depending on the resources available and the research question to be investigated, all have distinct advantages and limitations. Techniques, such as Northern Blot, fluorescence in situ hybridization and quantitative PCR (qPCR) examine the expression of one specific target gene. With microarrays, a capture-based sequencing approach, it is possible to study expression of tens of thousands of genes at the same time, and RNA sequencing allows the exploratory analysis of all transcripts in a cell without defining a set of target genes [311]. RNA sequencing and qPCR are the focus of this work (**Paper I**, **Paper II** and **Paper III**).

Quantitative PCR

qPCR, also called real-time PCR, is the standard method used for the quantification of gene expression and for validation of results from microarrays or RNA sequencing in laboratories across the world. RNA isolated from cells is reverse transcribed to cDNA. Specific primers allow for PCR amplification of short segments across individual transcripts from this cDNA. Ideally, the amplification product should cover an exon boundary to exclude confounding the quantification of transcripts by amplification of contaminating DNA. After each amplification cycle the resulting PCR product can be detected by direct (SYBR green) or indirect (TaqMan probes) fluorescent labelling. Based on the accumulative fluorescent signal detected during the PCR reaction, the original amount of a transcript in the sample can be quantified. Absolute quantification requires a standard curve while relative quantification involves the comparison to a control sample [312]. A great advantage of qPCR is that it is extremely sensitive and makes the detection of even lowly expressed gene possible. However, its great sensitivity can also result in substantial variability in results due to differing amplification efficiency, sample handling, or variations in the amount of input material. Therefore, researchers have traditionally normalized qPCR results to the expression of genes with constant expression, so-called housekeeping gene. The most used housekeeping genes are *GAPDH*, *BACTIN* and ribosomal RNA. It is important to note that the expression of common housekeeping genes has been shown to vary in different tissues, in disease context, and upon pharmacological treatment or other intervention [312, 313]. Therefore, the use of multiple housekeeping genes has been suggested and researchers have aimed to identify cell type and context specific housekeeping genes (for example [314-316]).

RNA sequencing

The use of next generation RNA sequencing (RNA-seq) has shaped our understanding of the transcriptome like no other technique. RNA-seq is used in studies covering all aspects of biological and medical research and even finds use in clinical diagnostics [317]. The main application of RNA-seq is the analysis of differential gene expression between samples, but methods have been developed to study a variety of additional RNA properties and functions, such as RNA modifications [318, 319], spatial transcriptomics [320], RNA interaction with RNA, DNA, or protein [321, 322], analysis of newly synthesized RNA [323], and the study of active translation [324]. Adaptations of library preparation protocols, sequencing platforms, and computational tools for data analysis of RNA expression develop at a rapid speed. Some central considerations for RNA-seq and an overview of the approach for computational expression analysis are shown in **Figure 9** [325-328]. Here, I would like to highlight the different RNA-seq adaptations used in **Paper I**, **Paper II**, and **Paper III**.

About 80% of the RNA isolated from human cells is ribosomal RNA (rRNA). To avoid high sequencing costs due to sequencing a large amount of uninformative RNA, rRNA needs to be removed prior to sequencing [329]. Isolating poly(A) messenger RNA (mRNA) using dT-oligos is the most common method used to remove rRNA efficiently (**Paper III**). However, an abundance of RNA molecules lacks poly(A) tails, such as small RNAs and TEs. For the analysis of RNAs without poly(A) tails, alternative rRNA depletion methods have been developed [330, 331]. In **Paper I** and **Paper II**, removal of rRNA with the help of specific DNA probes and digestion of DNA-RNA hybrid oligos by RNase H was employed to facilitate the study of ERVs, which do not uniformly have a poly(A) tail. As up to 20% rRNA remains after rRNA depletion by RNase H and a larger number of unique transcripts are present in the sample, total RNA-seq requires more sequencing reads for sufficient coverage (mRNA-seq: ~30M million; total RNA-seq >60 million) and is therefore more expensive.

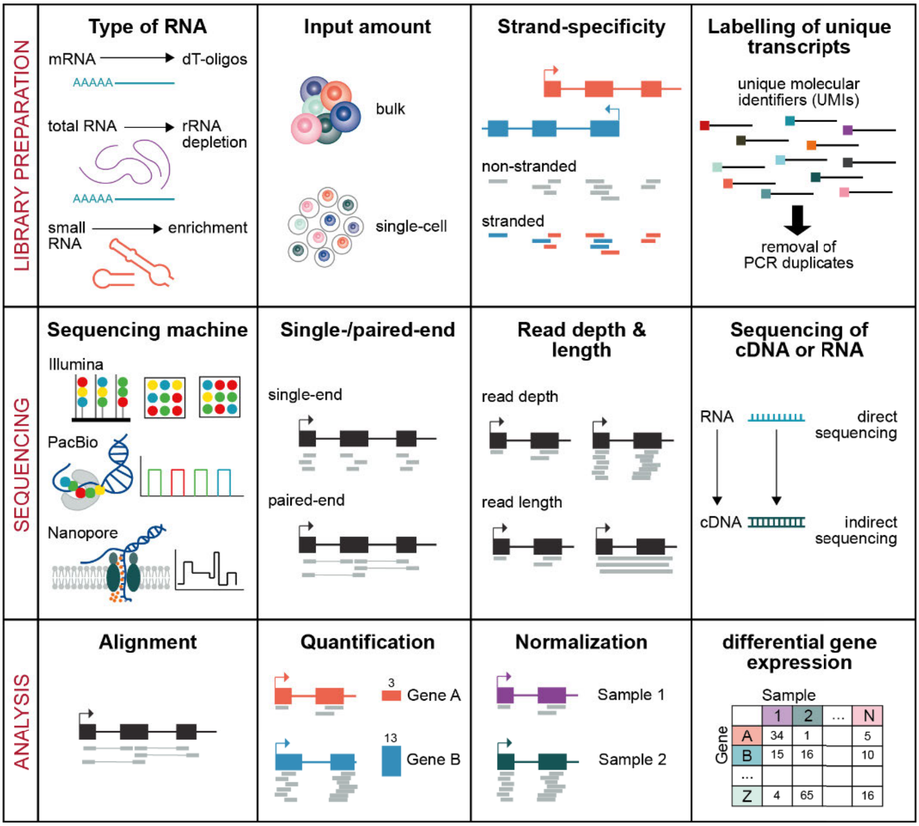

LIBRARY PREPARATION
Type of RNA
mRNA
dT-oligos
AAAAA
total RNA
rRNA depletion
AAAAA
small RNA
enrichment
Input amount
bulk
single-cell
Strand-specificity
non-stranded
stranded
Labelling of unique transcripts
unique molecular identifiers (UMIs)
removal of PCR duplicates
SEQUENCING
Sequencing machine
Illumina
PacBio
Nanopore
Single-/paired-end
single-end
paired-end
Read depth & length
read depth
read length
Sequencing of cDNA or RNA
RNA
direct sequencing
cDNA
indirect sequencing
ANALYSIS
Alignment
Quantification
3
Gene A
13
Gene B
Normalization
Sample 1
Sample 2
differential gene expression
Sample
1
2
...
N
Gene
A
34
1
5
B
15
16
10
...
Z
4
65
16

Figure 9: RNA sequencing overview. Based on the research question of interest, different options for library preparation should be chosen and the approach for sequencing of the libraries needs to be adjusted. Analysis of RNA sequencing follows four basic steps which can be achieved with the help of various computational tools.

In regular RNA-seq, information about the direction of transcription is lost, making it impossible to distinguish between two different genes that are transcribed from opposite strands at the same genomic location. To better facilitate our understanding of ERV expression, we employed a stranded RNA-seq approach in **Paper II** by degrading the second cDNA strand before PCR amplification and thereby only sequencing fragments originating from the first cDNA strand, which is complementary to the original RNA (dUTP method) [332].

Analysis of DNA methylation

DNA methylation might be one of the most extensively studied epigenetic marks and a range of methods have been developed for its analysis. Techniques for the study of DNA methylation can be divided into five groups; (i) quantification of global DNA methylation, (ii) methods utilizing restriction enzyme digestion, (iii) approaches involving affinity enrichment, (iv) conversion-based techniques, and (v) third-generation sequencing approaches (extensively reviewed in [333-336]).

(i) Total levels of 5mC or other DNA methylation marks can be quantified by mass spectrometry or immuno-dot blot. These techniques are ideal for comparing DNA methylation content of different species and cell types, or changes in global DNA methylation as a result of pharmacological intervention. (ii) Methylation-sensitive restriction enzymes cleave DNA at specific recognition sequences only in the absence or presence of DNA methylation, making it possible to selectively amplify methylated or unmethylated DNA. The most well-known restriction enzymes for the study of CpG methylation are *Msp*I and *Hpa*II. Both enzymes recognize the palindromic sequence CCGG,, but while *Msp*I cleaves DNA indistinctly, *Hpa*II is blocked by the presence of a methylation group at the inner cytosine [337]. (iii) Antibodies against DNA modifications allow the enrichment of methylated DNA fragments by DNA immunoprecipitation (MeDIP). Using MeDIP genome-wide DNA methylation profiles can be generated [338]. (iv) Most approaches that characterize DNA methylation at individual DNA bases involve a pre-treatment of DNA with sodium bisulfite. Bisulfite rapidly deaminates unmethylated cytosines to uracil, but the speed of this reaction is significantly slower for methylated cytosines. Making use of this discrepancy in reaction speed, it is possible to selectively convert unmethylated cytosines to uracil [339, 340]. Such converted DNA can be

PCR amplified without losing information about DNA methylation, which is essential for the commonly used amplification-based next-generation sequencing techniques. (v) More recently, third-generation sequencing approaches have been developed for direct quantification of DNA methylation, such as single molecule real-time sequencing (SMRT-seq) and nanopore sequencing.

Immuno-dot blot

In **Paper I** and **Paper IV** immuno-dot blots were used to study changes in global levels of 5hmC and 6mdA, respectively. For immune-dot blots, denatured DNA is bound and crosslinked to a positively charged nylon membrane. Using antibodies to specific DNA modifications the amount of the modification present in a sample can be visualized. While the method is semi-quantitative, it is inexpensive and convenient, and provides a good indication of global changes in DNA methylation. Immuno-dot blots have been used to study any DNA modification for which an antibody can be raised, including rare modifications such as 4mdC [73]. It needs to be noted, however, that the technique is reliant on specificity of the used antibody, which should be carefully validated, particularly for studies of rare DNA methylation marks [341].

Mass spectrometry

Total quantities of different DNA modifications in the same sample can also be studied by ultra high performance liquid chromatography coupled with mass spectrometry (UHPLC-MS/MS) (**Paper IV**) [342]. In contrast to immuno-dot blots, UHPLC-MS/MS is quantitative, measuring the percentage of modified bases over all nucleotides (e.g. 5mC/total C). However, the sample handling and accurate interpretation of UHPLC-MS/MS results requires training and expertise and the need to include modified DNA standards excludes the possibility to detect novel forms of DNA modifications. 4mdC and 6mdA have been analysed by UHPLC-

MS/MS, but other studies failed to detect either mark in mammalian DNA [89]. Furthermore, Brown and colleagues pointed out that sample contamination with bacterial DNA, in which both 4mdC and 6mdA are abundant, leads to overestimation of rare DNA modifications in mammals [75].

DNA immunoprecipitation

Methylated DNA immunoprecipitation (MeDIP) (**Paper IV**) was first developed in 2005 in an effort to study a larger proportion of 5mCs than is possible using restriction enzyme digestion, the methods commonly used at the time [338]. After fragmentation, DNA fragments, at which the DNA modification of interest is present, are pulled down using specific antibodies. Locus-specific DNA methylation at genes of interest can be analysed by downstream quantification by qPCR. Most often, global DNA methylation profiles are analysed by combining MeDIP with microarrays or next-generation sequencing. Since MeDIP allows for confirmation of the presence of DNA methylation anywhere on a DNA fragment, but not the exact position, fragment size also represent the limit of resolution for this technique. Standard protocols for MeDIP recommend sonication of DNA into fragments of sizes between 200 and 1000 bp [343, 344]. To account for biases during DNA fragmentation or amplification, the enriched fraction is commonly compared to an input control. MeDIP is an antibody-based technique and can thus be used for the study of all types of DNA methylation for which high-quality antibodies have been raised. However, researchers have found that antibodies used for MeDIP show nonspecific binding to repetitive elements [345]. To account for this nonspecific antibody binding, the inclusion of an unspecific IgG antibody as a control, in addition to an input control, is highly recommended.

