EPIGENETIC DEREGULATION OF *WT1* AND *AWT1* IN HEMATOLOGICAL MALIGANCIES

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Hereby states that,

Amy Lauren Guillaumet Adkins, has performed under our direction and supervision, within the Imprinting and Cancer group in the department of PEBC at IDIBELL and in association with the department of Biochemistry and Molecular Biology of the Universitat Autònoma de Barcelona; the experimental work entitled "EPIGENETIC DEREGULATION OF *WT1* AND *AWT1* IN HEMATOLOGICAL MALIGNANCIES". This work has been financed by FPU (Formación del Profesorado Universitario) del Ministerio de Eduación, Cultura y Deporte and a EMBO short-time fellowship.

The work accomplishes the adequate conditions in order to be defended in front of the corresponding Thesis Committee, to obtain the doctor degree from the Universitat Autònoma de Barcelona.

Signed:

Dr. David Nicholas Monk Dr. Anna Bassols Amy Lauren Guillaumet

Barcelona, 2013

Per l'Edu

for my familiy

des de Cal Guasquet

to Louisville, Kentucky

“Tell me and I forget

Teach me and I remember

Involve me and I learn”

Benjamin Franklin

“Sometimes the questions are complicated

And the answers are simple”

Dr. Seuss

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ABSTRACT

Intense research over the past decade has revealed that cancer is much an epigenetic as a genetic disease. The epigenetic deregulation can disrupt many cellular processes resulting in silencing of tumor suppressor genes, activation of oncogenes, genome instability and inappropriate imprinted gene expression. The latter process is the parental of origin monoallelic expression that is dictated by regions of differential methylation inherited from the germline, that when disrupted contributes to the initiation and progression of cancer. In hematological malignancies very little is known about the deregulation of imprinted transcripts. The *WT1* gene has various transcripts that act as a tumor suppressor and an oncogene depending on the cellular context, and for which paternal expression of the alternative *AWT1* and antisense isoforms has been reported. This allelic expression is postulated to be regulated by a tissue-specific differential methylation region (DMR), known as the WT1-antisense regulatory region. Interestingly, *WT1* expression is aberrantly up regulated in hematological malignancies and is routinely used as a molecular marker for minimal residue of disease in acute myeloid leukemia.

Herein, we have fully characterized the epigenetic landscape encompassing the *WT1* and *AWT1* promoter regions in an attempt to identify the mechanism leading to aberrant expression, and whether loss-of-imprinting is routinely observed. Using various molecular techniques we fail to identify allelic methylation within the *WT1* promoter interval, but we observe a temporal increase in methylation that is consistent with previous reports. Despite conclusive evidences for lack of imprinting, we found that the region frequently becomes hypermethylated in leukemias and lymphomas, and that hypermethylation of the *AWT1* promoter occurs in 100 % of AML cell lines despite the high expression levels. Further characterization of myeloid derived leukemia cell lines revealed that this cancer-associated hypermethylation is associated with a concomitant switch in histone modifications, from a permissive to a heterochromatic state. DNA methylation analysis in more than

169 primary leukemia samples revealed that this hypermethylated signature occurs in 89% of AML samples, independent of underlying mutations or translocation/fusion proteins, and is a promising molecular marker for AML, having a positive predictive value of 100% and a negative predictive value of 87.6%. Despite this extensive epigenetic profiling, both at the promoter and at the miRNA level, the only correlation we observe with *WT1/AWT1* expression in AML is the co-expression of the transcription factor, *GATA-2,* which binds to the enhancer located in the 3'UTR of *WT1*. This observation, lead us to hypothesize that GATA-2 induced expression of *WT1* initiating an auto-regulative mechanism in which WT1 interacts and recruits the DNA methyltransferases to degenerative WT1/EGR-1 binding sites within the *AWT1* promoter, leading to its hypermethylation.

RESUM

En l'última dècada, s'ha posat en evidència que el càncer és tant una malaltia genètica com epigenètica. La desregulació epigenètica pot afectar molts processos cel·lulars, com són el silenciament de gens supressors de tumors, l'activació d'oncògens, l'inestabilitat genòmica i/o la desregulació d'expressió de gens impremtats. Aquesta última, és l'expressió monoal·lèlica parental dictada a través de regions de metilació diferencial, heretades de la línia germinal. Aquests gens, en càncer es troben pertorbats, contribuint a la iniciació i progressió del càncer. En neoplàsies hematològiques es desconeix la desregulació d'aquests transcrits. El gen *WT1,* segons els seu context cel·lular, pot actuar com un suppressor de tumors o com un oncogen, a més a més, presenta unes isoformes d'expressió paterna, el transcrit alternatiu *AWT1* i l'antisense *WT1-AS*. Aquesta expressió al·lèlica es creu que és regulada per una regió diferencialment metilada (DMR), coneguda com la regió reguladora de *WT1* antisense. Curiosament, l'expressió de *WT1* està desregulada en neoplàsies hematològiques, i s'utilitza rutinàriament com un marcador residual de la malaltia en leucèmies mieloides agudes. S'ha caracteritzat l'epigenètica de les regions promotores de *WT1* i *AWT1*, per identificar el mecanisme que condueix a l'expressió aberrant, i si la pèrdua d'empremta n'és la causa. Utilitzant diverses tècniques moleculars no es va ser capaç d'identificar la metilació al·lèlica dins de l'interval del promotor de *WT1*, però tot i això s'observa un augment temporal en la metilació, coherent amb resultats publicats prèviament. Malgrat no trobar aquesta regió impremtada, s'observa que aquesta regió s'hipermetila amb freqüència en leucèmies i limfomes, i que la hipermetilació del promotor *AWT1* es produeix en el 100% de línies cel·lulars d'AML, malgrat els alts nivells d'expressió. A més a més, la hipermetilació s'associa amb un canvi concomitant en les modificacions d'histones, d'un estat permissiu a un estat d'heterocromatina. En l'anàlisi de metilació de DNA en mes de 169 mostres de leucèmia primària, es va observar una hipermetilació

en un 89% de les mostres de AML, independentment de mutacions i/o translocacions/fusions, a més a més de ser un marcador molecular prometedor per a la AML, amb un valor predictiu positiu de 100% i valor predictiu negatiu de 87,6%. Malgrat aquest perfil epigenètic extens, tant a nivell de promotor com a nivell de miRNA, l'única correlació que s'observa amb l'expressió de *WT1/AWT1* en AML és la co-expressió del factor de transcripció, GATA-2, que s'uneix a un enhancer situat al 3'UTR de *WT1*. Aquesta observació, porta a la hipòtesi que GATA-2 indueix l'expressió de *WT1/AWT1*, i aquest inicia un mecanisme autoregulador en què interactua WT1 i recluta les metiltransferases d'DNA als llocs d'unió de WT1/EGR-1 al promotor d'*AWT1* hipermetilant-lo.

ABBREVIATIONS

3'UTR: 3'-untranslated region.

5caC: 5'-carboxylcytosine.

5Fc: 5'-formylcytosine.

5hmC: 5-hydroxymethylcytosine.

5mC: 5-methylcytosine.

AID: Activation-induced cytidine deaminase.

ALL: Acute lymphoblastic leukemia.

AML: Acute myeloid leukemia.

ATCC: American type culture collection.

AWT1: Alternative *WT1* transcript.

AZA: 5-Azacytidine.

BAC: Bacterial artificial chromosome.

bp: Base pairs.

BCL-2: B-cell lymphoma 2.

BIS: Bisulfite

BL: Burkitt lymphoma

BM: Bone marrow.

BSA: Bovine serum albumin.

BWS: Beckwith-Wiedemann syndrome.

cDNA: Complementary DNA.

ChIP: Chromatin immunoprecipitation.

CLL: Chronic lymphocytic leukemia.

CLP: Common lymphocyte progenitor.

CML: Chronic myeloid leukemia.

CMP: Common myeloid progenitor.

CTCF: CCCTC binding factor.

CTLs: CD8+ cytotoxic T lymphocyte.

CpG: Cytosine-phosphate-guanine.

CoIP: Co-immunoprecipitation.

DAC: 5'-Aza-2'-deoxycytidine.

DLEU2: Deleted in lymphocytic leukemia 2.

DMEM: Dulbecco's modified eagle medium.

DMRs: Differentially methylated regions.

DMSO: Dimethyl sulfoxide.

DNA: Deoxyribonucleic acid.

DNMTs: DNA methyltransferases.

dNTPs: Deoxyribonucleoside trihphosphates.

DTT: Dithiothreitol.

ESCs: Embryonic stem cells.

EDTA: Ethylene diamide tetracetic acid.

EGFR: Epidermal growth factor receptor.

FAB: French-American-British classification.

FDA: Food and drug administration.

FL: Follicular lymphoma

FISH: Fluorescence in situ hybridization.

gDNA: Genomic deoxyribonucleic acid.

GMP: Granulocytic monocytic progenitor.

HATs: Histone acetyltransferases.

HDACs: Histone deacetylases.

HDMs: Histone demethylases.

HKMTs: Histone lysine methyltransferase.

HMTs: Histone methyltransferases.

HSCs: Hematopoietic stem cells.

IC_{50}: Half maximal inhibitory concentration.

ICF: Immunodeficiency centromere instability and facial anomalies syndrome.

ICRs: Imprinting control regions.

IGF1R: Insulin-like growth factor 1 receptor.

K: Lysine.

kb: Kilobases.

kDa: KiloDaltons.

L: Lysine.

LINE: Long interspersed nucleotide element.

LOI: Loss-of-imprinting.

LOH: Loss of heterozygosity.

LRES: Long-range epigenetic silencing.

LSC: Leukemic stem cell.

MDS: Myelodysplastic syndrome.

meDIP: Methylated DNA immunoprecipitation.

MEP: Megakaryocytic erythrocytic progenitor.

MGB: Minor groove binder.

MLL: Mixed lineage leukemia.

MRD: Minimal residual marker.

mRNA: Messenger RNA.

mUPD: Maternal uniparental disomy.

NAD^+: Nicotinamide adenine dinucleotides.

NCBI: National Center for Biotechnology Information.

NIH: National Institute of Health.

NFQ: Non-fluorescent quencher.

NK: Natural killer.

NPV: Negative predictive value.

ncRNAs: Non-coding RNAs.

PBS: Phosphate buffer saline.

PCR: Polymerase chain reaction.

Ph: Philadelphia chromosome.

PMSF: Phenylmethanesulfonylfluoride.

PRC2: Polycomb repressive complex 2.

PRMTs: Protein arginine methyltransferases.

PPV: Positive predictive value.

pUPD: Paternal uniparental disomy.

R: Arginine.

RB1: Retinoblastoma 1.

RISC: RNA-induced silencing complex.

rpm: Revolution per minute.

RNA: Ribonucleic acid.

RT-PCR: Reverse transcriptase polymerase chain reaction.

SAM: S-adenosylmethionine.

SIRT: Sirtuins.

SDS: Sodium dodecyl sulphate.

SNP: Single nucleotide polymorphism.

SOC: Super-optimal broth with catabolite repression.

sWT1: Small *WT1* transcript.

TAE: Tris-acetate buffer.

TET1: Ten-eleven translocation 1.

TGD: Thymine DNA glycosylase.

TP53: Tumor protein p53.

TRBP: Transactivation-responsive RNA-binding protein.

TEMED: N',N',N'-tetramethyl-1,2-diaminoethane.

TSG: Tumor suppressor genes.

TSS: Transcription start site.

UPD: Uniparental disomy.

UTR: Untranslated region.

UHRF1: Ubiquitin-like, with PHD and RING finger domains 1.

VEGF: Vascular endothelial growth factor.

VEGFR: Vascular endothelial growth factor receptor.

WAGR: Wilms' tumor, Anidria, Genitourinary abnormalities, mental Retardation.

WHO: World Health Organization.

WT1: Wilms' tumor 1.

WT1-ARR: Wilms' tumor antisense regulatory region.

WT1-AS: Wilms' tumor 1 antisense.

INTRODUCTION

1. CANCER.

According to the World Health Organization (WHO), cancer is a leading cause of death worldwide, accounting 7.6 million fatalities in 2008. It is estimated that deaths from cancer will rise to over 13 million in 2030. Therefore, extending our knowledge about the causes of cancer, interventions, prevention and management, are still very much needed.

1.1. CARCINOGENESIS.

Carcinogenesis is the process of transformation from a normal to a cancerous cell. Cancer arises when the homeostatic balance of a cell is disrupted, leading to an increase in cell proliferation. Carcinogenesis is a multistep process, where several events lead to the growth of a malignant tumor. These events reflect genetic and epigenetic alternations, where normal cells undergo several pre-malignant states before resulting in invasive cancer. Several types of genetic alterations affect cell growth such as point mutations, aneuploidy, chromosome translocations, as well as gene deletions and amplifications. These alterations can cause proto-oncogenes to become oncogenes and tumor suppressor genes to lose function. Although these are opposite functions that would be intuitively mutually exclusive for a single protein, evidence is emerging that one protein can exhibit both properties under different cellular conditions. For example, the Wilms' tumor 1 (WT1) protein has dual behaviour. In leukemia it acts like and oncogene, whereas in the childhood kidney cancer, Wilms' tumor, it acts like tumor suppressor. Therefore, the oncogenic function of WT1 depends upon the cell type in which it is expressed (Yang et al., 2007). In tumors, epigenetics alterations can also occur, leading to activation or silencing of genes (see section 3.1). Therefore, cancer is a extremely heterogeneous, and in most cases, an accumulative disease. The determinants of cancer are many and varied, including genetic predisposition, environmental influences, infectious agents, nutritional factors and radiation exposure among

others. In most human cancers, there are some common molecular, biochemical and cellular characteristics. In the year 2000, Doug Hanahan and Robert Weinberg, proposed six hallmarks of cancer: self-sufficiency in growth signals, evading growth suppressors, evasion of programmed cell death, enabling replicative immortality, inducing angiogenesis and activating invasion/metastasis. Recently, these two authors included two additional hallmarks that have emerged from evidences exposed over the last decade: reprogramming of energy metabolism and evading immune destruction (Weinberg et al., 2011) (Figure I.1).