DNA methylation array

Microarrays have found application for the study of DNA methylation in combination with several previously mentioned methods, such as restriction-enzyme digestion, DNA immunoprecipitation, and, most often, bisulfite conversion [334] (**Paper I** and **Paper III**). Nowadays, the Illumina Infinium beadchip microarray technology is by far the most used DNA methylation array. Following sodium bisulfite conversion, DNA is hybridized to a microarray that includes site- and methylation-specific CpG probes. Unmethylated (T) or methylated (C) cytosines are detected by fluorescence, either by single-base extension, which is blocked after mismatches (Infinium I), or by incorporation of fluorescently labelled nucleotides distinct for C/G or A/T (Infinium II) [346]. This method has been shown to achieve reliable and reproducible results and is applicable for high-throughput screening due to its lower cost compared to whole genome sequencing [333]. The first Infinium methylation microarray covered just over 27,000 CpG sites across the genome [347]. Since then, the quality of probes has continuously been assessed and badly performing probes were removed. Additionally, the number of CpG sites that are included has increase with each version of Illumina's microarrays with the newest MethylationEPIC v2 BeadChip covering 950,000 CpGs. Nevertheless, even the EPIC methylation array includes only 3% of the 28 million CpGs which are present in the human genome and does not cover all regulatory elements equally [348]. The use of microarrays for DNA methylation analysis also precludes a completely unbiased approach to investigating genome-wide DNA methylation, because the choice of which CpGs are included in the arrays is affected by probe performance and whether a particular CpG has been shown to be of interest in previous studies. Since many CpGs that are part of the EPIC array are associated with malignancies, this screening approach is often used for studies of DNA methylation in cancer. Furthermore, probes included in the array are specific for humans or mice, and do not allow for the analysis of DNA methylation in other species [349].

Next-generation sequencing of whole genome DNA methylation

To sequence genome-wide DNA methylation at base resolution, researchers have long made use of the reactivity of cytosine nucleotides with sodium bisulfite. In the presence of sodium bisulfite a deamination reaction occurs, converting cytosines to uracil, which will be converted to thymine in subsequent PCR or sequencing reactions (**Figure 10A**). This reaction is significantly slower for 5mC, resulting in the specific deamination of unmethylated cytosines [339, 340, 350]. In 1992, Frommer *et al.* first applied sodium bisulfite conversion followed by sequencing to study DNA methylation [351]. Since then, bisulfite sequencing in combination with next-generation sequencing has been considered the 'gold standard' for the characterization of genome-wide DNA methylation profiles. While 5fC and 5caC are deaminated to uracil, similar to unmethylated cytosine [39, 352], 5hmC becomes cytosine-5-methylenesulfonate upon treatment with bisulfite and is read as cytosine in subsequent sequencing reactions [353]. Thus, it is not possible to distinguish between 5mC and 5hmC with traditional bisulfite sequencing. Modifications of the standard bisulfite conversion protocol have been developed involving specific oxidation of either 5mC or 5hmC prior to bisulfite treatment and thereby resulting in deamination of the oxidized base to uracil. Two well-known techniques used to differentiate between 5hmC and 5mC are Tet-Assisted Bisulfite Sequencing (TAB-seq) [354] and oxidative bisulfite sequencing (oxBS) [355] (**Figure 10B**).

Despite its widespread use, whole genome bisulfite sequencing has multiple well-described biases. Extreme treatment conditions (high temperature and low pH), variations in cytosine content, and incomplete bisulfite conversion can lead to DNA degradation and PCR bias. Ultimately, these biases result in uneven coverage of the genome and overestimation of methylation [356, 357]. To improve whole genome DNA methylation sequencing, Vaisvila and colleagues have developed an enzyme-based conversion approach (EM-seq) [358] (**Paper II**), which produced higher and more even coverage of the whole genome and can be used with lower amounts of input DNA (picograms) [359, 360].

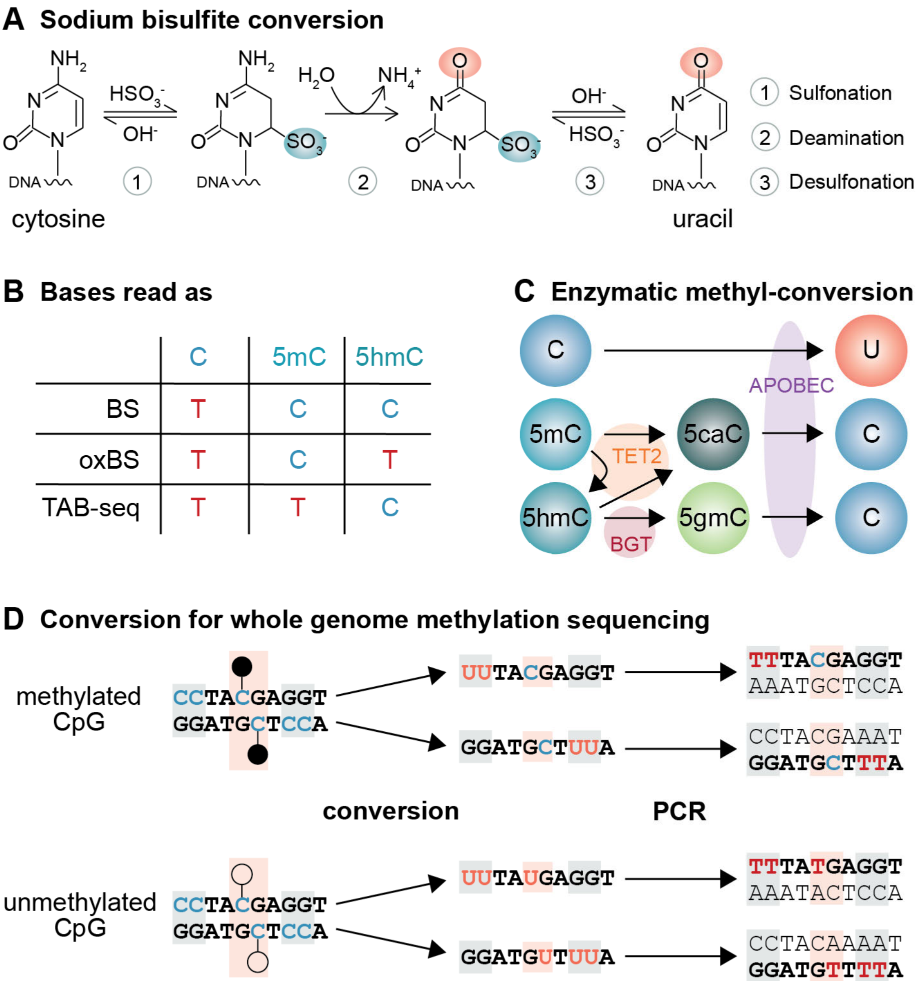

A Sodium bisulfite conversion
NH2
N
O
N
DNA
HSO3-
OH-
1
SO3-
H2O
NH4+
2
O
OH-
HSO3-
3
cytosine
uracil
1 Sulfonation
2 Deamination
3 Desulfonation
B Bases read as
C
5mC
5hmC
BS
T
C
C
oxBS
T
C
T
TAB-seq
T
T
C
C Enzymatic methyl-conversion
C
U
APOBEC
5mC
5caC
TET2
C
5hmC
BGT
5gmC
C
D Conversion for whole genome methylation sequencing
methylated CpG
CCTACGAGGT
GGATGCTCCA
UUTACGAGGT
TTTACGAGGT
AAATGCTCCA
GGATGCTUUA
CCTACGAAAT
GGATGCTTTA
conversion
PCR
unmethylated CpG
CCTACGAGGT
GGATGCTCCA
UUTAUGAGGT
TTTATGAGGT
AAATACTCCA
GGATGUTUUA
CCTACAAAAT
GGATGTTTTA

Figure 10: Whole genome methylation sequencing. (**A**) Conversion of cytosine to uracil by sodium bisulfite. (**B**) Base that is sequenced after conversion of 5-(hydroxy)methylated cytosine for three methylation-sensitive sequencing approaches. BS, bisulfite sequencing; oxBS, oxidative bisulfite sequencing; TAB-seq, Tet-Assisted bisulfute sequencing. C, cytosine; T, thymine (**C**) Conversion of (hydroxy)methylated cytosine in enzymatic methyl-conversion sequencing (EM-seq) by ten-eleven translocation 2 (TET2), β-glucosyltransferase (BGT), and APOBEC. U, uracil; 5mC, 5-methylcytosine; 5hmC, 5-hydroxymethylcytosine; 5caC, 5-carboxylcytosine; 5gmC, 5-(β-glucosyloxymethyl)cytosine. (**D**) Strand- and methylation-specific fragments after conversion (bisulfite or EM-seq) and PCR amplification.

The conversion of unmethylated cytosine to uracil in EM-seq is achieved by a two-step enzymatic conversion. TET2 and β-glucosyltransferase (BGT) oxidize 5mC to 5caC and 5hmC to glycosylated 5-(β-glucosyloxymethyl)cytosine (5gmC) (**Figure 10C**). These modified bases are protected from the following deamination reaction facilitated by APOBEC. This second step of conversion turns cytosines, 5fC, and 5caC into uracil. The read-out of EM-seq and bisulfite sequencing libraries is identical with unmethylated cytosines being read as thymine and 5mC/5hmC remaining cytosines.

Computational analysis of DNA methylation

Computational analysis of DNA methylation arrays, MeDIP sequencing and bisulfite sequencing/EM-seq differ in several key aspects [361].

For Illumina Infinium microarrays, site specific DNA methylation is quantified based on fluorescence intensity stemming from unmethylated and methylated probes. For each covered CpG, DNA methylation is represented as β-values [methylated intensity/(methylated + unmethylated intensity)]. Care should be taken to apply stringent filtering and normalization. For example, the performance of Type I and Type II probes as well as fluorescent intensities between probes for methylated and unmethylated CpGs can vary. Furthermore, probes including common SNPs are often removed [362, 363].

After MeDIP, sequenced fragments are aligned to a standard reference genome. As the quantity of reads represents the amount of DNA methylation, sites in the genome at which aligned reads are enriched in comparison to control samples are identified with peak-callers [364].

Alignment of bisulfite sequencing and EM-seq is complex due to the conversion of unmethylated cytosines to thymine. Conversion is affected by the presence of a methylation mark and only the unmethylated cytosine on one strand is converted, but not the complementary guanine. After PCR amplification, DNA methylation-sensitive conversion can lead to four possible sequences originating from the same genomic location (**Figure 10D**). Two approaches have been developed for the alignment of methylation-converted DNA. One method is the wildcard method, which changes all cytosine and thymine in the reference genome to a wildcard (Y) that either base can be aligned to without penalty. The second implemented approach permits the use of aligners commonly used for processing of whole genome sequencing. It involves changing all cytosines in the reference sequence and in all sequenced reads to thymine before alignment [365]. Multiple aligners for whole genome methylation sequencing have been published which differ in regard to speed, memory usage, mapping efficiency, and mapping accuracy (benchmarked by Gong *et al* [366]). After alignment, DNA methylation for each cytosine is quantified by summarizing the frequency of cytosine and thymine at each position (methylation = C/(C+T)). There are several tools used to extract information about DNA methylation from aligned methylation sequencing files. In **Paper II**, the Burrows-Wheeler Aligner (bwa-meth) was combined with MethylDackel to quantify DNA methylation per CpG. The downstream analysis of whole genome methylation sequencing is most commonly the identification of differential DNA methylation between samples. Various tools have been developed for the analysis of differentially methylated cytosines utilizing several statistical tests, such as logistic regression, logistic ratio test, modified t-test, and fisher's exact test [367]. Due to high sequencing costs or a lack of input material whole genome methylation sequencing is often limited by the omission of biological replicates. To accurately distinguish differential DNA methylation without replicates, it is recommended to use Fisher's exact test [336, 361] (**Paper II**). Furthermore, since differential methylation is explored at millions of cytosines, it is vital to apply a multiple testing correction.

Results and Discussion

In the following, results from this thesis work will be summarized and discussed in view of current scientific understandings and new discoveries. For extensive descriptions of the results and further discussion, please refer to the individual published papers and manuscripts included in this dissertation.