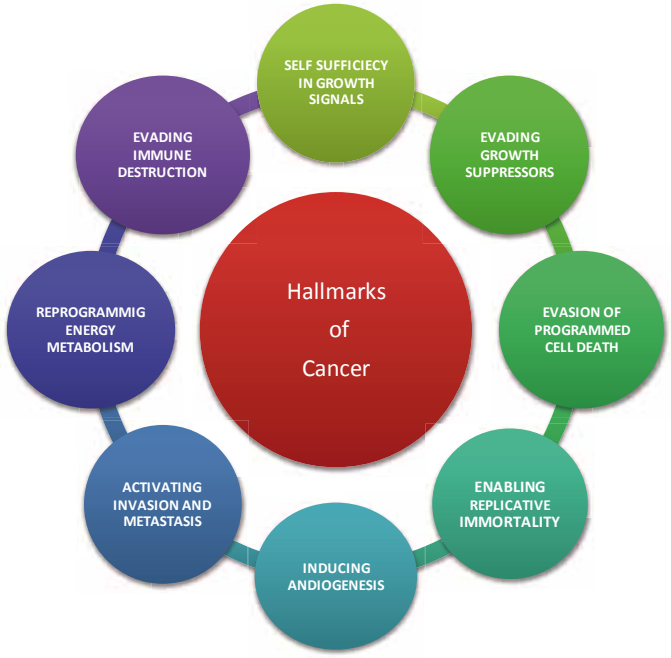


Figure I.1: Hallmarks of Cancer. Figure adapted from Hanahan and Weinberg, 2011.

1-*Self Sufficiency in Growth Signals.*

Normal cells require mitogenic growth signals in order to progress from quiescence to a proliferative state. This process is carefully controlled, ensuring a homeostasis in cell number and maintenance of normal tissue architecture and function. Normally, these growth signals are transmitted into the cell by the binding of ligands to the cell-surface receptors. Cancer cells can acquire the

capability to sustain proliferative signalling in a number of ways. They can produce growth factors themselves, resulting in autocrine proliferative stimulation. They can also send signals to stimulate normal cells within the supporting tumor-associated stroma, which reciprocate by supplying the cancer cells with various factors. Receptor signalling can also be deregulated by elevating the levels of receptor proteins located on the surface of the cancer cells. Growth factor independence may also take place due to the constitutive activation of components of signalling pathways operating downstream of these receptors, for example, epidermal growth factor receptor (EGFR), insulin-like growth factor 1 receptor (IGF1R) and vascular endothelial growth factor (VEGFR) (Witsch et al., 2010).

2-*Evading Growth Suppressors.*

Cancer cells have to override programs that negatively regulate cell proliferation, which in many cases depends on tumor suppressor genes. Tumor suppressor genes (TSG), such as *RB1* (Retinoblastoma 1) and *TP53* (Tumor protein p53), operate as a central control in the cell, deciding whether the cells have to proliferate or activate senescence and apoptotic programs. In cancer, these proteins are frequently mutated, affecting the pathway function and contributing to the continuous cancer cell proliferation (Sherr et al., 2002).

3-*Evasion of Programmed Cell Death.*

Apoptosis is the process of programmed cell death. Biochemical events lead to characteristic cell changes and death. These changes include cell shrinkage, chromatin condensation and chromosomal and DNA fragmentation. Apoptosis can be triggered by a variety of factors such as DNA damage, cellular stress, hypoxia and/or cytotoxic drugs. Key regulators of this process are the BCL-2 (B-cell lymphoma 2), p53 and RB1 proteins. In cancer cells, these are mutated,

therefore, giving the ability for cancer cells to evade apoptosis (Adams et al., 2007).

4-*Enabling Replicative Immortality.*

Telomeres are a region of repetitive nucleotides sequences that cap the ends of the chromosomes, which is essential for maintaining genomic integrity and stability. Telomeres are shorted with every replication, even though this process is delayed by telomerase, a DNA polymerase that has the ability to elongate them. In a normal cell, the telomere length decreases from replication to replication. In cancer cells, the telomerase is upregulated, resulting in the telomere maintenance and limitless replicative potential (Blasco et al., 2011).

5-*Inducing Angiogenesis.*

Like in normal tissues, tumors require sustenance in the form of nutrients and oxygen as well as an ability to evacuate metabolic waste and carbon dioxide. For tumors to grow in size and develop metastatic potential, they must undertake an "angiogenic switch" or angiogenesis by perturbing the local balance of proangiogenic and antiangiogenic factors. The vascular endothelial growth factor (VEGF) plays a central role in this process, giving rise to the growth of new blood vessels from pre-existing vessels (Hanahan et al., 1996).

6-*Activating Invasion and Metastasis.*

Metastasis is the spread of cancer cells from primary tumor sites to distant organs and tissues and remains the cause of more than 90% of death in solid tumors. The best-characterized alternation involved in the process of metastasis is the loss by carcinoma cells of E-cadherin, a key cell-to-cell adhesion molecule. E-cadherin is expressed in epithelial cells and acts as a suppressor of invasion and metastasis and its functional elimination results in an acquisition of metastatic ability (Talmadge et al., 2010).

7-*Reprogramming of energy metabolism.*

Cancer cells have to adjust the energy metabolism in order to proliferate in an uncontrolled way. In cancer cells a metabolic switch takes place, due to the hypoxia conditions, therefore, glycolysis is accentuated with an upregulation of glucose transporters and enzymes of the glycolytic pathway (Semenza et al., 2008).

8-*Evading Immune Destruction.*

The immune system plays a resisting or eradicating role in neoplasias. Therefore, the immune system acts as a significant barrier to tumor formation and progression. The innate immune system constantly kills cancer cells inside our bodies, and the immune response may act as a prognostic marker. For example, patients with colon and ovarian tumors with massively infiltrated $CD8^+$ cytotoxic T lymphocytes (CTLs) and natural killer (NK) have a better prognosis than those that lack such abundant killer lymphocytes (Pagès et al., 2010). But cancer cells can evade immune destruction by eliminating components for their destruction, for example, by suppressing the actions of cytotoxic lymphocytes (Mougiakakos et al., 2010).

In addition to these eight hallmarks of cancer described by Hanahan and Weinberg, it is also important to mention that both constitutional and acquired genetics also plays a key role in the development of this disease. These include chromosomal rearrangements, mutations, gene amplification, deletions, and loss of heterozygosity (LOH) all of which alter gene expression levels. Aberrant expression can also be achieved by disrupting the epigenetic regulation within cells.

2. EPIGENETICS.

Epigenetics was first described in 1942 by C.H. Waddington (Waddington, 1942) as "the causal interactions between genes and their products, which bring the phenotype into being", but today it is described as the study of heritable changes in gene expression that occur independent of changes in the primary DNA sequence. These heritable changes are regulated by different systems, DNA methylation, RNA-associated silencing, histone modifications and variants (Egger et al., 2004). These mechanisms have been shown to be indispensable in the regulation of tissue-specific gene expression, X-chromosome inactivation and genomic imprinting.

2.1. EPIGENETIC MECHANISMS.

Eukaryotic DNA is packaged into chromatin consisting of nucleosomes wrapping 146 base pairs of DNA around an octamer of four core histones (H2A, H2B, H3 and H4) (Luger et al., 1997) (Figure I.2). Historically, chromatin has been classified as either euchromatin or heterochromatin. Euchromatin is not condense permissive chromatin that may be transcriptionally active or inactive. Heterochromatin is condensed and transcriptionally silent. It can be in a permanent state of silence, known as constitutive heterochromatin, or potentially dynamic, known as facultative heterochromatin. Thus, there is a spectrum of chromatin states suggesting that chromatin is a highly plastic macromolecule. The chromatin structure can be modified by epigenetic mechanisms, which can be divided into four categories: DNA methylation, covalent histone modifications, non-covalent mechanisms (histone variants and nucleosome remodelling) and non-coding RNAs (ncRNAs). All of these modifications put together create the "epigenetic landscape" allowing the genome to display unique properties and distribution patterns in different cell types for its cellular identity (Sharma et al., 2010).

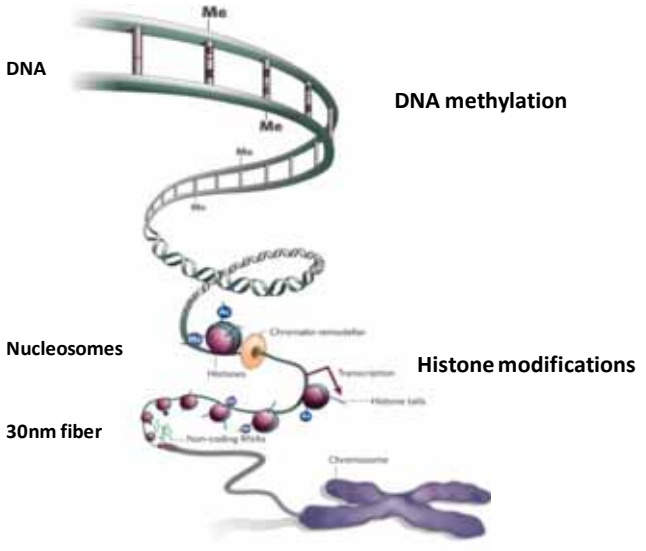


Figure I.2: Overview of the different levels of DNA organization and the epigenetic mechanisms that can modify it. Figure adapted from AACR Human Epigenome Task Force and EU, Nature, 2008.

2.2. DNA METHYLATION.

DNA methylation is the oldest epigenetic mechanism known to correlate with gene expression (Razin et al., 1980). It is present to varying degrees in all eukaryotes except yeast. This modification consists of the addition of a methyl group at cytosine residues of the DNA template. Cytosine bases that are located 5' next to a guanine and are methylated are known as CpG dinucleotides. The DNA methyltransferase (DNMTs) are a family of enzymes that catalyze either the *de novo* or maintenance methylation of hemimethylated DNA following DNA replication. They transfer a methyl group from the methyl donor S-adenosylmethionine (SAM) resulting in 5-methylcytosine (5mC) (Figure I.3).

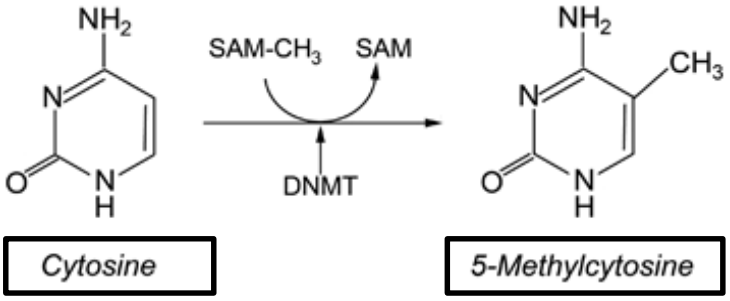


Figure I.3: Conversion of cytosine to 5-methylcytosine by DNMTs. DNMT transfers a methyl group (CH_3) from SAM to the 5'-carbon position of a cytosine.

5mC constitute 1% of all DNA bases, affecting about 70% of the CpG dinucleotides of the genome (Ehrlich et al., 1982). Most of these CpG dinucleotides are associated with repetitive elements such as centromers, retroelement repeats, microsatellite sequences and promoter regions. Among these DNA sequences, all except the promoter regions, known as the CpG islands, undergo a global methylation in the genome. These CpG islands are GC-rich unmethylated sequences associated with promoter regions of transcripts. Approximately 60% of human genes have CpG island promoters. Therefore, gene transcription can be regulated by the presence of absence of DNA methylation at these CpG elements. There are two basic ways in which DNA methylation can inhibit transcription. First, methylation can interfere with the interaction of transcription factors and other DNA binding proteins (Tate et al., 1993). Secondly, methylation can inhibit gene expression by recruiting methyl-CpG binding protein (MBD) (Boyes et al., 1991; Nan et al., 1998). In addition to regulating transcription, DNA methylation has many other important biological functions. It represses the expression of parasitic sequences, endogenous retrovirus and transposable elements, such as α-satellites, ALU-Yb8 and LINE-1, as well as maintaining genome stability (Walsh et al., 1998; Krueger et al., 2012). This is highlighted by the ICF syndrome

(immunodeficiency, centromere instability and facial anomalies), which is an autosomal recessive immune disorder, caused by mutations of DNMT3B. This rare syndrome is associated with hypomethylation of pericentromeric regions and the formation of fusion rosette chromosomes (Heyn et al., 2012). Mice with engineered inactivation of Dnmt3a/b also have multiple chromosomal aberrations (Dodge et al., 2005). The enzymes involved in establishing DNA methylation profiles are well known, however, despite the intense search, no direct DNA demethylase has been identified. Recently, the ten-eleven translocation 1 protein (TET1) has been shown to catalyzed the conversion of 5mC into 5-hydroxymethylcytosine (5hmC) suggesting a potential mechanism for passive demethylation (Tahiliani et al., 2009). Other enzymes, such as AID (activation-induced cytidine deaminase) and TGD (thymine DNA glycosylase) could also be involved in the demethylation of the DNA (Bhutani et al., 2010; Cortellino et al., 2011).