The role of TET2 in T cell acute lymphoblastic leukaemia

*The tumour suppressor TET2 is silenced by promoter DNA methylation in T-ALL (**Paper I**)*

TET2 is a known tumour suppressor gene that is regularly mutated in myeloid and lymphoid malignancies [104]. However, in a meta-analysis of 854 T-ALL patients across nine studies we found less than 4% of patients with mutations in any of the TET or IDH enzymes (**Figure 11A**) [368-376]. Mutations in *IDH1* or *IDH2* phenocopy *TET* loss of function mutations. As *TET2* dysregulation can occur by mutations or alteration of gene transcription, we further investigated *TET2* expression in primary T-ALL. *TET2* has been proposed to have two isoforms, *TET2a* and *TET2b*, (**Figure 11B**) with distinct functions [377]. We found that only the longer *TET2a* isoform, which contains a catalytic domain, is expressed in thymocytes or any other of the 27 analysed tissues (**Figure 11C** and **Paper I**, data not shown). Interestingly, *TET2a* expression was reduced in the majority of primary T-ALL and completely silenced in up to 17% of patients (**Figure 11C**). In two separate cohorts of T-ALL patients [378, 379], we found that the *TET2* promoter gained DNA methylation in comparison to primary thymocytes [380] (**Figure 11D**), suggesting that TET2 function could be lost in T-ALL by aberrant promoter DNA methylation instead of through mutations, as was seen in other haematopoietic malignancies.

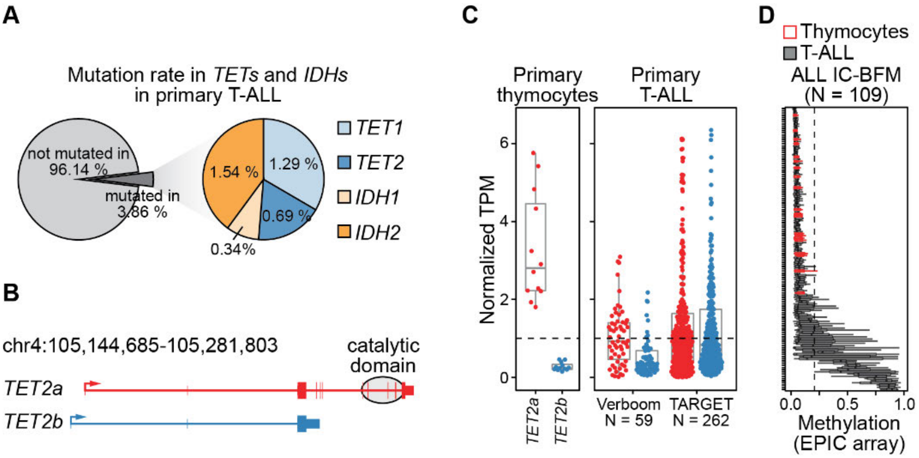


Figure 11: *TET2* is silenced and frequently methylated in primary T-ALL. (**A**) Mutations in genes encoding for ten-eleven translocation enzymes (TET) or isocitrate dehydrogenases (IDH) in primary T-ALL. (**B**) Two isoforms of *TET2*, *TET2a* and *TET2b*, are annotated in the human reference genome. (**C**) Expression of *TET2a* (red) and *TET2b* (blue) in primary thymocytes and primary T-ALL patients shown as normalized transcript per million (TPM). TPM < 1 (dotted line) is considered not expressed. (**D**) DNA methylation at the *TET2* promoter in primary thymocytes (red) and primary T-ALL samples (grey). Dotted line: 20% DNA methylation.

*TET2 silencing is associated with global DNA hypermethylation (**Paper I**)*

A more thorough analysis of published DNA methylation data (EPIC array) from primary thymocytes and 109 T-ALL patients [378, 381] revealed that patients with *TET2* promoter methylation above 70% were clearly distinct from primary thymocytes when separating samples by the 1000 most variable CpGs (**Figure 12A**). These *TET2* hypermethylated samples had not only gained DNA methylation at CGIs in general, but also showed a trend towards retaining DNA methylation at intergenic regions, which have been seen to lose 5mC in most cancers (**Figure 12B**).

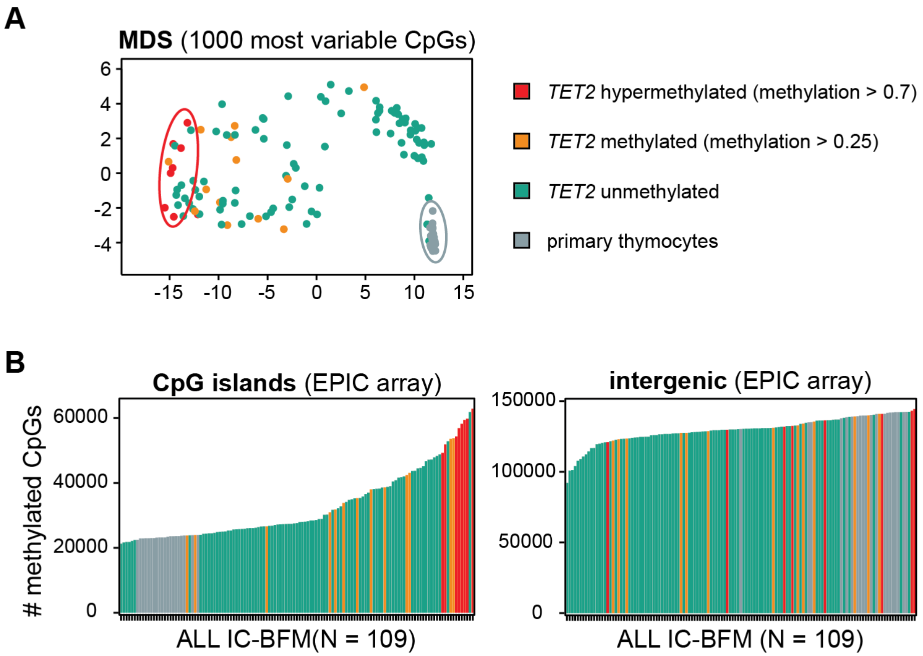


Figure 12: *TET2* promoter hypermethylation is associated with global gain of DNA methylation. (**A**) Multidimensional scaling (MDS) plot of the 1000 most variable CpGs in healthy thymocytes (grey) and primary T-ALL samples. (**B**) Total number of methylated CpGs at CpG islands (left) and intergenic regions (right) in healthy thymocytes (grey) and 109 primary T-ALL samples. (**A,B**) *TET2* promoter methylation indicated by colour.

A recent comprehensive study combining whole genome methylation and RNA sequencing data in T-ALL patient samples similarly found that global hypermethylation was associated with reduced expression and promoter methylation of *TET2* in 26% of patients [221]. Whether the loss of the DNA demethylating enzyme *TET2* precedes the global gain of DNA methylation in T-ALL or if it is a consequence of it, remains to be ascertained. Mouse models of *Tet2* knockdown and knockout have shown that loss of the catalytic domain of *Tet2* results in widespread gain of DNA methylation, especially at enhancers [103, 382]. On the other hand, TET1 and particularly TET3 can compensate for loss of TET2 as levels of 5mC and 5hmC are only slightly affected in haematopoietic stem cells

(HSCs) lacking TET2 [381, 383-385]. However, these studies analysed healthy cells and did not investigate the possibility that the amount of global DNA methylation could increase during malignant progression in cells lacking *TET2* [382]. While *TET2* mutations are common in clonal haematopoiesis in older age and do not necessarily result in progression to haematopoietic malignancies [386, 387], only biallelic and not monoallelic loss of function mutations of *TET2* have been shown to induce immunodeficiency, development of B and T cell lymphoma, and whole blood DNA hypermethylation [388, 389]. Lastly, the complete loss of *TET2* through transcriptional silencing could have differing functional consequences compared to loss of function mutations. Ito *et al.*, found that mice expressing a *Tet2* variant lacking its catalytic function developed predominantly myeloid cancers while complete loss of *Tet2* elicited progression to both myeloid and lymphoid malignancies [390], showing the possibility of a non-catalytic function of *TET2* that might be involved in T-ALL biology.

Pharmacological targeting of DNA methylation in T-ALL

While the underlying cause of DNA hypermethylation and the accompanying silencing of *TET2* is still to be investigated, the observed gain in DNA methylation in T-ALL cells nevertheless provides a target for treatment. Therefore, we further examined the potential of pharmacological intervention with vitC and HMAs in T-ALL cell lines.

*Vitamin C increases TET enzymatic activity (**Paper I**)*

With silencing of *TET2*, the enzyme primarily responsible for DNA demethylation is lost. By supplying vitC, a cofactor of all three TETs, it could be hypothesized that the activity of TET1 and TET3 can be increased to compensate for the loss of TET2. Thus, T-ALL cell lines with silenced (DND-41, LOUCY, TALL-1), or expressed *TET2* (CCRF-CEM, JURKAT, SUP-T1) were treated with vitC. Indeed, the addition of 100 µM vitC to cell culture medium for 24 hours promoted TET enzymatic activity as seen by a marked increase of 5hmC in all T-ALL cell lines (**Figure 13A**). We furthermore observed that cells expressing *TET2* had a markedly more pronounced increase of 5hmC upon vitC treatment (**Figure 13B**), suggesting that TET2 is the primarily active form of TETs in immortalized T-ALL cell lines.

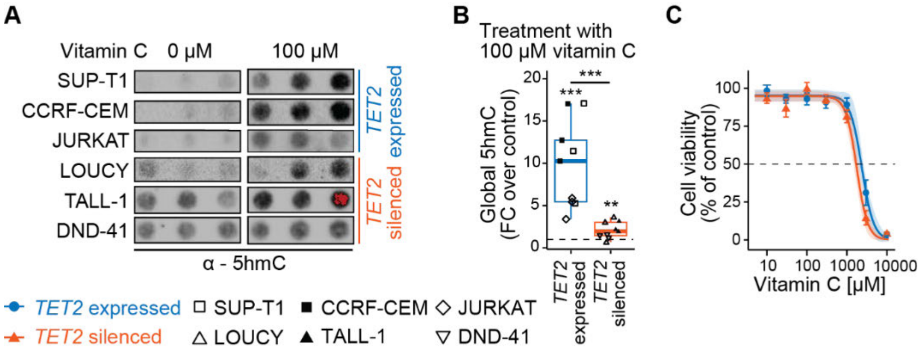


Figure 13: Vitamin C promotes TET activity at physiological concentrations and induces cell death at pharmacological concentrations. (**A**) Immuno-dot blot for 5-hydroymethylcytosine (5hmC) in T-ALL cell lines in untreated control cells and after treatment with 100 µM vitamin C for 24 hours. (**B**) Quantification of 5hmC in untreated control cells and after treatment with 100 µM vitamin C for 24 hours (immuno-dot blot (A)) shown as fold change (FC) over untreated control in *TET2*-expressed and *TET2*-silenced T-ALL cell lines. Comparison by student's t-test; ns, $p > 0.05$; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$. (**C**) Cell viability measured by alamar blue assay after treatment with increasing concentration of vitamin C. Data from biological triplicates in six T-ALL cell lines combined based on *TET2* expression status. Mean and standard error of the mean indicated.

While 100 µM vitC resulted in gain of 5hmC, this was not coupled to toxicity. Instead, cell death was only induced after pharmacological concentrations of > 1000 µM vitC (**Figure 13C**). Inhibition of vitC import through GLUT or SVCT transporters with the competitive inhibitor phloretin abolished the intracellular, epigenetic effect of vitC, but did not rescue cell viability (**Paper I**, data not shown).

Together, these results call into question whether the increased activity of the TET enzymes observed after treatment with vitC impacts cell viability. It should be taken into consideration that T-ALL cells were treated with vitC for only 24 hours and a possible functional response to epigenetic remodelling could be missed due to the assay's shortness. An increase of 5hmC can affect gene expression directly, as 5hmC is associated with active transcription [165], or indirectly, by preventing maintenance of DNA methylation during DNA replication [391]. In the latter case, complete DNA demethylation at positions marked by 5hmC would only occur after cell proliferation. In 24 hours, it is unlikely that the majority of treated T-ALL cells have undergone cell division and it is possible that a functional response to loss of DNA methylation at tumour suppressor genes could have been observed after an extended treatment-duration.