2.3. MECHANISM OF DNA METHYLATION.

DNA methyltransferases are the enzymes responsible for DNA methylation. In mammals, there are five members of this protein family: DNMT1, DNMT2, DNMT3A, DNMT3B and DNMT3L, with only DNMT1, DNMT3A and DNMT3B possessing methyltransferase activity. The catalytic members of the DNMT family are classified into either *de novo* DNMT (DNMT3A and DNMT3B) or maintenance DNMT (DNMT1) (Figure I.4). The *de novo* DNMTs are highly expressed in embryonic stem cells and downregulated after differentiation. The DNMT3L protein is expressed during gametogenesis and is essential for establishing maternal genomic imprinting despite being catalytically inactive (the full role of the DNML3L-DNMT3B complex in genomic imprinting will be discussed in section 2.7.1). The role of DNMT1 is to maintain DNA methylation through cell division, and it is most active during the S phase of the cell cycle, when the DNA is hemimethylated after semi conservative DNA replication. The

DNA methylation profile is copied from the parental to the new DNA strand through interaction with UHRF1 (Ubiquitin-like, with PHD and RING finger domain 1) (Boestick et al., 2007). In this way, methylation patterns are efficiently preserved throughout division. Recently, it has been shown in cells, that in absence of both DNMT3A and DNMT3B, DNMT1 cannot maintain methylation by itself as these show gradual loss of methylation, suggesting that the *de novo* DNMTs also possess limited maintenance function (Chen et al., 2003).

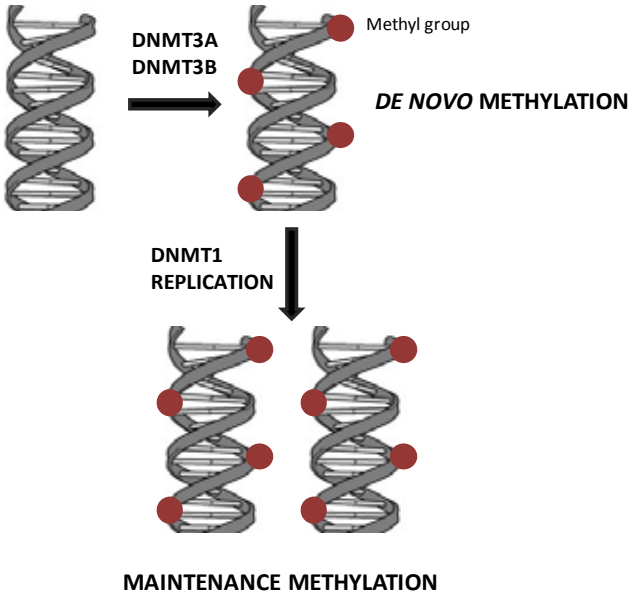


Figure I.4: Mechanisms of DNA methylation. Figure adapted from Wu et al., 2010.

2.4. 5-HYDROXYMETHYLCYTOSINE (5hmC).

5-hydroxymethylcytosine (5hmC) is relatively a new epigenetic modification, known as the 6th base of the genome. It was first identified in the T-even bacteriophage to protect the phage genome almost six decades ago (Wyatt et al., 1953). The enzymes responsible for the conversion of 5mC to 5hmC (Figure I.5) are members of the TET protein family.

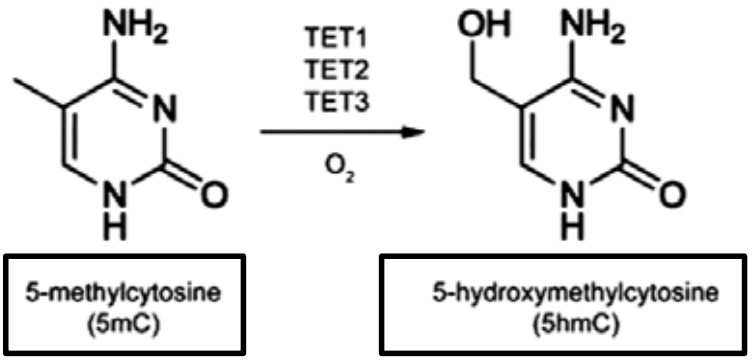


Figure I.5: TET proteins through the oxidation of 5-methylcytosine generate 5-hydroxymethylcytosine. Figure adapted from Ku et al., 2011.

The mammalian TET protein family contains three members, TET1, TET2 and TET3, which are 2-oxoglutarate and Fe(II)-dependent dioxygenases. TET1 and TET3 contain a CXXC zinc finger domain at the amino-terminus, which is a known DNA-binding domain. TET1 is mainly found in the developing pre-implantation embryos, whereas TET2 and TET3 are more ubiquitously expressed (Szwagierczak et al., 2010). The tissue distribution of 5hmC in mammals varies among different tissues, with the highest levels in the brain. Embryonic stem cells also have high levels of 5hmC that decrease significantly after differentiation (Globisch et al., 2010). Several biological functions of TET-mediated conversion (5mC to 5hmC) have been proposed. First, 5hmC might alter the function of chromatin environment by recruitment or displacement of

proteins, therefore, acting as a direct epigenetic signal. However, most binding proteins with high affinity for 5mC do not recognize 5hmC (Jin et al., 2010). Secondly, it might be involved in the demethylation of DNA, through either passive or active demethylation pathways. Since 5hmC is not maintained through DNA replication, it will be passive removed through cell division. As for the active pathway, 5hmC is an intermediate of the enzymatic pathway of demethylation, supported by the idea of the existence of 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) in the mammalian DNA (Williams et al., 2011). The exact pathway is depicted in figure I.6.

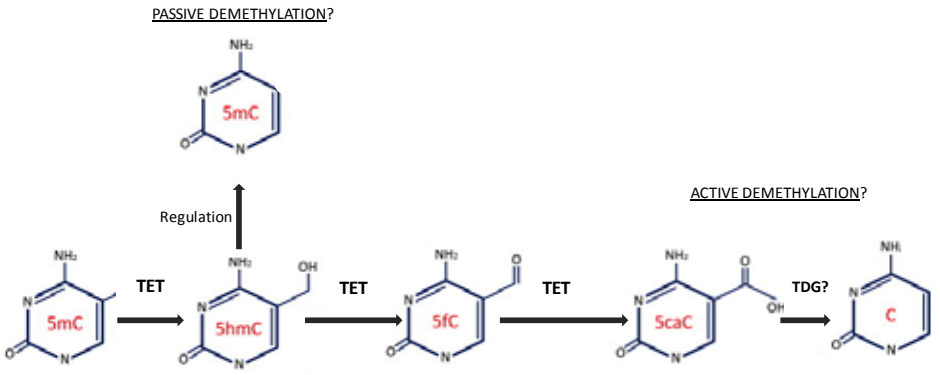


Figure I.6: Pathway of the TET conversion of 5mC to 5hmC. Figure adapted from Williams et al., 2011.

These proteins play important roles in transcriptional regulation, but many aspects still have to be elucidated. For example, TET1 indirectly facilitating the polycomb repressive complex (PRC2) chromatin binding by decreasing DNA methylation at PRC2 target genes. Another repression mechanism in which TET1 is involved is through the recruitment of the Sin3A co-repressor complex to gene promoters. As for the role in transcriptional activation, TET1 contributes by preventing DNA methylation by binding to strong CpG island ensuring that they remain unmethylated (Williams et al., 2011).

2.5. CHROMATIN MODIFICATIONS AND THEIR MECHANISM OF ACTION.

The core histones H2A, H2B, H3 and H4, group to form an octamer that is the minimal unit of chromatin, the nucleosome. Histone H1 is a linker histone that does not form part of the core nucleosome but binds to the linker DNA. The nucleosome is associated with 147bp of DNA which is wrapped 1.65 turns around the histone octamer, with the neighboring nucleosome separated by approximately 50bp of free linker-DNA. Theses histones contain a globular C-terminal and an unstructured N-terminal tail that can be subjected to a variety of posttranslational covalent modifications. There are about 50 know modifications that affect these amino-terminal tails, such as methylation, acetylation, phosphorilation, ubiquitylation and sumoylation (Figure I.7).

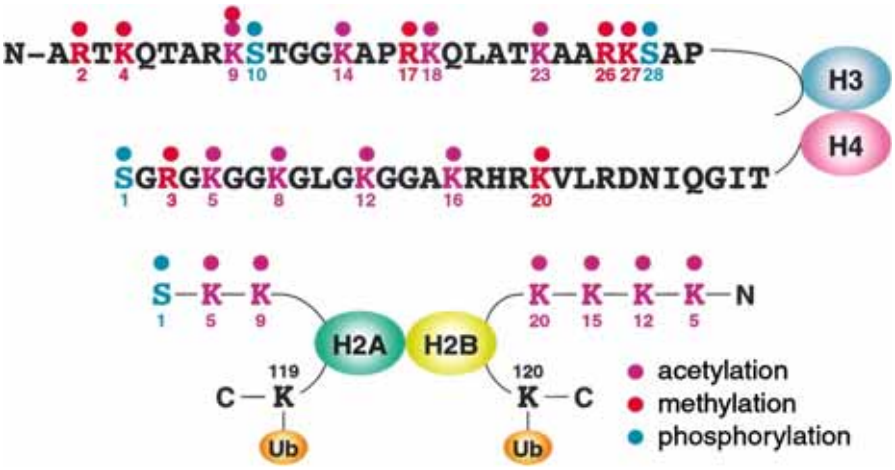


Figure I.7: The main post-translational modifications of the histone tails.

These histone tails are critical in maintaining chromatin structure and controlling gene expression without affecting or changing the DNA sequence. For this reason, all of these modifications are proposed to store the epigenetic memory inside the cell in the form of the “histone code” that determines the structure and activity of different chromatin regions (Jenuwein et al., 2001). The histone modifications can lead to either activation or repression

depending upon which residue is modified and the type of modifications present. Specific patterns of histone modifications are present with distinct cell types, and play key roles in determining the cell identity (Mikkelsen et al., 2007). Enzymes that can add or remove these covalent signals dynamically regulate these modifications. These include histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs), and histone demethylases (HDMs).

2.5.1. HISTONE ACETYLATION AND DEACETYLATION.

Acetylation of the histone N-terminal causes neutralization of the amino acid, weakening the electrostatic interactions between the DNA and the histones. This results in a more relaxed chromatin promoting gene expression. Acetylation of the lysine residue of histone H3 and H4 causes the opening of the chromatin, allowing transcription factors to bind to their promoters and activating gene expression. Acetylation is performed by the HATs, which can be divided into three different protein families: the MYST family (MOZ/YBF2/SAS2/TIP60), GNAT family (GCN5 *N*-acetyltransferase) and CBP/p300 family (Wang et al., 2007). The opposite reaction, the deacetylation, is performed by HDACs. There are numerous HDAC enzymes, classified in four catalytic groups. Class I HDACs consist of the nuclear localized HDAC 1,2,3 and 8; class II consist of HDAC 4,5,6,7,9, and 10 that are found in both the nucleus and cytoplasm; class III consists of sirtuins (SIRT 1-7) found in nucleus and cytoplasm; and class IV consists solely of HDAC 11 found in the nucleus. Class I and Class II have related mechanisms, which do not require cofactors, whereas Class III and IV require NAD^+ (nicotinamide adenine dinucleotides) as part of its catalytic mechanism (Glozak et al., 2007) (Figure I.8).

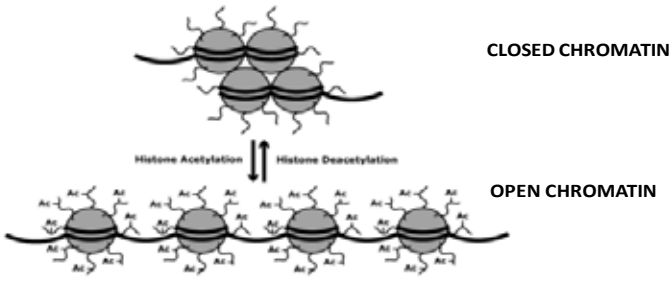


Figure I.8: Regulation of gene expression by histone acetylation/histone deacetylation. Figure adapted from McIntyre et al., 2007.

2.5.2. HISTONE METHYLATION AND DEMETHYLATION.

Methylation as a histone covalent modification is more complex than any other, since it can occur on either lysine (K) or arginines (R), and it can active or repress genes depending on which residue is methylated. Lysines can be mono-, di-, or trimethylated. Out of the many know methylated sites six have been well characterized: five on H3 (K4, K9, K27, K36 and K79) and one on H4 (K20). Methylation at H3K4, H3K36 and H3K79 has been linked to active transcription, and the remainder to repression. Histone lysine methyltransferase (HKMTs) can be divided into two main groups; those with SET-domains and the non SET-domain containing proteins. The SET domain harbors the enzymatic activity. All of the HKMTs proteins have SET-domains, such as EZH2, MLL, SUV39H1 among many others, except DOT1L. Similar to the DNMTs, all of the HKMT catalyzes the transfer of a methyl group from a SAM substrate (Bannister et al., 2011). Arginines can be mono- or dimethylated on H3 (R2, R17 and R26) and on H4 (R3). Arginine methylation is implicated in positive and negative regulation of transcription. There are two classes of arginine methyltransferase (PRMTs), type-I (PRMTs 1, 3, 4, 6 and 8) and type-II (PRMTs 5, 7, and 9). Like HKMTs, these enzymes transfer a methyl group from SAM but to arginines (Bannister et al., 2011). Until recently, it was thought that histone lysine and arginine methylation were permanent modifications. The first lysine demethylase to be identified was LSD1 (Shi et al., 2004). Other

enzymes such as the Jumonji-domain (JMJD)-containing proteins have also been recently identified to demethylate lysines (Masammaparast et al., 2010), and JMJD6 has been shown to act as both an arginine demethylase and a lysyl-hydroxylase (Chang et al., 2007).

2.6. INTERPLAY OF DNA METHYLATION AND HISTONE MODIFICATIONS.

DNA methylation and histone modifications have their own role in the regulation of gene expression, but they can interact with each other to determine expression, chromatin organization and cellular identity. This interaction seems to work in both directions: histone methylation can help direct DNA methylation patterns, and DNA methylation can serve as a template for histone modification after DNA replication. These connections are obtained though the direct interaction of DNA and histone methyltransferases (Cedar et al., 2009). For example, HMTs such as SUV39H1 can direct DNA methylation to specific targets by directly recruiting DNA DNMTs (Lehnertz et al., 2003). Recently, a new concept has been proposed known as the chromatin "interactome" were nucleosome-interacting proteins can recognize distinct chromatin modification patterns, which are influenced by the DNA sequence, the configuration of the histone octamer and also combination of the histone and DNA modifications (Bartke et al., 2010).