*Treatment with vitamin C induces cell death through creation of reactive oxygen species (**Paper I**)*

The observed toxicity at pharmacological levels of vitC in the absence of cellular import of vitC indicates that the formation of extracellular ROS plays a role in the response of T-ALL cells to vitC treatment. Consequently, we treated cells with vitC in the presence of catalase. Catalase converts hydrogen peroxide (H_2O_2) to water and oxygen, thereby removing the main source of ROS generated during the oxidization of vitC to DHA [392, 393]. The addition of catalase to treatment with 3000 µM vitC for 24 hours removed all ROS (**Figure 14A**) and rescued cell viability completely in the six tested T-ALL cell lines (**Figure 14B**).

The finding that vitC induces cytotoxicity in T-ALL cell lines primarily through ROS is in line with earlier results in various other cancer types in *in vitro* and animal models [394-398]. Chen *et al.* showed that ten different cancer cell lines were sensitive to pharmacological concentrations of vitC though H_2O_2 associated cell death, while all four normal cell types that were assayed remained unaffected [274]. Their results suggest that cancer cells, which naturally have elevated levels of ROS, might be more susceptible to ROS-mediated cell death than healthy cells [399, 400]. Indeed, several studies confirmed that increased oxidative stress in cancer cells after vitC treatment causes apoptosis in response to DNA damage and activation of cell cycle regulators such as p21 [401-405].

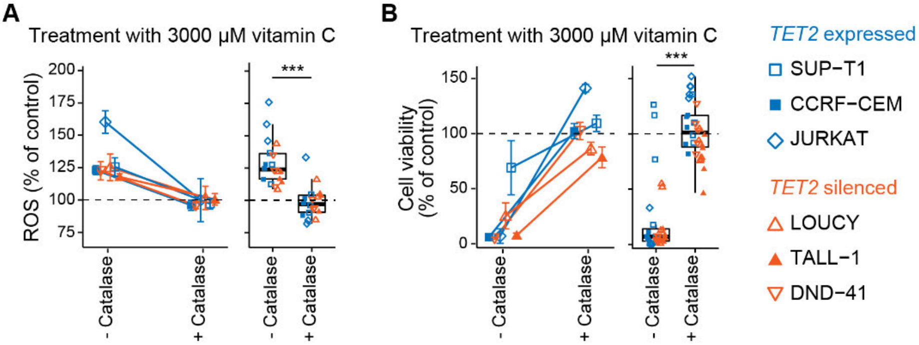


Figure 14: Reactive oxygen species cause cell death upon treatment with vitamin C. (**A**,**B**) Total levels of reactive oxygen species (intra- and extracellular) (**A**) and cell viability (**B**) relative to an untreated control upon treatment with 3000 µM vitamin C for 24 hours in the absence or presence of catalase in six T-ALL cell lines. Mean and standard deviation for triplicates for each cell line (left) and box plot for all cell lines combined are shown. Comparisons by student's t-test; ns, $p > 0.05$; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

*Silenced and methylated TET2 is reactivated upon treatment with hypomethylating agents (**Paper I** and **Paper II**)*

Apart from promoting TET activity through vitC, the aberrant gain of DNA methylation seen in T-ALL can be directly targeted through removal of 5mC by HMAs. In **Paper I**, we found that treatment with the DNMT inhibitor AZA resulted in expression of *TET2* in T-ALL cell lines in which

TET2 was previously silenced (DND-41, LOUCY) (**Figure 15A**). This gain in *TET2* expression was more pronounced after AZA treatment was removed and cells were cultured for an additional five days in the absence of AZA. The stable activation of *TET2* is especially noteworthy as most genes either do not become expressed after loss of DNA methylation or are rapidly silenced once treatment is withdrawn [13, 284, 406].

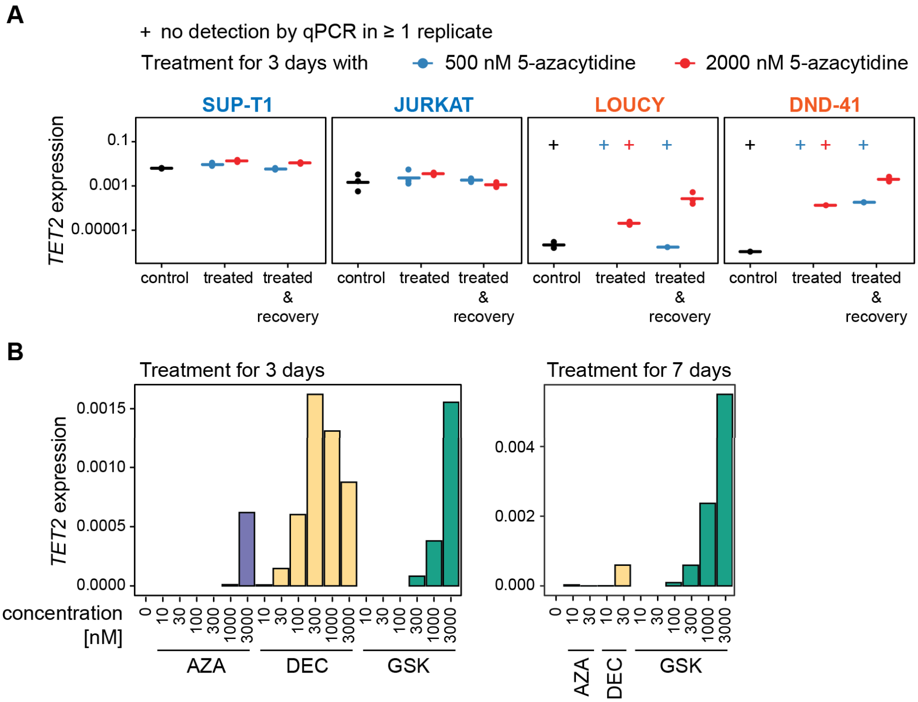


Figure 15: The DNA demethylase *TET2* becomes stably expressed upon treatment with hypomethylating agents. (**A**) Expression of *TET2* relative to housekeeping gene *GAPDH* measured by qPCR after treatment with indicated concentrations of 5-azacytidine (AZA). T-ALL cell lines with expressed (SUP-T1, JURKAT) or silenced (LOUCY, DND-41) *TET2* were treated with AZA for 72 hours (treated) followed by drug withdrawal for 5 days (recovery). Failure to detect any expression by qPCR in one or more replicates is indicated (+). (**B**) Expression of *TET2* relative to housekeeping gene *GAPDH* measured by qPCR in LOUCY cells after treatment with increasing concentrations of AZA, 5-aza-2′-deoxycytidine (DEC), or GSK-3685032 (GSK) for 3 (left) and 7 days (right).

While different HMAs can have varying effects on gene expression [407], *TET2* was consistently re-expressed after treatment with three different DNMT inhibitors, AZA, DEC, and GSK (**Figure 15B**).

*Cytotoxicity in response to hypomethylating agents is associated with DNA damage (**Paper I** and **Paper II**)*

Not only does treatment with AZA activate *TET2* expression, an initial screening of six T-ALL cell lines with expressed (CCRF-CEM, MOLT-3, SUP-T1) or silenced *TET2* (DND-41, LOUCY, TALL-1) (**Paper I**) showed that cells, which had lost *TET2* were significantly more sensitive to treatment with AZA for three days (p = 0.013) (**Figure 16A**). A repeat of this experiment in **Paper II** including an additional four cell lines and two additional HMAs (DEC and GSK) did not show the same significance for three-day treatment (**Paper II**, data not shown). Only when treatment was extended to seven days could a trend be discerned that *TET2* silenced cell lines were more sensitive to AZA (p = 0.002) and DEC (p = 0.0003). There was no detectable difference between T-ALL cell lines when treated with GSK for seven days (**Figure 16B**). Previous analyses of patient data show that mutations in *TET2* are associated with a higher response rate to HMAs in AML and MDS but do not indicate an improved overall prognosis [408, 409]. However, mutations result in reduced levels of functional TET2 whereas silencing by promoter methylation, as seen in T-ALL, causes a complete loss of the protein and can therefore have different consequences. A recent study, for example, showed that only a complete loss of *TET2* in the form of bi-allelic loss-of-function mutations sensitised AML patients to AZA [410].

We further aimed to investigate the molecular effects of HMA treatment in T-ALL. For JURKAT, LOUCY, and SUP-T1 cells cytotoxicity was coupled to a marked loss of DNMT1 in response to AZA and DEC at concentrations as low as 100 nM and 10 nM, respectively. As seen before, GSK resulted only in minor degradation of DNMT1 protein [234] (**Figure 16C** and **Paper II**, data not shown).

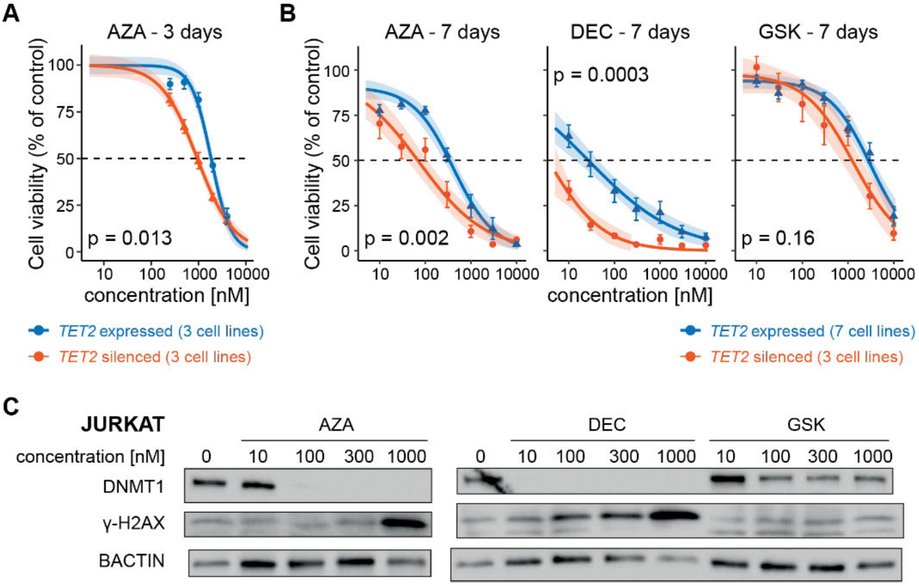


Figure 16: Toxicity of 5-azacytidine and 5-aza-2′-deoxycytidine is mediated by DNA damage. (**A**) Cell viability relative to an untreated control measured by alamar blue assay for T-ALL cell lines treated with increasing concentrations of 5-azacytidine (AZA) for 3 days. Biological triplicates for each included cell line. *TET2* expressed: CCRF-CEM, MOLT-3, SUP-T1; *TET2* silenced: DND-41, LOUCY, TALL-1. (**B**) Cell viability relative to an untreated control measured by alamar blue assay for T-ALL cell lines treated with increasing concentration of AZA, 5-aza-2′-deoxycytidine (DEC), or GSK-3685032 (GSK) for 7 days. Biological triplicates for each included cell line. *TET2* expressed: ALL-SIL, CCRF-CEM, HPB-ALL, MOLT-3, MOLT-4, PEER, SUP-T1; *TET2* silenced: DND-41, LOUCY, TALL-1. (**C**) Western blot for DNMT1, phosphorylated H2AX (γ-H2AX), and beta actin (BACTIN) for JURKAT cells treated with indicated concentrations of AZA, DEC, or GSK for 3 days. (**A**,**B**) p-value calculated by Kolmogorov–Smirnov test.

Additionally, treatment with AZA and DEC but not GSK resulted in an accumulation of phosphorylated H2AX (γ-H2AX), an indicator for DNA damage (**Figure 16C**), suggesting that toxicity of AZA and DEC is heavily mediated by DNA damage [250]. The observed loss of DNMT1 and DNA damage after treatment with AZA and DEC could be associated with each other since DNMT1 has a known role in the DNA damage response [411]. DNMT1 localizes to DNA double strand breaks and has been seen to contribute to the DNA damage response independently of its catalytic function as a DNA methyltransferase, possibly through interaction with the ATR effector kinase CHK1. Furthermore, knockout of *DNMT1* impacts DNA mismatch repair and results in further genomic instability [412-414].

*Only extreme loss of DNA methylation affects cell viability in the absence of DNA damage (**Paper II**)*

Despite not prompting a DNA damage response, GSK induced cytotoxicity, particularly if treatment was maintained for seven days (**Figure 16B**). Therefore, GSK, which is a reversible selective DNMT1 inhibitor, provides the opportunity to study the consequences of acute loss of DNA methylation on T-ALL cells in the absence of DNA damage. Analysing global DNA methylation after incubation with AZA, DEC, or GSK for three or seven days in four T-ALL cell lines showed that GSK prompted a stronger and more consistent loss of DNA methylation than the other two HMAs (**Figure 17A**). In untreated cells, total DNA methylation differed between different T-ALL cell lines by up to 18% (ALL-SIL: 86% and JURKAT: 68% global DNA methylation). However, after treatment with 3000 nM GSK for seven days, the amount of DNA methylation was more similar at 18 – 26% (**Figure 17A**).

As DNA methylation can have varying functional consequences dependent on genomic location, we further analysed locus-specific DNA demethylation. Overall, all genomic locations showed comparable amounts of mean DNA methylation content in all cell lines before and after treatment. Only promoters overlapping CGIs were less methylated in untreated cells and retained only as little as 8% global CpG methylation after treatment with GSK for seven days (**Figure 17B**). Our data further allowed us to correlate cell viability with total DNA methylation. Across all analysed cell lines treated with GSK, toxicity could be seen only after global DNA methylation had been reduced to 30% or less (**Figure 17C**).

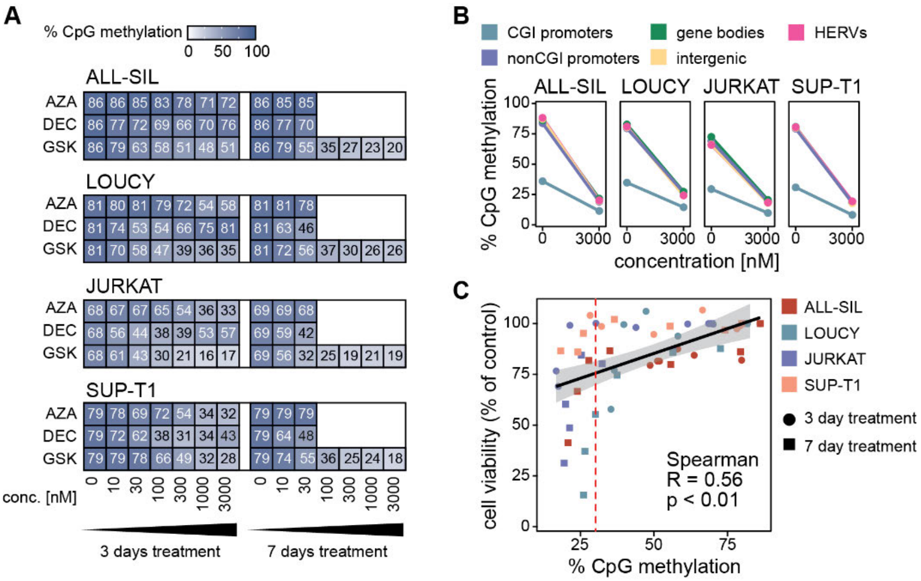


Figure 17: The selective and reversible DNMT inhibitor GSK causes robust DNA demethylation. (**A**) Mean global CpG methylation measured by low-coverage whole genome methylation sequencing in four T-ALL cell lines treated with increasing concentrations of 5-azacytidine (AZA), 5-aza-2′-deoxycytidine (DEC), or GSK-3685032 (GSK) for 3 and 7 days. (**B**) Mean global CpG methylation at different genomic locations measured by low-coverage whole genome methylation sequencing in four T-ALL cell lines treated with 0 or 3000 nM GSK for seven days. Promoters (1000 bp up- and 500 bp downstream of transcription start sites) are divided based on whether or not they overlap with CpG islands (CGI). (**C**) Correlation between cell viability and global CpG methylation after treatment with GSK for 3 or 7 days in four T-ALL cell lines. 30% DNA methylation marked by red dotted line.

As the alamar blue assay cannot differentiate between toxicity and decreased cell growth, the observed effect could also indicate a failure to proliferate for cells that have lost DNMT1 activity. However, as we observed apoptosis but no cell cycle arrest for treatment with 3000 nM GSK for three days by Annexin V and PI staining (**Paper II**, data not shown), it is likely that there is a lowest limit below which further removal of DNA methylation becomes cytotoxic. The finding that

DNMT1 knockout and the accompanying loss of DNA methylation is toxic in human ESCs strengthens our hypothesis of cell death occurring in response to extreme DNA demethylation [415].

Studies with GSK in mouse ESCs showed a similar reduction of global DNA methylation as T-ALL cell lines, from 70% to 18% [416]. Furthermore, knockdown of *DNMT1* and loss of DNA methylation in THP-1 cells prevented tumour growth in mouse models [417]. Studies on inducible *DNMT1* knockdown models in human differentiated and cancer cells reported toxicity and global loss of DNA methylation, which was comparable to the extent of DNA demethylation reported here [418, 419]. These studies describe that promoters and gene bodies are enriched for demethylated CpGs, which we did not observe in T-ALL cell lines. We employed an approach of low-coverage whole genome methylation sequencing that provides an approximation of genome-wide and locus-specific DNA methylation. In contrast, the previously described studies [418, 419] estimated DNA methylation by Illumina BeadChip 450K array. Array probes are enriched at promoters and gene bodies [348, 420] and are likely to offer higher resolution information. It is possible that this could explain the discrepancies of these findings to ours.

*Widespread loss of DNA methylation results in activation of methylation-sensitive genes (**Paper I** and **Paper II**)*

Promoter-specific DNA demethylation by HMAs has been suggested to activate the gene expression of tumour suppressor genes [421]. In **Paper I**, we saw a general upregulation of gene expression across four T-ALL cell lines in response to 2000 nM AZA with between 910 and 2163 genes being upregulated in LOUCY and JURKAT cell, respectively. However, these genes were not enriched for tumour suppressors (**Paper I**, data not shown). Our data from **Paper I** clearly showed that AZA treatment results in upregulation of gene expression, but this study had some notable limitations. AZA is known to be primarily incorporated into RNA and could thereby affect gene expression independently of DNA methylation.

Furthermore, information on changes of DNA methylation upon treatment is missing and precludes analysis of the functional impact of DNA methylation on transcription. Lastly, the short timeframe for treatment (72 hours) might not allow for the assessment of all changes in gene expression, as regulation of transcription has been shown to be delayed in response to changes of the DNA methylation profile by HMAs [407]. In **Paper II**, these issues were addressed by treatment with DEC and GSK for up to seven days and the sequencing of whole methylome and transcriptome in matched samples.

Treatment with 10 nM DEC for three days and 300 nM GSK for three and seven days all resulted in global DNA demethylation which was evenly distributed across all chromosomes (**Figure 18A**). While 10 nM DEC reduced mean DNA methylation from 84% to 69% in LOUCY and from 82% to 70% in SUP-T1 cells, treatment with GSK for seven days led to a more extensive loss of DNA methylation to 31% global CpG methylation in both cell lines. The widespread reduction in DNA methylation is reflected in the observation that up to 50% of promoters became demethylated upon treatment with GSK (**Figure 18B**). DEC showed a more arbitrary response with most promoters losing, but a notable number of promoters also gaining DNA methylation ($\geq$ 25% DNA methylation difference, adjusted $p < 0.01$). A comparison of short-term (three days) and long-term (seven days) treatment with 300 nM GSK revealed that the vast majority of changes in DNA methylation occurred after three days and the number of differentially methylated promoters increased only slightly after treatment for an additional four days (**Figure 18B**). Despite extensive DNA demethylation, only a few genes showed increased expression upon loss of promoter methylation (**Figure 18C**). These results confirm previous studies which have shown only minor differences in expression caused by inhibition of DNMT1 through pharmacological means or by siRNA. These studies suggested that other epigenetic mechanisms, such as heterochromatic histone modifications, play a role in continued gene inactivation after removal of DNA methylation [13, 418], which should be further explored.

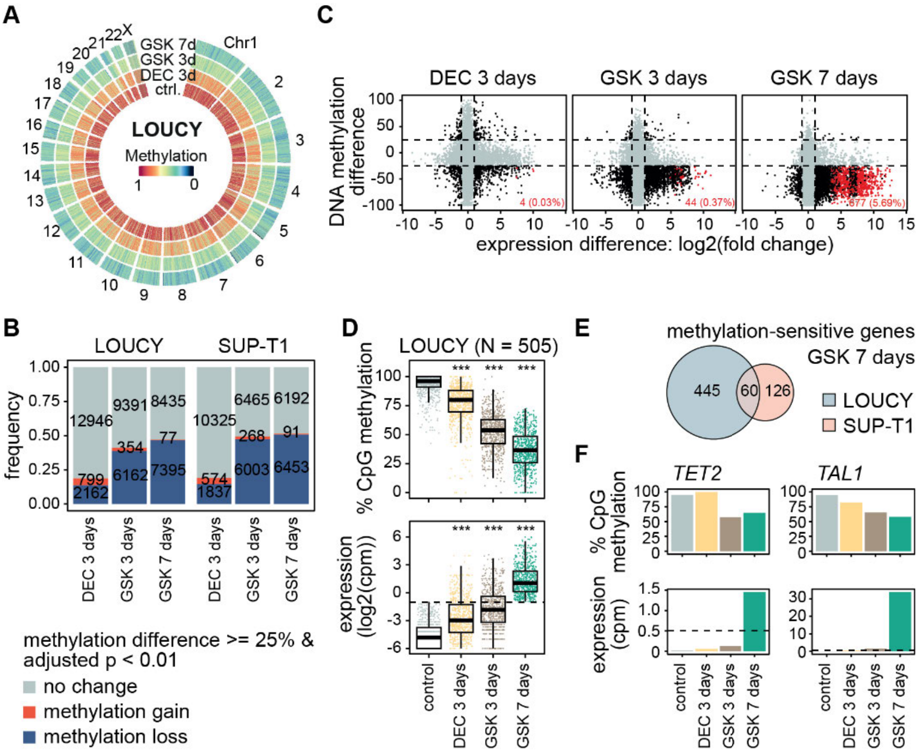


Figure 18: Activation of methylation-sensitive genes after DNA demethylation. (**A**) CpG methylation across the whole genome in 100kb bins for LOUCY cells treated with 10 nM 5-aza-2′-deoxycytidine (DEC) for 3 days, or 300 nM GSK-3685032 (GSK) for 3 or 7 days compared to an untreated control (ctrl). (**B**) Total number of differentially methylated promoters for LOUCY and SUP-T1 cells treated with 10 nM DEC for 3 days, or 300 nM GSK for 3 or 7 days. (**C**) DNA methylation difference at promoters and corresponding gene expression difference as log2(fold change) compared to an untreated control in LOUCY cells treated with 10 nM DEC for 3 days, or 300 nM GSK for 3 or 7 days. Significant upregulation marked in red. (**D**) Mean CpG methylation at promoters (upper) and gene expression as log2(counts per million (cpm)) (lower) of genes defined as methylation-sensitive in LOUCY cells treated with 300 nM GSK for 7 days. Cpm of 0.5 indicated as dotted line. Wilcoxen signed-rank test; *, $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. (**E**) Venn diagram showing the overlap of genes defined as methylation-sensitive after treatment with 300 nM GSK for 7 days between LOUCY and SUP-T1 cells. (**F**) Mean CpG methylation at promoters (upper) and gene expression as cpm (lower) for selected genes in LOUCY cells after treatment with 10 nM DEC for 3 days, or 300 nM GSK for 3 or 7 days.

To identify genes whose expression is directly linked to promoter DNA methylation, we defined a set of genes as methylation-sensitive if they (i) were not expressed in untreated control cells (counts per million (cpm) < 0.5), (ii) lost DNA methylation upon treatment (methylation difference compared to control $\leq 25\%$, adjusted $p < 0.01$), and (iii) were upregulated after treatment (cpm ≥ 0.5, expression fold change > 2, adjusted $p < 0.05$). After a seven-day treatment with GSK, 505 genes in LOUCY and 186 genes in SUP-T1 cells were defined as methylation-sensitive. Analysis of DNA methylation and expression for these genes in all samples revealed that more than 40% of DNA methylation was lost after three days, but gene expression only increased enough to cross our cutoff of cpm ≥ 0.5 after seven days of treatment (**Figure 18D**), suggesting a delayed transcriptional response after loss of DNA methylation. Pappalardi *et al.* described a similar finding in AML cells using GSK [234]. They observed DNA demethylation after two days of treatment and gene upregulation after up to four days.