2.7. GENOMIC IMPRINTING.

In diploid organisms, the maternal and paternal alleles of autosomal genes are expressed at similar levels. However, in eutherian mammals and marsupials a group of genes, known as imprinted genes are only expressed from one allele, resulting in the monoallelic expression determined by the parent-of-origin. A gene that is expressed from the maternal allele is said to be paternally imprinted, whereas one that is expressed only from the paternal allele is

maternally imprinted. Therefore, genomic imprinting differs from the classical point of view of Mendelian genetics. This monoallelic expression is the end result of various epigenetic mechanisms, such as differential DNA methylation, histone modifications and non-coding RNA. Therefore, these transcripts constitute a particularly interesting example of epigenetic regulation, since in an individual cell, there are active and repressed alleles of the same gene. These genes play an important role in normal development, fetal growth, nutrient metabolism and adult behavior. Imprinted genes tend to be clustered together, and are not found in isolation throughout the genome. Genes within an imprinting cluster share many regulatory elements and often have similar developmental and tissue-specific patterns of expression. These clusters are regulated by imprinting control regions (ICRs) that manifest as differentially methylated regions (DMRs), which can act as chromatin insulators and/or promoters of functional ncRNAs.

2.7.1. THE LIFE CYCLE OF IMPRINTS.

Imprinted genes are established during the development of germ cells into sperm or eggs. After fertilization, these imprints are maintained in the developing organism and throughout adult life. In the germ cells of the new organism, these imprints are erased, as a result of genome-wide demethylation and re-established with respect to the gender of the fetus. This remethylation take place at different times in the male and female germ cells. The paternal-specific methylation occurs during the period between mitotic arrest and birth (Davis et al., 1999; Li et al., 2004), whereas the maternal-specific methylation is established after birth during the oocyte growth phase prior to ovulation (Lucifero et al., 2002). The *de novo* methylation that occurs in the germline of imprinted DMRs is performed by DNMT3A and the stimulatory protein, DNMT3L (Bourc'his et al., 2001; Kaneda et al., 2004). In the developing oocyte, the methylation associated with H3K4 is removed by

the germline-specific lysine demethylase, AOF1/KDM1B (Ciccone et al., 2009; Proudhon et al., 2012). The resulting unmodified H3K4 residues are then recognized by the ADD domain of the DNMT3L/DNMT3 complex, resulting in DNA methylation (Ooi et al., 2007; Zhang et al., 2010). Once established, the germline DMRs are protected from TET-1 associated pre-implantation epigenetic remodeling by specific factors such as ZFP57 and PGC7/STELLA (Quenneville et al., 2011; Nakamura et al., 2007)(Figure I.9).

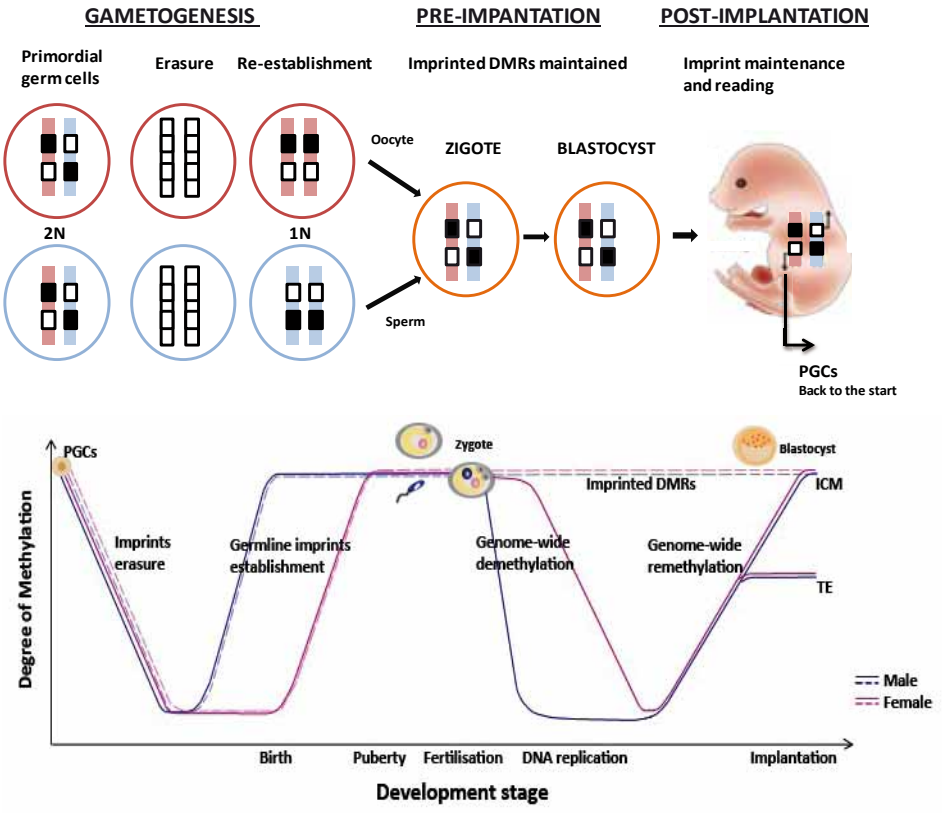


Figure I.9: Upper image is the erasure, establishment and maintenance of methylation imprints at imprinting centers during germ cell and embryonic development. Bottom image represents the DNA methylation life cycle. PGC (primordial germ cells), ICM (inner cell mass) and TE (trophoectoderm).

Figure adapted from Ishida et al., 2012.

2.7.2. PROPERTIES OF THE IMPRINTING MECHANISM.

Imprinted genes are associated with DMRs, where the repressed allele is methylated, and the permissive allele is unmethylated. These DMRs can regulate a number of imprinted genes within a cluster. Imprinted DMRs can be sub-classified into whether the methylation is acquired in the germline or during somatic development. Germline DMRs have methylation differences between egg and sperm and are maintained after fertilization. In somatic DMRs, the methylation is still parental-specific, but acquired during the development of the fetus. Maternal germline DMRs are found at CpG rich promoters, and paternal germline DMRs are associated with low density of CpG dinucleotides within intergenic regions. An example of maternally methylated DMR is the *KvDMR1* gene (Figure I.10). The maternally inherited chromosome is methylated, whereas the paternally inherited chromosome is unmethylated, acting as a promoter for a non-coding antisense RNA, *KCNQ1OT1* (Smilinich et al., 1999; Du et al., 2004). This long ncRNA recruits the HMTs G9a and EZH2, responsible for the repressive histone modifications H3K9me2 and H3K27me3, respectively, to the maternal allele of surrounding genes, ensuring their allelic silencing in *cis* (Pandey et al., 2008).

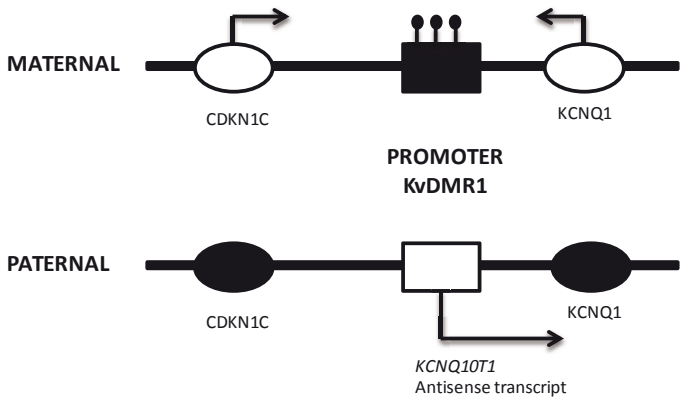


Figure I.10: Imprinted *KVDMR1* domain on chromosome 11p15. The *KCNQ1* gene contains a promoter for the long antisense transcript *KCNQ1OT1,* oriented in an antisense direction to the *KCNQ1* transcript. The antisense is paternally expressed.

The best-studied paternal methylated DMR is located within the *IGF2-H19* locus (Figure I.11). The *IGF2* is located 90kb away from the maternally expressed H19 transcript. These two genes share enhancer sequences downstream of *H19.* The DMR is upstream from the transcription start site (TSS) of H19, containing binding sites for the methylation-sensitive insulator protein, CTCF (CCCTC binding factor), which binds to the unmethylated DNA. When CTCF is present on the maternal allele of the H19-DMR, it facilitates higher-order chromatin folding that blocks the enhancer interactions with the *IGF2* promoter, juxtaposing the enhancers next to the *H19* promoter. On the paternal allele, the DMR is methylated, CTCF cannot bind and the enhancer is able to activate the *IGF2* promoter.

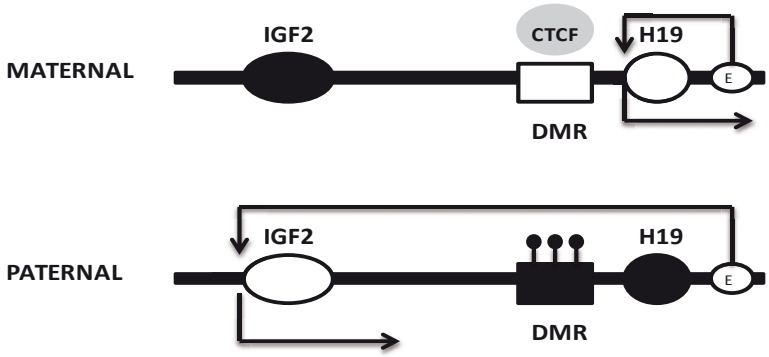


Figure I.11: Imprinted *IGF2/H19* domain on chromosome 11p15. The paternal *IGF2* is expressed while maternal allele is silent, while the *H19* has a reverse pattern. Silencing of the paternal allele is achieved by DNA methylation on the DMR. The maternal allele lacks methylation and allows the CTCF to bind and prevent transcriptional enhancers, located downstream of *H19,* from activating *IGF2* transcription. Image is adapted from Edwards et al., 2007.

2.8. MicroRNAs.

MicroRNAs belong to a class of non-coding RNAs among many others, such as piRNAs and snoRNA. MicroRNAs (miRNAs) were first discovered in *Caenorhabditis elegans* as post-transcriptional regulators of genes involved in developmental timing (Lee et al., 1993). MicroRNAs are small non-coding RNA molecules, typically 19-24bp which regulate the expression of target mRNAs, both at transcriptional and translational level by perfect or partial base-pairing in the 3'-untranslated region (3'UTR) of the target mRNAs (Lagos-Quintana et al., 2001). To date there are more than 1,000 miRNAs (Hassan et al., 2012) that are expressed in a tissue-specific manner and control a wide range of physiological roles that span from cell proliferation, apoptosis, differentiation, development, regulation, aging and metabolism. Consequently, a deregulation of microRNAs, have been found to be involved in a wide range of diseases, including cancer, diabetes, immune or neurodegenerative disorders (Calin et al., 2002; Landthaler et al., 2004; Poy et al., 2004). In order to obtain a mature miRNA from larger transcripts, two processing events have to occur. Firstly, the pri-miRNA is transcribed by RNA polymerase II, and this is cleaved into a hairpin, forming a pre-miRNA by ribonuclease III/Drosha and the dsDNA binding protein DGCR8/Pasha. Secondly, to obtain the mature miRNA, exportin-5, a RAN-GTP dependent cargo transporter, translocates the pre-miRNA from the nucleus to the cytoplasm. Once in the cytoplasm, DICER1 associated to TRBP2 (transactivation-responsive RNA-binding protein 2) processes it into a mature miRNA duplex. Afterwards, the protein complex RISC (RNA-induced silencing complex) binds to the duplex and determines the fate of the miRNA and its target mRNA. One strand of the miRNA duplex is stably associated with RISC complex that then targets the mRNAs for degradation (Figure I.12).

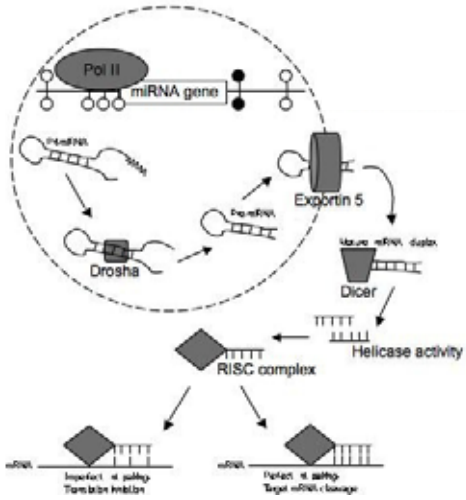


Figure I.12: Overview of the microRNA biogenesis.

As mention previously, miRNAs control many different physiological processes, one of them being hematopoiesis. In the hematopoietic system, a common progenitor cell can give rise to different lineages, and the process of commitment is governed by the expression of several genes including miRNAs. Therefore, specific miRNAs can control the myeloid and lymphoid differentiation. For example, miR-223 can control whether the common myeloid progenitor will differentiate into a granulocyte or a monocyte (Johnnidis et al., 2008), and miR-150 is involved in controlling the transition from pro-B to pre-B cell (Zhou et al., 2007).

2.8.1 TOOLS TO PREDICT SPECIFIC MicroRNAs.

Many different bioinformatics web sites predict microRNA targets. The most commonly utilized are miRanda, miTarget, PicTAr and TargetScan. A single miRNA can have many different target genes depending on: its seed sequence, with specificity depending upon the 5' sequence of the miRNA, 3' region pairing, conservation of the site, untranslated region (UTR), the number of potential target sites with a mRNA molecule and free energy of the complex.