While several hundred genes for both cell lines were methylation-sensitive, only 60 were shared between LOUCY and SUP-T1 cells (**Figure 18E**). Furthermore, tumour suppressor genes were not enriched in methylation-sensitive genes in either cell line. This finding suggests that DNA demethylation by HMAs is a cell-type specific process and does not uniformly target tumour suppressor genes. Individual tumour suppressor genes, such as *TET2*, *PAX5*, *PAX6* and *FBLN2*, but also the oncogene *TAL1* were activated after treatment with 300 nM GSK for seven days in LOUCY cells (**Figure 18F**). *PAX5* and *FBLN2* are known tumour suppressor genes that have previously been shown to be silenced by DNA methylation in a cancer context [422, 423]. However, *TAL1* is a T-ALL specific oncogene known to drive tumour progression. Thus, treatment with HMAs and the connected changes in gene expression can both inhibit or promote tumour growth and should be carefully evaluated before use.

*Only few endogenous retroviruses become activated in response to DNA demethylation (**Paper I** and **Paper II**)*

Apart from the activation of tumour suppressor genes, HMAs have been suggested to induce an immune response by activation of TEs including ERVs, LINEs, and short interspersed nuclear element (SINEs) [283, 284].

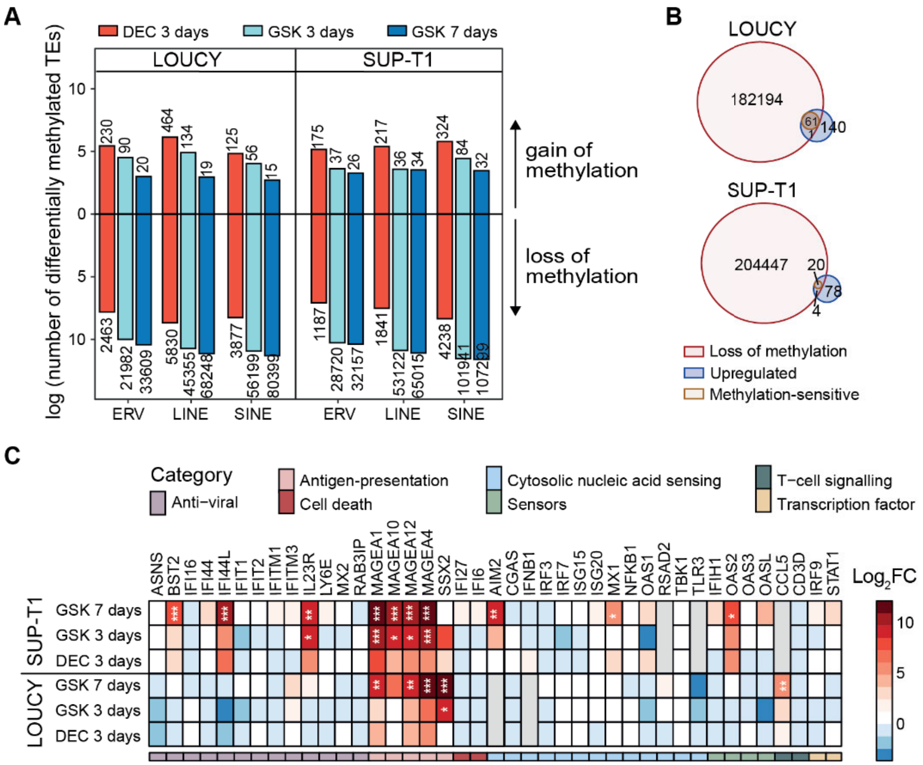


Figure 19: Activation of transposable elements after HMA treatment. (**A**) Number of differentially methylated transposable elements (TEs) in LOUCY and SUP-T1 cells after treatment with 10 nM 5-aza-2′-deoxycytidine (DEC) for 3 days, or 300 nM GSK-3685032 (GSK) for 3 or 7 days compared to an untreated control. Differential methylation defined as ≥ 25% absolute methylation difference and adjusted p < 0.01. (**B**) Overlap of endogenous retroviral elements (ERVs) that lose ≥ 25% DNA methylation and gain expression. (**C**) Differential expression of genes involved in immune response in LOUCY and SUP-T1 cells after treatment with 10 nM DEC for 3 days, or 300 nM GSK for 3 or 7 days.

In **Paper I**, we found that treatment with AZA results in upregulation of ERVs across several ERV families (**Paper I**, data not shown). The results warranted a deeper analysis of TE re-expression upon HMA treatment in **Paper II**.

As 50% of our genome consists of TEs [424], DNA methylation across TEs reflected global 5mC content with mean DNA methylation across TEs being 86% and 82% in LOUCY and SUP-T1 cells, respectively. Treatment with DEC as well as GSK resulted in extensive loss of DNA methylation at all three classes of TEs and identification of thousands of differentially methylated individual TEs across ERVs, LINEs and SINEs (**Figure 19A**). However, only a minority of ERVs was upregulated after treatment with 300 nM GSK for seven days and most upregulated ERVs were not differentially methylated (**Figure 19B**) demonstrating that most ERV sequences in the genome have either mutated and cannot be expressed anymore or are regulated by epigenetic mechanisms other than DNA methylation. Indeed, evolutionarily young ERVs, which have been integrated into the genome more recently, are suggested to be regulated by DNA methylation while older ERVs can be found in heterochromatic regions marked by H3K9me3 [424].

Despite the limited number of upregulated ERVs, a few immune-related genes gained expression after treatment (LOUCY: 24 genes; SUP-T1 16 genes, **Paper II**, data not shown). Particularly *MAGE* genes showed clear upregulation (**Figure 19C**). It is unclear, however, whether *MAGE* genes are activated by ERV expression as part of an interferon gamma response or by DNA demethylation of the genes themselves. Several members of the *MAGE* gene family have been shown to be regulated by DNA methylation as hypomethylation of their promoters leads to transcription in cancer cell models and patients [425, 426]. The example of the *MAGE* genes highlights the challenges faced when analysing cellular responses to HMA treatment and ERV activation. Differences in gene expression could be evoked by expression of retroviral elements or by direct demethylation of immune genes. Furthermore, ERVs have been suggested to act as regulatory element that induce expression of innate immune genes independently of their own active transcription [427] providing an additional layer of complexity.

*Summary of **Paper I** and **Paper II***

In summary, **Paper I** and **Paper II** have given evidence that the tumour suppressor *TET2* is downregulated or silenced in primary T-ALL and is associated with a general increase in DNA methylation. Furthermore, T-ALL cell lines are sensitive to the use of HMAs, but primarily respond to DNA damage induced by AZA and DEC. Lastly, the consequences of DNA demethylation in the absence of DNA damage were explored with the use of a novel DNMT inhibitor. Treatment with GSK revealed a critical lowest value for DNA methylation in T-ALL cell lines and identified a limited number of genes which are regulated by DNA methylation. The matched datasets of whole genome DNA methylation and total RNA-seq provide a valuable resource for further exploration of the biology of DNA methylation in T-ALL.

DNA methylation and gene expression in healthy thymocytes

To understand the significance and function of DNA methylation in T-ALL, DNA methylation in healthy thymocytes, needs to be well described. In **Paper III**, DNA methylation and gene expression in matched samples from six human thymocyte subpopulations were characterized. Using this data as reference, the possibility of DNA methylation as a driver of T-ALL oncogenesis can be further explored. **Paper III** also describes the differences between males and females during thymocyte development and investigates a possible involvement of the X chromosome in establishing sex bias in T cells and immunity. In this work I will summarize key results of **Paper III** with relevance to the biology of T-ALL and DNA methylation.

*Dynamic DNA methylation at key driver genes in thymocyte development (**Paper III**)*

Studies in mice have found that DNA methylation changes dynamically during thymocyte development [167, 428]. Our analysis of DNA methylation (Illumina EPIC array) and mRNA-seq in primary human thymocytes revealed that loss of DNA methylation occurs in parallel with initiation of gene expression of key genes of T cell development (**Figure 20**).

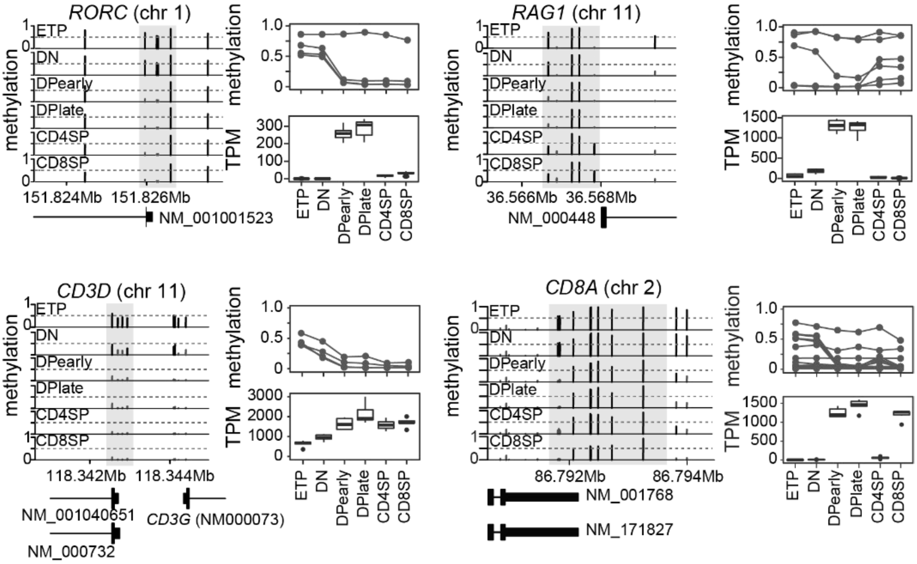


Figure 20: Loss of DNA methylation co-occurs with gain of gene expression. DNA methylation from EPIC array data over chosen exemplary genes. DNA methylation of CpGs inside grey boxes for each thymocyte subpopulation are additionally represented in line plots. Corresponding gene expression is shown by boxplots as transcript per million (TPM). ETP, early thymic progenitors; DN, double negative; DP, double positive; SP, single positive.

For example, *RORC*, *RAG1* and *CD8*, which become expressed after β-selection in DP cells, are methylated in earlier thymic subpopulations (ETP, DN) but lose this DNA methylation when gene expression is induced. While gain of expression is associated with a loss of DNA methylation, gene silencing is not necessarily accompanied by gain of DNA methylation as can be seen for *RORC* and *CD8*. Both genes are not expressed in later stages of thymocyte development (CD4/CD8 SP and CD4 SP cells, respectively) but do not show a matching increase in DNA methylation at those developmental stages (**Figure 20**). Indeed, others have seen that loss of 5mC is more common than gain of DNA methylation during T cell development (see *CD3* in **Figure 20**) and that most driver genes do not regain DNA methylation when they are silenced [429]. One of the few exceptions is *RAG1*, which is demethylated in DP cells and gains DNA methylation at the SP stage during which it is not expressed (**Figure 20**). Together, these results indicate that the process of DNA demethylation and the TET enzymes might have a more vital function during thymocyte development than *de novo* DNA methylation by DNMT3A and DNMT3B.

*T-ALL patients can be re-classified based on cell type of origin (**Paper III** and unpublished)*

Currently, cytosolic and surface markers are used to group T-ALL patients and efforts are being made to predict prognosis and treatment response based on this classification. Broadly, T-ALL subgroups correspond to the cell type that the disease originated from. In practice, these classifications are often limited to defining ETP-ALL cases based on early findings that ETP-ALL is associated with a higher risk, which have now been refuted. Accordingly, T-ALL patients are often divided into three groups: ETP-ALL, nearETP-ALL, and nonETP-ALL [430]. Using RNA-seq data from primary thymocytes as a comparison, patients could be more clearly classified by cell type of origin.