3. EPIGENETICS AND CANCER.

The classical view of cancer is that it arises from a single cell through a series of mutations by activating oncogenes and inactivating tumor suppressor genes. Each mutation, leads to a selective overgrowth of a population of tumor cells that contain properties related to invasiveness, metastasis ability and therapeutic resistance (Figure I.13).

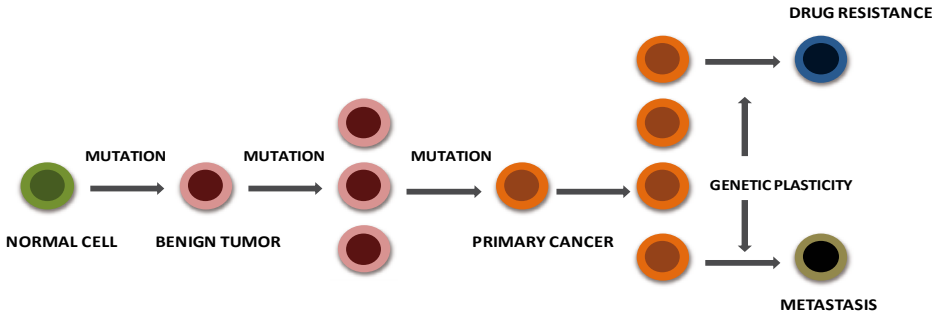


Figure I.13: Clonal genetic model of cancer. Figure adapted from Feinberg et al., 2006.

Classical genetics cannot explain the entire process of carcinogenesis. There is evidence that epigenetics plays a key role in contributing to this malignancy. Therefore, existing models of cancer development have accommodated this phenomenon, resulting in the epigenetic progenitor model of cancer. According to this model, cancer arises in three steps: epigenetic disruption of progenitor cells, an initiating mutation, genetic and epigenetic plasticity (Figure I.14).

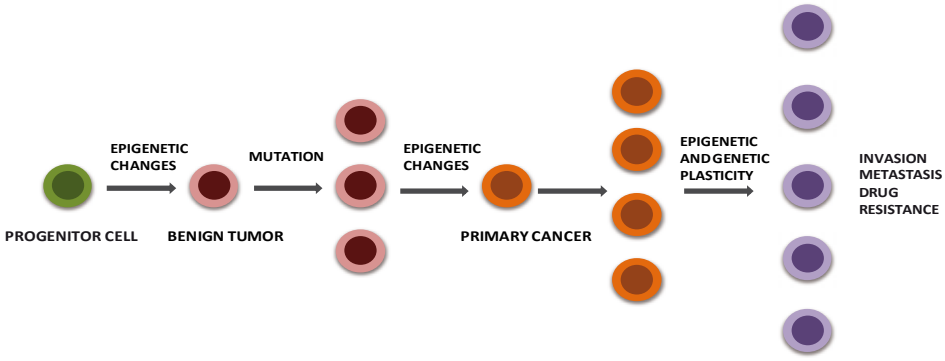


Figure I.14: The epigenetic progenitor model of cancer. Figure adapted from Feinberg et al., 2006.

The epigenetic disruption of progenitor cells is considered to be the first step involved in the process of carcinogenesis. This disruption could perturb the normal balance between undifferentiated progenitor cells and differentiated committed cells, either in number or in their capacity of aberrant differentiation, which provides a common mechanism for unifying neoplasia. The second event implicates an initiating mutation in the disrupted progenitor cells. Finally, the third step involves the genetic and epigenetic plasticity, an enhanced ability to stably evolve in malignant phenotype, with both widespread genetic and epigenetic disruption (Feinberg et al., 2006).

3.1. EPIGENETIC MODIFICATIONS IN CANCER.

3.1.1. DNA METHYLATION CHANGES IN CANCER.

In cancer, many epigenetic changes occur. One of the most altered is the DNA methylation profile. Global hypomethylation of the genome leads to many changes: chromosomal instability, promoting chromosome rearrangement and activation of pro-oncogenes, reactivation of transposable elements that can lead to their activation and translocation to other genomic regions and a loss-of-imprinting (LOI). At the same time, hypermethylation occurs at the promoters of tumor suppressor genes leading to an inactivation of many transcripts that possess key roles within the cell. This aberrant disruption of DNA methylation is partly due to inappropriate expression of the DNMT enzymes. Overexpression of DNMT1 leads to hypermethylation, while the global hypomethylation correlates with the expression of an abnormal DNMT3B isoform, DNMT3B4, which lacks the conserved methyltransferases motifs (Saito et al., 2002).

3.1.2. DNA HYPERMETHYLATION.

Historically, the first TSG reported to undergo transcriptional silencing through DNA methylation of the CpG island promoter was the *RB1* gene (Greger et al., 1989; Sakai et al., 1991). Since these seminal publications many other TSGs have been shown to undergo methylation-mediated downregulation in cancer involved in a wide range of cellular processes including the cell cycle ($p16^{INK4a}$), DNA repair (BRCA1), cell-to-cell interaction (E-Cadherin), apoptosis and angiogenesis (VHL & TFPI2) (Herman et al., 1994; Merlo et al., 1995; Herman et al., 2003; Esteller et al., 2000). Indeed this central set of commonly hypermethylated promoters is known as the CpG island hypermethylated phenotype (CIMP) (Toyota et al., 1999; reviewed in Curtin et al., 2011). Hypermethylation profiles of TSGs can be specific to a cancer types; therefore, each tumor has its own "hypermethylome", which are reported to be similar in sporadic tumors and in inherited cancer syndromes (Esteller et al., 2006). How these hypermethylation events occur are still to be unraveled, but there are some pointers that help to explain their occurrence. For example, the CpG sequences maybe associated with an underlying chromatin signature. For instance, gene promoters that possess bivalent histone marks, such as the presence of H3K4me2/3 and H3K27me3 on the same nucleosome are 14 times more likely to be hypermethylated than CpG islands associated with H3K4 methylation alone (Ohm et al., 2007). In addition, the affected CpG islands can be located within a chromosomal domain subjected to long-range epigenetic silencing (LRES) that initiates from single loci and spreads over many kilobases (Frigola et al., 2006).

3.1.3. ABERRANT HISTONE MODIFICATIONS IN CANCER.

High-throughput sequencing has enabled genome-wide mapping of chromatin changes occurring during tumorgenesis (ENCODE project-Washington University; Thurman et al., 2012). These state-of-the-art techniques have confirmed biochemical observation that initially revealed gross loss of acetylated H4-lysine 16 (H4K16ac) and H4-lysine 20 trimethylation (H4K20me3) (Fraga et al., 2005). Loss of acetylation is mediated by HDACs, which are found to be overexpressed and/or mutated in cancer. The main HDAC implicated in cancer belongs to the Sirtuin family, with SirT1 being reported to be upregulated in cancer. In addition to the global loss of H4K20me3, cancer cells can also suffer widespread loss of H3K4me3 (Hamamoto et al., 2004), and gains of the repressive marks H3K9me3 and H3K27me3 (Jones et al., 2007) all of which maybe a cause or consequence of DNA hypermethylation.

3.1.4. DEREGULATION OF MicroRNAs IN CANCER.

Studies comparing miRNA expression profiles in tumors and corresponding normal tissues indicate widespread changes in expression during tumorgenesis (Lu et al., 2005). MiRNAs can function as tumor suppressors or oncogenes, depending upon their target genes. Changes in miRNA expression could be achieved through various mechanisms: chromosomal abnormalities, genetic mutations to miRNA binding sites and epigenetic alterations to the miRNA promoter. Alternatively, mutations in proteins involved in miRNA biosynthesis, such as TRBP2, an essential partner of the DICER1 complex, can impair the miRNA processing and therefore miRNA expression (Melo et al., 2009). The miR-127, which has tumor-suppressor properties and is embedded in a CpG island, can be directly silenced by promoter hypermethylation (Saito et al., 2006). DNA-demethylating reagents and/or histone deacetylase inhibitors can reverse changes in miRNA expression. For example, re-expression of miR-127

in T25 bladder cancer cell lines was accomplished with DNA demethylation drugs and HDAC inhibitors (Saito et al., 2006). In myeloid and lymphoid malignancies, the deregulation of miRNAs are also involved in the pathogenesis of these diseases. The first convincing evidence of the role of miRNAs in human cancer was found in the pathogenesis of chronic lymphocytic leukemia (CLL) (Calin et al., 2002). The miR15a and miR-16-1 are located in 13q14 region, a domain frequently deleted in chronic lymphocytic leukemias. These deletions ablate a ncRNA mapping between exon 2 and 5 of the *DLEU2* gene, which is the precursor RNA for the miR15a/16-1 (Calin et al., 2006). The role of miR-15a/16-1 as a tumor suppressor gene in CLL was established by showing an inverse correlation between their expression and the anti-apoptotic gene *BCL-2* in leukemic cells (Cimmino et al., 2005). Overexpression of the miR-15a/16-1 cluster in the MEG-01 cell line, leaded to a downregulation of 3301 genes and an upregulation of 265 genes, with a subsequent proteomic analysis revealing that 27 different proteins change by at least four-fold. Among these proteins were BCL-2 and WT1 (Calin et al., 2008). A study performed on acute myeloid leukemia (AML) patients with predominantly intermediate and poor cytogenetics identified a miRNA expression profile that correlated with molecular abnormalities including t(11q23), trisomy 8 and FLT3-ITD mutations (Garzon et al., 2008).

3.1.5. DEREGULATION OF 5-HYDROXYMETHYLCYTOSINE IN CANCER.

The TET enzymes that convert 5mC to 5hmC are also deregulated in cancer. The TET1 enzyme was originally discovered in some leukemia patients due to a chromosomal translocation with the *MLL* gene (Lorsback et al., 2003). The TET2 protein is frequently mutated in myeloid malignancies and is associated with a DNA hypermethylated phenotype (Abdel-Wahab et al., 2009; Figueroa et al., 2010; Weissmann et al., 2011). To date, mutations in the TET3 protein

have not been shown to be involved in cancer, presumably due to any mutations being lethal to the cell.

3.1.6. DEREGULATION OF GENOMIC IMPRINTING IN CANCER.

In cancer, epigenetic disturbances also occur to imprinted genes causing a deregulation of its expression. The earliest indication that genomic imprinting is involved in cancer comes from cytogenetic studies of two human neoplasms: hydatidiforms mole and ovarian teratomas. Hydatidiform mole is a malignant trophoblastic tumor caused by the abnormal fusion of two complete sets of the paternal genome. Ovarian teratomas are benign tumors that arise from two complete sets of the maternal genomes. These tumors indicate that an imbalance between maternal and paternal genomes causes neoplasia. Therefore, the earliest and abundantly observed alterations in human cancer are LOI due to epigenetic deregulation (Jelinic et al., 2007). The involvement of imprinting in the origin of cancer is supported by the existence of genetic diseases with altered expression of these transcripts, for which there is an increased cancer risk. The most common imprinting syndrome with increased risk of childhood cancer is Beckwith-Wiedemann syndrome (BWS) that is associated with aberrant allelic DNA methylation on chromosome 11. Hypermethylation of the *H19*-DMR is observed in ~5-10% of BWS cases, and this specific subgroup has an increased risk of developing Wilms' tumor, hepatoblastoma and adrenal cancers (Weksberg et al., 2001). This could be due to either the biallelic overexpression of *IGF2*/miR-483 (Veronesse et al., 2010) or the silencing of *H19*/miR-675 (Tsang et al., 2010). The direct role of LOI in adult cancer is less clear, but several late-onset cancer show LOI, specifically *CDKN1C/p57*^{KIP2} in pancreatic cancer (Sato et al., 2005), *DIRAS3* in oligodendroglial tumors (Riemenshneider et al., 2008) and *L3MBTL3* in myeloid malignancies (Li et al., 2004), suggesting these are *bona fide* tumor suppressor genes.

3.1.7. EPIGENETICS, CANCER DIAGNOSIS AND PROGNOSIS, EPIGENETIC THERAPY.

As mention above, DNA methylation and histone modification patterns are associated with the development and progression of cancer, and have the capacity of reversible changes. This has lead to the possibility of epigenetic therapy as a treatment option, and as a use of potential clinical biomarkers for cancer. The main goal of epigenetic therapy is to restore normal DNA methylation patterns and histone modification aberrations that occur in cancer using epigenetic drugs. To date, the only specific agents targeting the epigenetic molecules are DNA methylation and histone deacetylase inhibitors. DNA methylation inhibitors include two types: nucleoside analogues and non-nucleoside analogues. Nucleoside analogues have a modified cytosine ring that is attached to either the ribose or deoxyribose moiety. These are then metabolized by kinases that convert the nucleosides into nucleotides for incorporation into DNA and/or RNA. Therefore, DNA methylation is inhibited when the compounds are incorporated into DNA. The most commonly used inhibitors are: 5-Azacytidine (AZA) and 5-Aza-2'-deoxycytidine (DAC) (Figure I.15).

Figure I.15: DNA methylation inhibitors, nucleoside analogues: 5-Azacytine and 5-Aza-2'-deoxycytidine.

These two drugs are viewed as mechanistically similar hypomethylation agents, with similar mechanisms of action on DNA-mediated markers of activity, but different effects on cell viability, protein synthesis, cell cycle and gene expression have been demonstrated (Hollenbach et al., 2010). Others

nucleoside analogue do exist, such as 5-Fluoro-2'-deoxycytidine, 5,6-Dihydro-5-azacytidine and Zebularine. Non-nucleoside analogues are small-molecule inhibitors that inhibit DNA methylation by binding directly to the catalytic region of the enzyme, without it incorporating into the DNA.

Histone deacetylase inhibitors are divided into four groups: short-chain fatty acids, hydroxamic acids, cyclic tetrapeptides and benzamides. The exact mechanism with which these drugs mediate antitumor activity has not been elucidated, even though many hypotheses have been made as to which cellular pathway are involved (Yoo et al., 2006).