The six primary thymocyte subtypes were extracted from thymus samples from six participants, three females and three males, and mRNA was sequenced. Principal component analysis separated the samples along the thymocyte development trajectory. Notably, ETP and DN cells clustered together and were not distinguishable from each other in this analysis (**Figure 21A**). Analysis of DNA methylation (EPIC array) in the three female participants showed that the three groups of early (ETP, DN), double positive (DPearly, DPlate), and single positive (CD4SP, CD8SP) cells could be clearly separated from each other by DNA methylation. However, inside each of these groups, samples clustered by participant and not cell type (**Figure 21B**), again showing that there are only minor differences between ETP and DN cells.

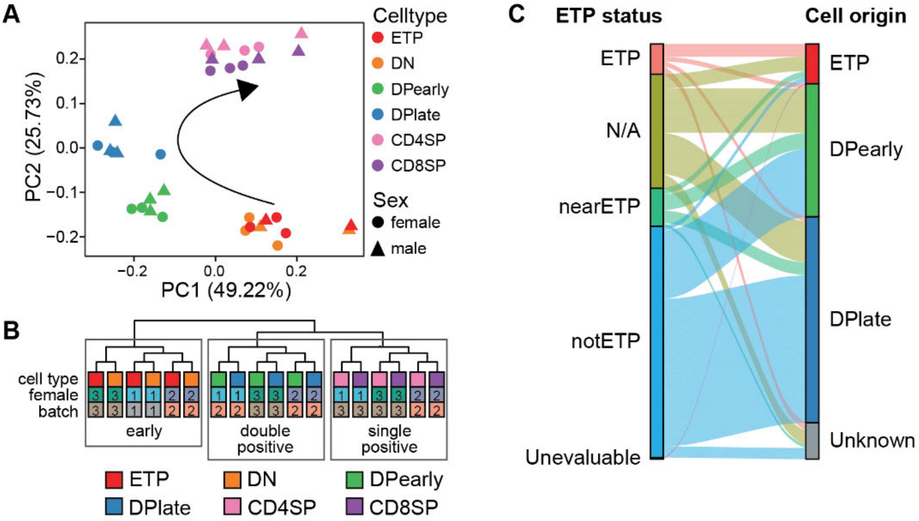


Figure 21: Classification of T-ALL patients based on cell type of origin. (**A**) Principal component (PC) analysis of RNA sequencing data from six thymocyte subpopulations from six participants. Cell type is indicated by colour and sex of the participant by shape. (**B**) Clustering of the six primary thymocyte subpopulations from three female participants based on the 1000 most variable CpGs from EPIC array DNA methylation data. Cell type, participant and batch/array are indicated. (**C**) Re-classification of 264 T-ALL patients, for which RNA sequencing and T-ALL subgroup based on ETP status were publicly available, into groups according to cell type of origin. ETP, early thymic progenitors; DN, double negative; DP, double positive; SP, single positive; N/A, no classification possible.

Based on RNA-seq from these samples, we aimed to re-classify patients from a large cohort including 264 paediatric T-ALL patients [374]. Despite immunophenotypic data being available for all patients, 29% of patients could not be assigned to either ETP-, nearETP-, or nonETP-ALL based on cytosolic and surface markers. We found that cell type of origin in this N/A patient group reflected a balanced mixture of ETP, DPearly, and DPlate cells (**Figure 21C**, unpublished). The nearETP subgroup of patients was also assigned to a mixture of all ETP, DPearly, and DPlate cells and did not seem to uniformly reflect any cell type of origin. Overall, the marker-based ETP-ALL and nonETP-ALL classification seemed to be associated with the expected cell types of origin, ETP and DP, respectively. As RNA sequencing data from patients is not always readily available, it is encouraging that the cheaper and more easily applied marker-analysis accurately reflects cell type of origin for most patients. Nevertheless, as DNA methylation shows distinct differences between thymocyte subpopulations, it can be assumed that a thorough analysis of DNA methylation in T-ALL requires previous classification of samples by cell type of origin to provide the correct DNA methylation profile as reference.

*Females express a higher dosage of X-linked tumour suppressor genes (**Paper III**)*

The inclusion of three male and three female participants in our study allows for a sex-aware analysis of gene expression in thymocytes. Whereas gene expression was highly similar between the sexes overall (**Figure 21A**), the main source of genetic differences between males and females can be found on the X chromosome. In XX-female, the majority of genes on one of the X chromosomes are silenced to compensate for dosage differences compared to XY-males. This inactivation of the X chromosome is incomplete and around 15% of genes are expressed from both X chromosomes in females regardless; so-called 'escape genes' (**Figure 22A**). Previous studies have seen that escape from XCI can be variable between tissues [141]. **Paper III** therefore characterizes escape from XCI and its effect on thymocyte biology based on sex-biased expression, DNA methylation, and allele-specific expression from a female who had inactivated the same parental X chromosome in all cells. In total, 69% of all expressed X-linked genes could be classified as escape or inactivated genes (**Paper III**, data not shown).

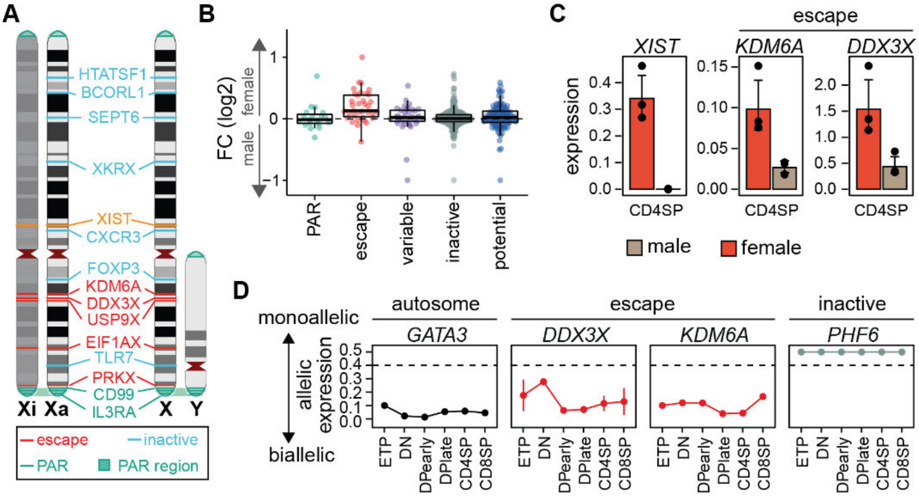


Figure 22: Female-biased expression of tumour suppressor genes escaping X inactivation. (**A**) Schematic representation of expression from the X chromosome. Escape genes are expressed from both the active (Xa) and inactive X (Xi), while inactive genes are solely expressed from Xa. Genes in the pseudoautosomal region (PAR) are expressed from both X chromosomes and the Y chromosome in males. (**B**) Gene expression in thymocytes as log2 of fold change (FC) female over male. Genes are divided based on previous classification of X inactivation status. (**C**) Gene expression measured by qPCR and normalized to housekeeping gene *GAPDH* in male and female CD4 single positive thymocytes. (**D**) Allelic expression of selected genes across the six thymocyte populations: early thymic progenitors (ETP), double negative (DN), early and late double positive (DP), and CD4 and CD8 single positive (SP) cells. Cut-off for monoallelic expression at 0.4 indicated as grey dotted line.

In the context of T-ALL, tumour suppressor genes that escape from XCI in females are of particular interest. The 'EXITS (escape from X inactivation by tumour suppressors) hypothesis' proposes that the presence and expression of an additional copy of X-linked tumour suppressor genes protects female patients from developing cancer. While just one mutation is sufficient for loss of a gene in males, the same gene needs to be mutated or inactivated on both X chromosomes in females [431]. As T-ALL shows a profound sex-bias with around 70% of patients being male, the EXITS hypothesis in the context of T-ALL is intriguing to investigate. Comparing expression of X-linked genes in thymocytes between male and female participants showed a higher expression of many previously annotated escape genes in females (**Figure 22B**). Our results reflect the expected 1.2 – 1.6-fold higher expression of escape genes from XX-cells. Among these female-biased genes were known tumour suppressor genes, such as *KDM6A* and *DDX3X* (**Figure 22C**).

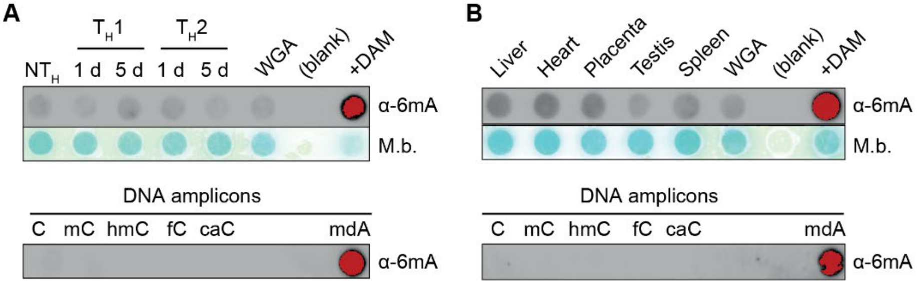
Higher expression of individual genes on the X chromosome is an indication for XCI escape but could also stem from upregulation of the gene on the active X chromosome alone. Therefore, we further integrated single nucleotide variants, called from whole exome sequencing, to analyse allele-specific expression in one female participant that had inactivated the same parental X chromosome in all cells. Both *KDM6A* and *DDX3X* showed clear biallelic expression across all thymocyte subtypes (**Figure 22D**) and are therefore candidate male-specific tumour suppressors. Loss of the H3K27me3 demethylase KDM6A is known to drive cancer progression and has been shown to be inactivated in several malignancies, including T-ALL [432, 433]. On the other hand, *DDX3X*, a member of the family of DEAD-box helicases involved in RNA metabolism, can act as both a tumour suppressor and oncogene in different contexts [434, 435] and its possible role in T-ALL remains to be investigated. Surprisingly, we found *PHF6* to be expressed from only one allele (monoallelic) (**Figure 22D**). PHF6 has been extensively studied as a driver of T-ALL progression [436-438] and was found to be mutated almost exclusively in male T-ALL patients [439]. As only one allele of *PHF6* is expressed in all thymocyte subpopulations in males and females, any loss of function mutation in this gene should be equally detrimental in both sexes. These results highlight the importance of characterizing sex-biased and allele-specific expression in healthy thymocytes in order to further illuminate the involvement of the X chromosome in T-ALL biology.

The biology of DNA N6-methyladenine in human T cells

Not only cytosine methylation, but also methylation of the N6 of adenine bases (6mdA) has been suggested to exist in human DNA and be involved in cancers [85, 440]. As the study of 6mdA in mammals in still limited and accompanied with methodological challenges [75], we started by investigating 6mdA in the context of T cells, which are known to undergo extensive epigenetic changes during activation and differentiation [166, 441] and have been reported to contain relatively high amounts of 6mdA [442].

*No N6-methyladenine detected in mammalian DNA including human T cells (**Paper IV**)*

Naïve CD4$^+$ T cells were isolated from human peripheral blood mononuclear cells and differentiated *in vitro* into Th1 and Th2 T helper cells. However, by immuno-dot blot analysis, we did not detect any changes in total 6mdA content during this differentiation process (**Figure 23A**). Even more striking, no sample in four biological replicates and using two different antibodies showed a stronger signal than whole genome amplified (WGA) DNA, which does not contain any DNA modifications (**Figure 23A** and **Paper IV**, data not shown). As we failed to find any 6mdA in primary human T cells, we expanded our analysis to DNA from five different tissues in human and mice. However, 6mdA could not be identified in any of the analysed tissues above the negative WGA control (**Figure 24B** and **Paper IV**, data not shown).


A
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NT_H
1 d
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Liver
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Testis
Spleen
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α-6mA
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DNA amplicons
C
mC
hmC
fC
caC
mdA
α-6mA

Figure 23: No detection of N6-adenine methylation in mammals by immune-dot blot. (**A**) Immuno-dot blot for adenine methylation (6mA) in naïve (NT_H) and differentiated T helper cells (T_H1 and T_H2). (**B**) Immuno-dot blot for 6mA in five human tissues. (**A,B**) Whole genome amplified (WGA) DNA and DNA treated with DNA adenine methylase (DAM) were included as negative and positive controls, respectively. Antibody specificity was confirmed by the inclusion of DNA amplicons including unmodified cytosine (C), methyl-C (mC), hydroxymethyl-C (hmC), formyl-C (fC), or carboxyl-C (caC). Loading was confirmed by methylene blue (M.b.) staining.

Apart from immuno-dot blots, mass spectrometry is a highly sensitive method to detect global amounts of DNA modifications. Others have highlighted the risk of contamination of reagents used in mass spectrometry with bacterial DNA [75] which could explain the vastly differing total amounts of 6mdA reported in different studies using mass spectrometry (**Paper IV**, data not shown).

*Detection of N6-methyladenine in mammals is confounded by bacteria contamination and extensive methodological challenges (**Paper IV**)*

Several well-designed studies have reported the existence of 6mdA in mammalian DNA using a range of techniques, such as immune-dot blots, MeDIP-seq, mass spectrometry, and more recently SMRT-seq and nanopore sequencing. In **Paper IV**, we critically re-evaluated published datasets using these methods of detection of 6mdA in mammalian DNA.

We found that contamination of cell lines with *mycoplasma* resulted in artefactual detection of 6mdA, as this DNA modification is abundant in bacterial DNA (**Figure 24A**). The possibility of false positive results through the presence of *mycoplasma* needs to be carefully considered in any study of 6mdA as primary human tissues and particularly cancer samples are often infected with *mycoplasma* [443]. We showed that antibodies raised against 6mdA bind to unmodified adenine in the absence of 6mdA (**Paper IV**, data not shown). Furthermore, previous work described the nonspecific binding of antibodies to repetitive sequences in the genome [345]. Indeed, comparison of the binding profile of 6mdA in MeDIP-seq data from genomic DNA and a matched WGA sample showed that most peaks reported for genomic DNA overlapped WGA, which should be devoid of any 6mdA, and therefore represent false positive peaks (**Figure 24B**). Additionally, artefactual signals in MeDIP-seq can arise from contamination by RNA since adenines in RNA are frequently methylated. We found that alignment with a splice-aware aligner (STAR) confirmed the presence of spliced reads in two published MeDIP-seq datasets (**Figure 23C**).

Recently, use of the third-generation sequencing techniques SMRT-seq and nanopore sequencing has gained popularity for the analysis of DNA modification. In bacterial DNA, where 6mdA is abundant, both techniques are highly precise for base-specific identification of 6mdA [444, 445]. However, we show that SMRT-seq tends to overestimate 6mdA in the presence of 5mC, a much more common DNA modification in mammals (**Paper IV**, data not shown). Another recent study has similarly failed to detect 6mdA in PBMCs or glioblastoma samples using SMRT-seq [446].

The accuracy of nanopore sequencing, which we did not investigate in our study, is determined by the analysis software that is used. While precision of 6mdA detection in bacteria using nanopore sequencing is normally around 70%, it can be improved by training analysis models on data in which the presence of 6mdA has been confirmed by other analysis methods such as MeDIP-seq [447]. However, considering the rarity of

6mdA in mammals and the inaccuracies of MeDIP-seq that were identified here, it is questionable if such an improvement by cross validation would be applicable in humans.

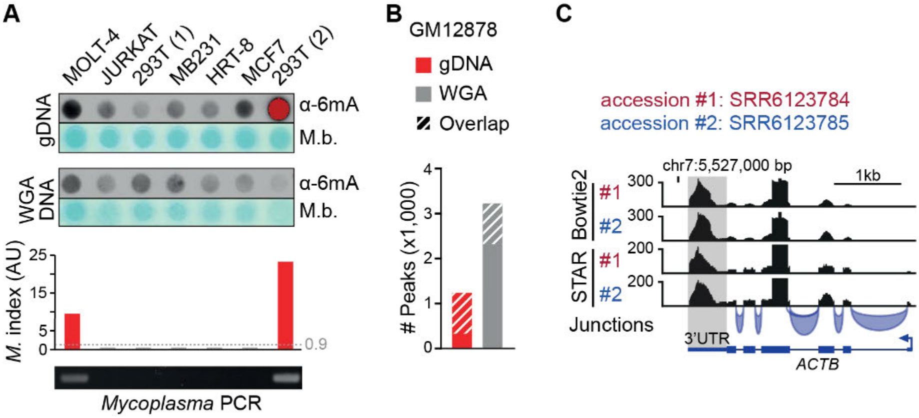

Figure 24: Artefactual detection of 6mdA. (**A**) Immuno-dot blot for adenine methylation (6mA) in genomic DNA (gDNA) from immortalized cell lines and negative controls of whole genome amplified (WGA) DNA (top). *Mycoplasma* contamination detected by fluorescent assay and PCR are shown below each sample. For the fluorescent assay, a *mycoplasma* index (*M.* index) above 0.9 (dotted line) is considered as positive detection of *mycoplasma*. (**B**) Number of peaks from DNA immunoprecipitation sequencing (MeDIP-seq) of genomic DNA (gDNA) and matched WGA DNA. Overlap between peaks from both samples are marked. (**C**) MeDIP-seq signal tracks for reads aligned using indicated aligners. Splice junctions are shown for sample #2 (SRR6123785).

Nevertheless, it has been suggested that methylated adenine bases from RNA could be incorporated into DNA through the nucleotide salvage pathway [448]. Although there is no incorporation of recycled RNA bases into DNA in healthy cells, depletion of the adenosine deaminase ADAL or overexpression of the adenylate kinase AK1 has been shown to promote false incorporation of 6mdA [449]. Since expression of many genes is heavily dysregulated in cancers, we cannot exclude the possibility that 6mdA is present in cancer cells, but current analysis techniques are not sensitive enough to distinguish any true 6mdA events from false positives. More recent studies have confirmed our findings and the presence of 6mdA as a true epigenetic mark in mammals remains unresolved [89, 446, 450, 451].

Concluding Remarks and Future Perspectives

As the pursuit of improved and more targeted therapies for cancer continues, the idea of targeting the epigenome of malignant cells has gained increasing interest. While the search for driver mutations and other genetic aberrations has previously been the focus of many studies on cancer biology, we have learned that epigenetic changes can dysregulate cellular function in a similar way to mutations. The methylation and subsequent silencing of a gene, such as reported for *TET2* in this work, results in loss of its enzymatic function, phenocopying loss-of-function mutations in *TET2*. In recent years, there have been an increasing number of studies reporting cases of tumour suppressor gene silencing through DNA methylation in both somatic and germline cells. Irregularities in histone modifications and chromatin conformation have likewise been investigated. These studies are most likely only the first steps on the long journey towards revealing the consequences of aberrant epigenomic profiles in cancer biology. As we have shown, not all epigenetic changes necessarily impact gene expression and the analysis of multiple layers of epigenetic regulation should be integrated to unravel the complex mechanism of transcriptional regulation in cancer. Furthermore, the role of DNA and histone modifications distal to genes and regulatory elements remains less well defined. The characterization of DNA methylation and gene expression in healthy thymocytes in this thesis provides a reference for further analysis of the role of DNA methylation in T-ALL in particular.

In contrast to genetic changes, epigenetic changes in cells can be readily modified by chemical agents. The possibility of correcting the epigenetic drivers of cancer was the motivation behind the extensive research effort on drugs targeting DNA methylation, such as vitC and HMAs over the past three decades. Similarly, many clinical trials have been conducted on the use of epigenetic drugs in haematopoietic malignancies, including T-ALL, and solid cancers. However, the recurring development of resistance to HMAs in MDS and AML patients and our inability to predict response to these therapies also highlights the need for further investigation of their mechanism of action. This PhD thesis illuminates the function of HMAs in T-ALL in a controlled *in vitro* setting using immortalized cell lines, but further *in vivo* studies on primary T-ALL samples are needed to unravel the validity of these findings in a more complex system.

Our finding that increased DNA methylation in a group of T-ALL patients could sensitize them to treatment with HMAs and the possibility that loss of *TET2* might be involved in either the gain of DNA methylation or the response to therapy has been corroborated by some very recent studies, strengthening the importance of further investigation of this novel avenue of therapeutic intervention in T-ALL. From a clinical perspective, the clear separation of T-ALL patients based on DNA hypermethylation, either genome-wide or at specific loci, needs to be further refined and associated with response to HMAs. On a more mechanistic level, the question remains whether *TET2* methylation is the origin of or a marker for high levels of DNA methylation. Targeted addition or removal of DNA methylation at the *TET2* promoter could, for example, shed more light on the role of *TET2*.

In summary, this work underlines the relevance of DNA methylation in T-ALL and offers the foundation for further research into epigenetic modifications in the biology and treatment of this disease.

Acknowledgements

I have enjoyed these last years of working towards a PhD degree like no other time in my life so far. This is largely thanks to all the wonderful people who have accompanied me along this way and so, I would like to say ***Thank You*** to everyone that has been there during this time.

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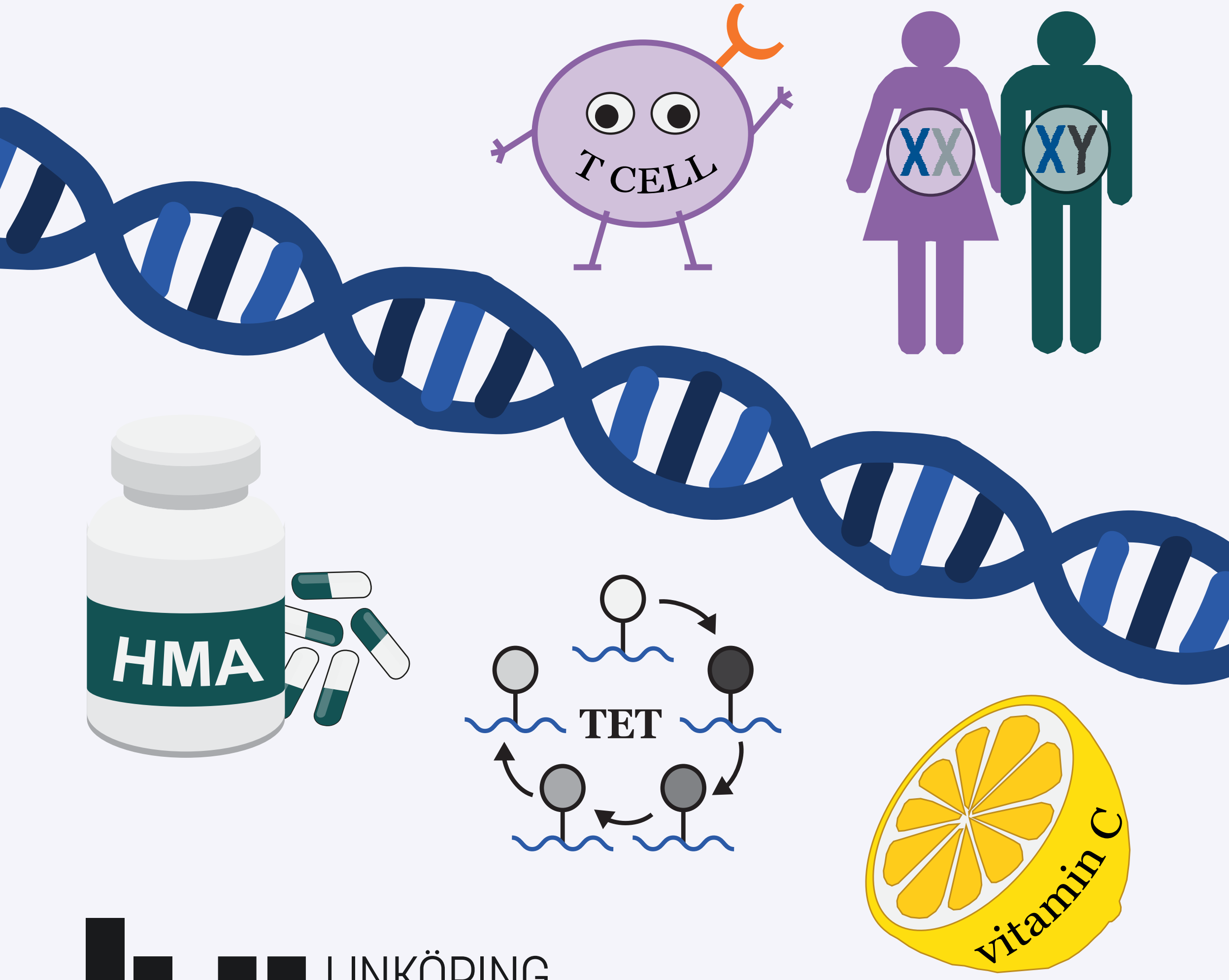
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