3.1.8. DNA METHYLATION AS A BIOMARKER FOR CANCER.

Since aberrant epigenetic profiles are observed in the pathogenesis of cancer they may act as novel biomarkers for diagnosis, prognosis and prediction of response therapies. The use of such biomarkers is more plausible than using genomic rearrangements/mutations since DNA methylation defects occur at higher rates in tumors. The use of DNA hypermethylation, rather than hypomethylation is a more feasible marker, since it is technically much easier to detect gain of methylation rather than a reduction. In fact, due to the release of tumor DNA into biological fluids it is possible to use the detection of hypermethylated sequences as a non-invasive technique. Despite large efforts by the epigenetics community, no methylation diagnoses have yet been approved by the US Food and Drug Administration (FDA), however, there are many promising candidates. In prostate cancer, the hypermethylation of *GSTP1* is an accurate marker for diagnosis (Esteller et al., 1998), and *MGMT* hypermethylation is also a reliable marker for glioblastomas (Esteller et al., 2000). Hypermethylation can be used for disease prognosis. Recently, a comprehensive study of childhood acute lymphoblastic leukemia identified over 20 genes where the methylation profile predicts disease relapse (Milani et al., 2010). DNA methylation can also be used for predicting response to

chemotherapeutic treatment, such as the hypermethylation at the *MGMT* gene in glioma. When this gene is hypermethylated it is associated to greater sensitivity to alkyating agents, leading to drug resistance (Esteller et al., 1999).

4. THE BLOOD SYSTEM: PHYSIOLOGY AND DISEASE.

4.1. HEMATOPOIESIS AND BLOOD LINEAGES.

Hematopoiesis is the biological process by which human blood cells are produced; these cells originate from the hematopoietic stem cells (HSCs) that are located in the bone marrow (BM). HSCs have the capacity of self-renewal, asymmetric cell division and are multipotent, meaning that they can differentiate into all types of blood cells. In order to achieve this, many key factors are involved, such as transcription factors, cytokines and even the cellular microenvironment. The multipotent HSCs generates all the mature blood cells through successive differentiation into oligolineage progenitors including the common myeloid progenitor (CMP) and the lymphoid-primed multipotent progenitor (LMPP). The CMPs generate granulocyte/monocytic progenitors (GMPs), that differentiate into granulocytes or monocytes/macrophages, and megakaryocytic/erythrocytic progenitors (MEPs), that differentiate into megakaryocytes or erythrocytes. The LMPPs generate the common lymphocyte progenitor (CLP) and this the B-lymphocytes, T-lymphocytes and NK killer cells (Figure I.16).

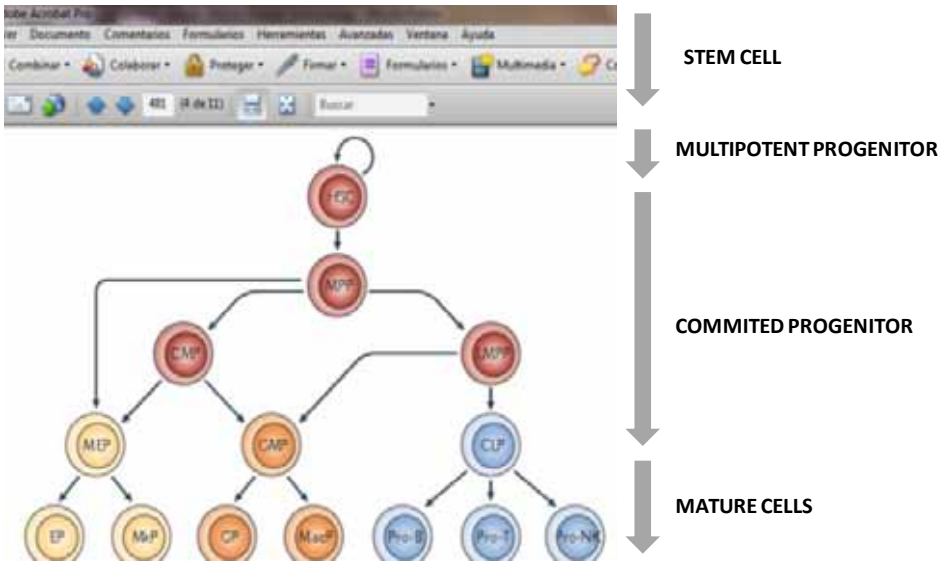


Figure I.16: Hierarchy of differentiation in the hematopoietic system. Figure adapted from Cedar et al., 2011.

4.2. HEMATOLOGICAL MALIGNANCIES, TUMORS OF THE HEMATOPOIETIC AND LYMPHOID TISSUES.

Hematological malignancies are types of cancer that affect the blood, bone marrow and lymph nodes. These are a highly heterogeneous group of diseases showing different genetic, transcriptional, phenotypical and clinical characteristics (Swerdlow et al., 2008). Genetic changes that take place at different stages of maturation of the hematopoietic lineages play an essential role in the development of these malignancies. These genetic changes include mutations, translocations, deletions and amplifications that result in gene activation or repression. Epigenetic changes also contribute to the development of these diseases (Esteller, 2008). Hematological malignancies can derive from either of the two main blood lineages: the myeloid and the lymphoid. Many different classifications for these neoplasms have been used. Historically, these diseases have been divided by the localization of the disease: in the blood (Leukemia) or the lymph nodes (Lymphomas). For the leukemias, these were then divided into acute or chronic types depending on

their clinical course. In 1976, the French-American-British (FAB) classification was proposed for the acute leukemias: lymphoblastic leukemia (L1-L3), myeloid leukemia (M0-M7) and myelodysplastic syndrome. This FAB classification is based on morphology, cell maturation, the cytochemical and immunophenotype. However, the world health organization classification (WHO) has now superseded the FAB classification. This classification combines morphology, cytochemistry, immunophenotype, genetics and clinical features of these diseases.

In order to facilitate the explanation of such malignancies, herein we are going to focus on the historical classification of leukemias, since this research project is focused on these types of malignancies.

4.3. LEUKEMIA.

The term "leukemia" comes from the Greek word "Leukos" and "Heima" meaning "white blood". Leukemia is clinically and pathologically subdivided into a variety of large groups according to the kind of blood cell affected. Leukemia can be divided into myeloid or myelogenous leukemia or lymphoblastic or lymphocytic leukemia:

1- Myeloid or myelogenous leukemia: the cancerous change occurs in cells that give to red blood cells, platelets and white blood cells and not lymphocytes.
2- Lymphoblastic or lymphocytic leukemia: the cancerous change arises from the cells that are committed to forming lymphocytes.

A second division of leukemias is based on its acute and chronic forms:

1- Acute Leukemia: is distinguished by the fast increase of immature blood cells. Immediate treatment is required due to the rapid progression and accumulation of the malignant cells, which eventually collapse the bone marrow. These malignant blood cells spill into the

bloodstream and spread into other organs of the body. Acute forms of leukemia are the most common forms of leukemia in children. Taking together the two classifications there are two main diseases: Acute Myeloid Leukemia (AML) and Acute Lymphoblastic Leukemia (ALL).

2- Chronic Leukemia: is described by the excessive build up of relatively mature, but still abnormal, white blood cells. After months or years, these malignant cells are produced at a much higher rate than normal cells, resulting in many abnormal cells in the blood. Chronic leukemia mostly occurs in older people, but can affect any age group. Taking together the two classifications there are two main diseases: Chronic Myeloid Leukemia (CML) and Chronic Lymphoblastic Leukemia (CLL).

Leukemia is a clonal disease, which means that it originates from a single leukemic stem cell. A small pool of leukemic stem cells (LSCs) supports the continued growth and propagation of the disease (Hundy et al., 2005). The origin of a LSC is still not clear; it could be a HSC cell with capacity of self-renewal or a progenitor cell that has gained the capacity of self-renewal.

4.3.1. ACUTE MYELOID LEUKEMIA.

Acute myeloid leukemia is characterized by an aberrant proliferation and accumulation of immature myeloid progenitor cells know as leukemic blasts that can affect the bone marrow, peripheral blood and other tissues. Leukemic blasts have a block in differentiation but retain the ability of proliferation and survival. AML is rare in childhood and the incidence increases with age. Approximately two-thirds of all cases occur in people aged over 60 years. It is the most common type of leukemia in adults, yet continues to be the lowest survival rate of all leukemias. Although rates have improved remarkably in the younger age group, the prognosis in older patients continues to be very poor (Deschler et al., 2006). The majority of cases of AML are associated with non-

random chromosomal translocations that often result in gene rearrangements (Look et al., 1997). AML is the most extensively cytogenetically characterized human neoplastic disorder, representing 33% of all hematological malignancies, and 27% of all malignant disorders with chromosomal abnormalities reported (Mitelman et al., 2007). A wide variety of different studies have provided evidence for the central role of gene rearrangements in the initiation of leukemia. Therefore, cytogenetics is one of the most important prognostic factor for predicting remission rate, relapse and overall survival. In table I.1, are listed the most prevalent and recurrent balanced translocations in AML.

Table I.1: The most common balanced translocations in AML.

Chromosomal translocation	Fusion-protein	Frequency (%)
t(8;21)(q22;q22)	RUNX1-RUNX1T1	4.3
t(15;17)(q22;q21)	PML-RARA	4.1
der(11q23)	MLL fusions	2.4
inv(16)(p13q22)	CBFB-MYH11	2.3
t(9;22)(q34;q11)	BCR-ABL1	0.7
inv(3)(q21q26)	RPN1-EVI1	0.6
t(6;9)(p22;q34)	DEK-NUP214	0.3
t(1;22)(p13;q13)	RBM15-MKL1	0.2
t(8;16)(p11;p13)	MYST3-CREBBP	0.1
t(7;11)(p15;p15)	NUP98-HOXA9	≤0.1
t(12;21)(p12;q11)	MN1-TEL	≤0.1
t(16;21)(p11;q22)	FUS-ERG	≤0.1

Many of the gene rearrangements involve a locus encoding a transcriptional activator, leading to expression of a fusion protein that retains the DNA-binding motifs of the wild-type protein. The fusion partner is a transcriptional protein that is capable of interacting with a corepressor complex. The fusion protein alters expression of target genes necessary for myeloid development,

laying the groundwork for leukemic transformation (Martens et al., 2010). The conventional treatment for AML consists in two phases: induction and consolidation. Induction aims to achieve complete remission by reducing the number of leukemic cells at an undetectable level, and consolidation aims to maintain complete remission by eliminating any residual undetectable disease, which includes transplantation of autologuous or allogenic stem cells (Ferrara et al., 2012). For standard therapy, Daunorubicin and Cytarabine are recommended for newly diagnosed AML (Burnett, 2012). New drugs are continually being developed for AML, which target the cytogenetic and genetic abnormalities observed in this disease. As for the epigenetic therapy, hypomethylating agents such as AZA and DAC are used in high-risk myelodysplastic syndrome, but not AML, and are associated with an improved outcome (Ferrara et al., 2011).

4.3.2. CHRONIC MYELOID LEUKEMIA.

Chronic myeloid leukemia is a clonal myeloproliferative disorder, which results from an acquired genetic change in a pluripotential stem cell. The disease is characterized by a gross overproduction of neutrophils and their precursors, with more or less preserved differentiation. This disease can have three clinical phases. A relatively benign “chronic phase” followed by an ominous “accelerated phase” and, finally, an almost invariably fatal acute leukemia phase termed “blast crisis”. The annual incidence of CML worldwide is around one per 100,000 with presentation most commonly in the fifth and sixth decades of life. The diagnosis is increasingly made in asymptomatic patients having routine blood test. The hallmark of CML cells is the presence of Philadelphia chromosome (Ph), t(9;22)(q34;q11) chromosomal translocation. Over 95% of CML cases are positive for Ph chromosome. The Ph translocation causes the fusion of the *ABL* proto-oncogene from chromosome 9 to the interrupted end of the breakpoint cluster region *BCR* of chromosome 22. The

chimeric *BCR-ABL* gene encodes a protein with considerably greater tyrosine kinase activity than the normal counterpart and contributes to the transformation process by activating constitutively JAK/STAT, Ras/Raf/MEK/ERK and PI3K/AKT signal transduction pathways (Ren et al., 2005). In chronic phase of CML, cells in the progenitor pool have increased proliferation due to overexpression of *BCR-ABL.* Progression to blast crisis with production of leukemic stem cells requires complex additional events including proliferation and self-renewal capacity, by avoiding cell death, a block in differentiation and bypassing the normal immune responses. CML patients are routinely treated with Imatinib, which inhibits the ABL1 tyrosine kinase activity and induces apoptosis. However, with time, resistance to Imatinib often develops, and other additional potential therapeutic targets are needed (Ferdinand et al., 2012).

4.3.3. EPIGENETICS MARKS DEREGULATED IN ACUTE MYELOID LEUKEMIA.

As previously mentioned, AML is associated to non-random chromosomal translocations, which contributes to the leukemogenesis of the disease. These chromosomal aberrations lead to fusion proteins that can aberrantly regulate gene transcription through chromatin modifications within the hematopoietic system. Fusion proteins, such as PML-RARα, AML1-ETO and MLL fusions, are examples of how these fusion proteins can deregulate epigenetic modifications, either directly or indirectly, leading to the development of AML. This aberrant recruitment of the epigenetic machinery by the oncofusion proteins to the promoters of key differentiation genes plays a central role in disrupting normal gene function. As a result, the normal patterns of histone epigenetic marks and DNA methylation are altered in leukemic blasts. These oncofusion proteins aberrantly recruit epigenetic modulators, the mechanisms by which they act, depends on what type of oncoprotein is formed. The main

histone marks deregulated in AML are histone acetylation and methylation. For example, the MLL protein which contains a SET domain responsible for the H3K4 methyltransferase activity, and is a key regulator of the expression of *HOX* genes during development (Cosgrove et al., 2010), undergoes partial tandem duplications, amplifications and many translocations in ALL (the most frequent are MLL-AF4 and MLL-ENL) and in AML (the most frequent are MLL-AF9 and MLL-AF6) causing a deregulation of the *HOX* genes (Muntean et al., 2012). Also, genome-wide promoter DNA methylation patterns revealed unique AML subgroups that also predicted clinical outcome (Figueroa et al., 2010). Interestingly, DNMT1, DNMT3A and DNMT3B expression are increased in AML and may play a significant role in inducing aberrant regional hypermethylation (Mizuno et al., 2001). Recently, mutations have been found in two isocitrate dehydrogenases, *IDH1* and *IDH2*, which disrupt the function of TET2. The *IDH1/2* and *TET2* mutant AML cells display global hypermethylation and a gene-specific methylation signature, contributing to the AML pathogenesis (Figueroa et al., 2010).

4.3.4. EPIGENETICS MARKS DEREGULATED IN CHRONIC MYELOID LEUKEMIA.

Epigenetic deregulation also occurs in CML. Several research groups have investigated stage-specific DNA methylation events in CML patients, in order to better understand the molecular mechanisms responsible for cancer progression. An increase in DNA methylation levels occur during the progression from chronic phase to blast crisis, including at the promoters of *ABL1*, *CALC1*, *HIC1* and estrogen receptor genes (Asimakopulos et al., 1999; Millis et al., 1996; Issa et al., 1997). The *HOXA4* gene is frequently found to be hypermethylated in CML and is correlated with poor prognosis (Strathdee et al., 2007).

5. THE WILMS' TUMOR GENE (*WT1*).

This gene was originally found in a subset of patients with Wilms' tumor or nephroblastoma, a pediatric kidney malignancy that was first described by Max Wilms in 1899. It affects about 1:10.000 children, usually under the age of 5 years, and is the cause of 7.5% of all childhood tumors. This disease is also found to be associated with other congenital abnormalities such as WAGR (Wilms' tumor, Aniridia, Genitourinary abnormalities, mental Retardation), the Denys-Drash syndrome, Frasier syndrome and Beckwith-Wiedemann syndrome. The Wilms' tumor gene was first isolated in the 1990 from pedigree studies of children with WAGR and LOH in Wilms' tumor. The Wilms' tumor locus was mapped to chromosome position 11p13 (Gressler et al., 1990).

5.1. THE *WT1* GENE.

The *WT1* gene spans more than 50 kb and contains 10 exons generating RNA transcripts of approximately 3.2kb in length and has a GC-rich promoter region lacking TATA box and CCATT boxes (Gressler et al., 1992). The mRNA translates into a nuclear transcription factor. The N-terminus domain of the protein is rich in proline and glutamine and defines functional domains such as RNA-recognition, self-association and transcriptional regulatory domains. The carboxyl terminal of the protein contains four Cys2His2 zinc fingers, each one encoded by exons 7-10, (Figure I.17), for specific DNA-binding, and resembling those in the zinc-finger transcription factor, EGR1. The zinc fingers bind to DNA target sequences that have the consensus sequence 5´-GCGTGGGAGT-3'.

Figure I.17: Schematic representation of the WT1 protein domains.

Many different isoforms exits for this transcription factor, and it is estimated that it can have over 36 different isoforms generated by alternative splicing,

RNA editing and utilization of an alternative first exon (Hohenstein et al., 2006). The most studied and common splicing events, which give rises to four main protein isoforms are: *WT1* isoform A (-17AA/-KTS), *WT1* isoform B (+17AA/-KTS), *WT1* isoform C (-17AA/+KTS) and *WT1* isoform D (+17AA/+KTS) (Figure I.18). The protein ranges from 52 to 54kDa in size (Scharnhorst et al., 2001). The first splicing site effects exon 5 leading to the presence or absence of 17 amino acids (+17/-17). The second alternative-splicing site, affects exon 9, disrupting the zinc-finger domain of the protein. This results in the possible insertion of three amino acids: lysine, threonine and serine (+KTS/-KTS), located in the middle of the protein transcriptional activation domain. This KTS insertion is results in altered spacing between the third and fourth zinc fingers, changing the DNA recognition site and decreasing the DNA-binding ability**.**

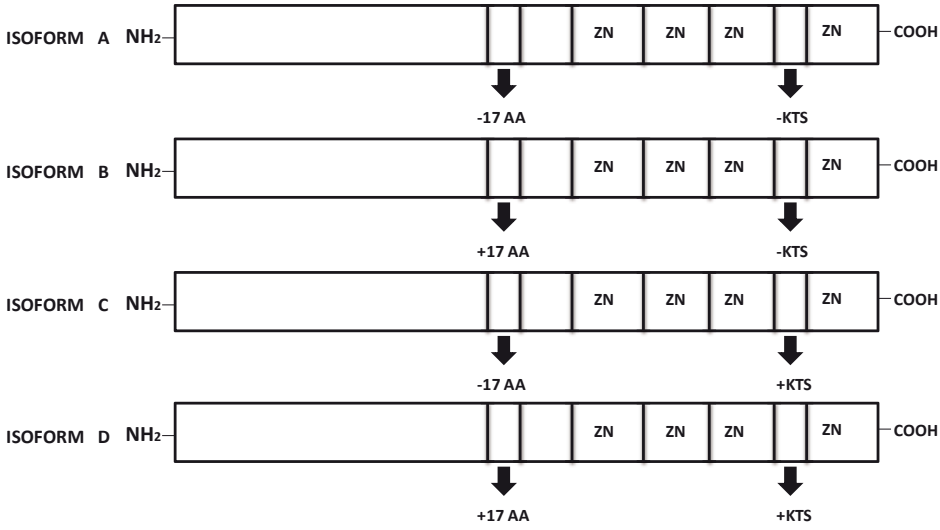


Figure I.18: Scheme of the *WT1* different isoforms, figure adapted from Kramarzova et al., 2012.

As previously mentioned, other splice forms do exist, but due to their rarity have not been as extensively investigated as the four main protein isoforms. In

addition to alternative splicing, a CUG upstream translational start site generates four larger WT1 proteins of 60-62KDa (Bruening et al., 1996).

5.2. *AWT1* ISOFORM.

Recently, a new isoform has been identified, *AWT1* (Alternative *WT1* transcript) or *sWT1* (small *WT1* transcript). This new transcript is generated from an alternative promoter and gives rise to an alternative first exon. The promoter region is located in intron 1 of *WT1* containing a translation start codon ATG, located at nucleotide 242 (Hossain et al., 2006). It encodes a 32kDa protein lacking the first 147 amino acids of the N-terminal of the WT1 protein, therefore, lacking the repressor domain and the RNA recognition motif of the full-length protein. The AWT1 protein also exhibits the 17aa and KTS splicing events present in the WT1 isoforms. This transcript was initially identified in human cDNA library and subsequently in human fetal kidney by RT-PCR (Dallosso et al., 2004). Since this novel transcript lacks the repression domain of the protein, the DNA-binding capacity of the protein is different, activating expression of genes that are repressed by the full-length isoform, such as *Cyclin E* and *IGF-1R* genes. The protein encoded by the shorter isoform is expressed in fetal kidney and in testis, and not in adult kidney, bone marrow or leukocytes. Moreover, this isoform is aberrantly expressed in AML and CML, suggesting it may have oncogenic properties (Ishikawa et al., 2011).

5.3 *WT1-AS* ANTISENSE TRANSCRIPT.

In addition to the many isoforms of *WT1* and *AWT1*, there is an overlapping antisense transcript that does not translate into a protein. This antisense ncRNA was first identified and mapped to the human 11p13 locus in 1990 (Huang et al., 1990). This antisense RNA was originally named WIT-1, and has been proposed to regulate the expression of *WT1/AWT1* (Malik et al., 1995).

5.4. IMPRINTING OF *AWT1* AND *WT1-AS.*

The antisense transcript, *WT1-AS,* and the alternative isoform *AWT1* have been reported to be paternally expressed in kidney samples. These postulated imprinted transcripts originate from a region of hemi-methylation, *WT1-ARR* (*WT1*-antisense regulatory region), which was inferred to be methylated on the maternal allele, as Wilms' tumor samples with maternal LOH of 11q13 were hypomethylated compared to normal kidney samples (Dalloso et al., 2004; Malik et al., 2000). Interestingly, in Wilms' tumor without LOH, but with *WT1-ARR* hypomethylation, these transcripts are biallelically expressed, indicating LOI. Subsequent Southern blot analysis on BWS patients with and without uniparental disomy (UPD) for chromosome 11 revealed that paternal UPD samples showed hypomethylation of the *WT1-ARR* region compared to the samples without UPD (Dalloso et al., 2004). Therefore, the *WT1-ARR* region has been classified as a *cis*-acting transcriptional silencer element for *WT1-AS* and *AWT1*. This region has also been shown to be a methylation-sensitive binding site for the boundary protein CTCF in EMSA experiments, however, *in vivo* confirmation of allelic CTCF binding has not been reported (Hancock et al., 2007).

5.5. *WT1* EXPRESSION PATTERN AND DEVELOPMENTAL ROLE.

Physiological *WT1* expression is restricted to a few types of cells and tissues. During embryogenesis, it plays a key role in the development of gonads, kidney, spleen, pericardium and in a specific population of neurons in the spinal cord. Postnataly, *WT1* is expressed in the podocytes of the kidney by lining the glomerular filtration units, Sertoli cells of the testis, and granulose cells of the ovary and the uterus (Pritchard-Jones et al., 1990; Armstrong et al., 1992). The physiological isoform pattern of the four isoforms A[-/-]: B[+/-]: C[-/+]: D[+/+] seems to be stable during the development of *WT1* expressing

tissues. However in Wilms' tumors the ratios change compared to normal fetal kidney (Haber et al., 1991).

5.6. WT1 TARGET GENES.

The presence of the four zinc fingers at the C-terminal portion of WT1 suggest it is a potent transcription regulator and transfection experiments have confirmed it can act has either an activator or a repressor depending of the cellular and promoter context (Reddy et al., 1995). Among the targets are genes important for cellular growth and metabolism, receptors, other transcription factors, enzymes, epithelial extracellular components and others. These are listed in the following table I.2, (adapted from Yang et al., 2007).

Table I.2: WT1 target genes.

ACTIVATION EFFECT	
TARGET	**REFRENCE**
Amphiregulin	Lee et al., 1999
Insulin growth factor II	Nichols et al., 1995
Platelet derived growth factor	Wang et al., 1993
c-Myc	Hans et al., 2004
p21	Englert et al., 1997
E-cadherin	Hosono et al., 2000
Wnt-4	Sim et al., 2002
Bcl-2	Hewitt et al., 1995

REPRESSOR EFFECT	
TARGET	**REFRENCE**
Colony stimulating factor-1	Harrington et al., 1993
Insulin growth factor II	Nichols et al., 1995
Platelet derived growth factor	Wang et al., 1993
Transforming growth factor-beta	Dey et al., 1994
Androgen receptor	Zaia, et al., 2001
Epidermal growth factor receptor	Englert et al., 1995

REPRESSOR EFFECT	
TARGET	**REFRENCE**
Insulin receptor	Webster et al., 1997
Insulin growth factor I receptor	Werner et al., 1993
Retinoic acid receptor alpha	Goodyer et al., 1995
c-Myb	McCann et al., 1995
c-Myc	Hewitt et al., 1995
Cyclin E	Loeb et al., 2002
PAX-2	Ryan et al., 1995
Human telomerase reverse transcriptase	Oh et al., 1999
Ornithine decarboxylase	LI et al., 1999
Thrombospondin 1	Dejong et al., 1999
Bcl-2	Mayo et al., 1999

5.7. POST-TRANSLATIONAL MODIFICATIONS AND INTERACTING PARTNERS OF WT1.

Proteins after their translation can be subject to chemical modifications, known as post-translational modifications. These include acetylation, phosphorilation, and ubiquitination among others. These modifications can regulate the activity state, stability and subcellular localization of proteins. The WT1 protein can be phosphorilated at zinc fingers 2 and 3 (Ser 365 and Ser 393) causing the modulation of the transcriptional activity and nuclear localization (Ye et al., 1996; Sakamoto et al., 1997). Several interacting proteins partners with WT1 have been identified by using of yeast two-hybrid assays, co-immunoprecipitation or GST pull down-assays. Following is table I.3 listing some of the interacting proteins that form complexes with WT1.

Table I.3: WT1 interacting proteins and their functions (adapted from the Scharnhorst et al., 2001).

PROTEIN	FUNCTION OF INTERACTION	REFERENCE
WT1	Required as a transcription regulation by WT1.	Englert et al., 1995
p53	WT1 modulates the transcriptional regulatory properties of p53 and suppresses p53-mediated apoptosis.	Scharnhorst et al., 2000
p63	Unknown	Scharnhorst et al., 2000
p73	Blocks DNA binding by WT1.	Scharnhorst et al., 2000
SF1	Cooperates to increase transcription of Müllerian inhibiting substance	Nachtigal et al., 1998
U2AF65	Putative splicing regulation function.	Davies et al., 1998
WTAP	Putative splicing regulation function.	Little et al., 2000
PAR4	Blocks transcriptional activation and growth suppression by WT1	Johnstone et al., 1996
ClAO1	Blocks transcriptional activation by WT1	Johnstone et al., 1998
HSP70	Cooperate to inhibit cell proliferation	Maheswaran et al., 1998
DNMT1	WT1 recruits DNMT1 to the PAX2 promoter	Xu et al., 2011
EZH2	WT1 recruits EZH2 to the PAX2 promoter	Xu et al., 2011

5.8. ROLES OF *WT1*: TUMOR SUPPRESOR GENE OR ONCOGENE?.

The roles that the WT1 protein plays are different depending on circumstances; therefore it can behave as a tumor suppressor gene, an oncogene, a transcriptional repressor and a post-transcriptional regulator. These different roles, which are opposite and conflicting with one another, makes this gene a complex one, especially since there are so many different isoforms. The WT1 up-regulation or lack of expression depending on the tissue and the time point of deregulation can lead to the development of a neoplasm. As a tumor suppressor gene: As described before, this gene was initially discovered as a tumor suppressor gene in Wilms' tumor, a pediatric kidney cancer. Inherited mutations followed by subsequent inactivation due to an inactivation of the other allele, leading to the development of Wilms' tumor.

This event is known as de the Knudson's "two hit" model for the genetics of tumor suppressor genes, which is mutually exclusive with imprinted expression, for which a single "hit" is sufficient for silencing.

As an oncogene: Over the last few years, much data has been accumulated suggesting a role of *WT1* in both hematological and non-hematological origin cancers. These tumors arise in tissues that do not normally express WT1, indicating an oncogene function. Importantly, mutations in the *WT1* gene are rarely detected in solid tumors, arguing against loss of tumor suppressor function (Huff, 2011).

5.9. *WT1* IN HEMATOPOIESIS AND IN HEMATOLOGIC NEOPLASMS.

5.9.1. EXPRESSION OF *WT1* IN HEMATOPOIESIS.

In normal human bone marrow, *WT1* expression is restricted to multipotent progenitor cells, CD34+, uncommitted dormant CD34+CD38-, and lineage-committed proliferative CD34+CD38+ of the bone marrow. Approximately 1% of CD34+ BM cells, which accounts for 1-4% of normal BM cell population, express *WT1* (Hosen et al., 2002). Experimental studies using ectopic expression of *WT1* in CD34+ cells have shown that it confers growth arrest and reduced colony-forming capacity, blocking the proliferative capacity of cells (Svedberg et al., 2001). Therefore, expression of *WT1* is limited to early progenitors of the blood system, suggesting that this gene plays a critical role in hematopoietic development, controlling proliferation and/or differentiation in HSC cells (Baird et al., 1997).

5.9.2. *WT1* EXPRESSION IN LEUKEMIA.

WT1 is aberrantly expressed in AML, CML, MDS and ALL. The lack of *WT1* expression in CLL, hairy cell leukemia and plasma cell leukemia, suggest that *WT1* is restricted to immature leukemias. In adult AML, *WT1* expression does

not correlate with age or cytogenetic risk group (Schmid et al., 1997; Karakas et al., 2002; Bergmann et al., 1997).

5.9.3. *WT1* ISOFORMS EXPRESSION IN LEUKEMIA.

As mention previously, WT1 has 36 potential protein variants. These are produced from the same DNA template as a result of alternative transcription initiation, alternative pre-mRNA, mRNA editing and alternative translation initiation. Recently, Kramarzova and colleagues, using a real-time PCR quantification approach, have catalogued the isoform pattern in leukemias. They concluded that childhood and adult AML and MDS express unique *WT1* isoform profiles, but with no relationship between the different profiles and morphologically or cytogenetically subtypes (Kramarzova et al., 2012). The *AWT1* transcript arises from an alternative exon 1, and lacking the N-terminal of the repression domain of the full length WT1. Hossain and colleagues were the first to describe the *AWT1* transcript being overexpressed in leukemia as well as having oncogenic properties (Hossain et al., 2006). Other groups have also reported *AWT1* to be overexpressed in AML and CML, indicating that the *AWT1* expression is preferentially in immature cells (Lopotová et al., 2011). Moreover, the expression of *AWT1* is not implicated in prognosis and does not stratify AML into a distinct entity (Ishikawa et al., 2011).

5.9.4. EFFECTS OF *WT1* IN LEUKEMIA.

WT1 is expressed in stem cells in the bone marrow and not in normal mature blood cells. Chemical induction of differentiation by sodium butyrate or 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in K562 and HL-60 leukemic cell lines, were *WT1* is highly expressed, results in a dramatic down-regulation, further suggesting *WT1* is associated with immature leukemic cells (Phelan et al., 1994; Sekiya et al., 1994). Other reports indicate that ectopic expression of various *WT1* isoforms delays the *in vitro* differentiation of leukemia cell lines

K562 and HL-60 (Inoue et al., 1994). Studies utilizing antisense oligonucleotides and/or siRNAs depletion in various leukemia cell lines have revealed that removing *WT1* results in inhibition of proliferation and rapid induction of apoptosis (Algar et al., 1996; Elmaagachi et al., 2005). These observations strongly support the view that *WT1* is highly expressed in cells were there is a less differentiated phenotype, promoting cell growth, proliferation, survival and blockage of differentiation in leukemia. In a study performed on mice, bone marrow cells over-expressing *WT1* carrying the *AML1/ETO* fusion rapidly induced AML. Neither *WT1* overexpression nor the fusion protein was able to induce leukemia on their own, indicating that several alterations involved in proliferation and differentiation are needed to develop leukemia (Nishida et al., 2005).

5.9.5. *WT1* MUTATIONS IN LEUKEMIA.

King-Underwood and colleagues first reported *WT1* mutations in acute leukemia more than a half a decade ago. Their investigations lead to the observation that these mutations occur in 15% of AMLs and 20% of biphenotypic leukemias, but are rarely occurred in ALLs (King-Underwood et al., 1998). In AML, mutations occur in both childhood and adult forms of the diseases and correlate with low response to chemotherapy, leading to a shorter relapse-free and overall survival. These mutations include missense mutations, deletions and insertions that truncate the WT1 protein. Many of the mutations have been described in exon 7 and exon 9. In exon 7, frame shift mutations occurs resulting in a truncated form of the protein, and in exon 9 missense mutations occurs destabilizing the third helix zinc finger leading to the disruption of the DNA binding. In support of a role for the *WT1* mutants conferring drug resistance, a study done on the K562 cell line showed that wild-type *WT1* is a negative regulator of the multidrug resistance-1 MDR1 protein. Thus, a loss of the negative regulation of MDR1, as a consequence of a

mutation of the WT1 protein, results in elevated levels of MDR1, therefore, conferring drug resistance (McCoy et al., 1999).

5.9.6. CLINICAL IMPLICATIONS AND APPLICATIONS OF *WT1* IN LEUKEMIA PATIENTS.

5.9.6.1. MINIMAL RESIDUAL DISEASE.

One of the major concerns after cancer treatment is that complete eradication of the malignant cells has not occurred. Therefore, there is a need to have specific parameters that detect residual malignant cell during clinical remission. The overexpression of *WT1* occurs in many types of leukemia, and its high expression has enabled it to be a molecular marker for minimal residual disease (MRD) (Lopotová et al., 2011). Expression profiling is routinely performed during, and after treatment, where high levels of *WT1* are indicative of worse outcome and overall survival (Bergmann et al., 1997; Kreuzer et al., 2001; Trka et al., 2002).

5.9.6.2. FUTURE CLINICAL APPLICATIONS OF *WT1*: IMMUNOTHERAPY.

Since *WT1* has demonstrated to be overexpressed in leukemia, it is an ideal tumor antigen. Several groups have evaluated the ability of selective CD8+ cytotoxic T lymphocytes (CTLs) to eradicate leukemic cells expressing WT1 protein (Casalengo-Garduño et al., 2010). To date, several phase 1 clinical trials with WT1 peptide based vaccination have been initiated (Van Driessche et al., 2012). In a murine model, it has been demonstrated that mice immunized with the WT1 peptide rejected challenges by WT1-expressing cancer cells and survived with no signs of auto aggression to normal organs that physiologically expressed WT1 (Oka et al., 2007), emphasizing the potential of this targeted treatment.

MATERIALS AND METHODS

1. TISSUES AND DNA RESOURCES.

1.1. NORMAL TISSUES.

Control DNA samples were obtained from various sources, depending on the type of sample required. Cord blood and placenta samples from healthy newborns were obtained from the Department of Neonatology, Hospital Sant Joan de Déu, Barcelona (Dr. Iglesias-Platas). Peripheral blood from healthy adult volunteers were obtained from PEBC-IDIBELL.

DNA samples from centenarians were obtained from the PEBC-IDIBELL (Dr. Esteller). DNA samples from normal adult kidneys were from the Department of Biochemistry and Molecular Biology from Hospital Vall d'Hebron, Barcelona (Dr. Meseguer). Written consent was obtained from all individuals or parents. DNA from the podocyte cell line was obtained from the Children's Renal Unit and Academic Renal Unit from the University of Bristol, UK (Dr. Saleem).

1.2. COHORT OF HEMATOLOGICAL MALIGNANCIES SAMPLES.

Three different cohorts of hematological malignancies were studied during this project. The first, with 41 adult acute myeloid leukemia, listed in Table M.1, were classified according to the French-American-British classification (FAB), with corresponding *GATA-2* and *WT1* expression data, was from the Laboratory of Genetics, Division of Oncology, Center of Applied Medical Research from the University of Navarra, Pamplona (Dr. Odero and Dr. Calasanz).

Table M.1: List of AML samples.

Type and Name	Number of samples
M0: AML with minimal differentiation	3
M1: AML without maturation	13
M2: AML with granulocytic maturation	9
M3: Acute promyelocytic leukemia	0
M4: Acute myelomonocytic leukemia	6
M5: Acute monoblastic/monocytic leukemia	5
M6: Acute erythroid leukemia	4
M7: Acute megakaryoblastic leukemia	1

The second cohort comprised of 327 different hematological malignancy samples classified according to the 2008 World Health Organization (WHO) outlines. They were from the tumor bank of the Institute of Human Genetics Pathology department of Christian-Albrecht, University of Kiel, Germany (Dr. Siebert). The following tables M.2 and M.3 catalogue the samples used, classifying them according to the lineage and the presence of characteristic mutation and cytogenetic aberrations:

Table M.2: List of myeloid neoplasms.

Myeloid neoplasms	Number of samples
Myeloproliferative neoplasms (MPS)	
Chronic myeloid leukemia without the *JAK2* mutation	11
Chronic myeloid leukemia with the *JAK2* mutation	1
Polycythaemia vera with *JAK2* mutation	1
Essential thrombocythaemia with *JAK2* mutation	1
Acute myeloid leukemia (AML)	
AML with balanced translocations/inversions	44
AML inv(3)	10
AML inv(6)	8
AML t(15;17)	7
AML t(9;11)	5
AML t(10;11)	2
AML inv(3) previous MDS	3
AML with *NMT1* mutation	2
AML myelodysplasia-related changes	1
AML with differentiation del(5q)	5
AML with differentiation del(5q) and 8+	1
AML complex	20

Table M.3: List of lymphoid neoplasms.

Lymphoid neoplasms	Number of samples
Precursor lymphoid neoplasms	
B-cell lymphoblastic with t(9;22)	9
B-cell lymphoblastic with *MLL* t(4;11)	6
B-cell lymphoblastic with t(12;21)	2
B-cell lymphoblastic with hyperploidy	9
T-cell lymphoblastic	1
Mature B-cell neoplasms	
Chronic lymphocytic normal karyotype	47
Chronic lymphocytic leukemia 11q-	7
Chronic lymphocytic leukemia 13q-	13
Chronic lymphocytic leukemia 11q- 13q-	12
Burkitt lymphoma	30
Follicular lymphoma	69

The third cohort consisted of 8 samples of childhood acute lymphoblastic leukemia and 5 samples for childhood acute myeloid leukemia collected at the Hospital Sant Joan de Déu, Barcelona (Dr. Català).

2. CELL CULTURE TECHNIQUES.

2.1. LEUKEMIA AND LYMPHOMA CELL LINES.

All leukemia and lymphoma cell lines were obtained from the PEBC-IDIBELL or the American Type Culture Collection (ATCC). The only exception was the UoC-MII AML megakaryoblastic cell line carrying 4 copies of the *MLL* gene, a kind gift from the Section of Hematology/Oncology, Department of Medicine, University of Chicago, USA. The cell lines used are listed in table M.4.

Table M.4: List of leukemia and lymphoma cell lines.

DISEASE TYPE	CELL LINE	CHROMOSOMAL ALTERATION
ACUTE MYELOID LEUKEMIA	MV4;11	t(4;11) *MLL-AF4* fusion
	SKNO-I	t(8;21) *RUNX1-AML1* fusion
	MOLM 13	t(9;11) *MLL-AF9* fusion
	KASUMI	t(8;14) *AML-ETO* fusion
	KG1a	Derived from the KG-1 cell line
	HL-60	Amplification *MYC* and *NRAS*
	NB4	t(15;17) *PML-RARA* fusion
	HEL-R	Erythroleukemia cell line
	UoC-MII	*MLL* amplification
CHRONIC MYELOID LEUKEMIA	K562	t(9;22) *BCR-ABL* fusion
ACUTE LYMPHOBLASTIC LEUKEMIA B-CELL PRECURSOR	KOPN-8	t(11;19) *MLL-ENL* fusion
	REH	t(12;21) *ETV6-RUNX1* fusion
	SD-1	t(9;22) *BCR-ABL* fusion
	SEM	t(4;11) *MLL-AF4* fusion
	RS4;11	t(4;11) *MLL-AF4* fusion
	TOM-I	t(9;22) *BCR-ABL* fusion
	NALM-20	t(9;22) *BCR-ABL* fusion
	TANOUE	t(8;14) *MYC-IGH* fusion
	JVM2	t(11;14) *BCL1/CCND1-IGH* fusion
ACUTE LYMPHOBLASTIC LEUKEMIA T-CELL PRECURSOR	MOLT 16	t(8;14) *MYC-TRAD* genes altered
	MOLT 4	hypertetraploid
	JURKAT	*TP53* mutation
LYMPHOMAS	RAJI	t(8;14) *MYC-IGH*
	GRANTA-519	t(11;14) *BCL1/CCND1-IGH* fusion
	L1236	Hodgkin lymphoma (hypodiploidy with 17% polyploidy)
	U937	*TP53* mutation
	KMH2	Hodgkin lymphoma (hypodiploidy with 3,5% polyploidy)
	HLDM2	Hodgkin lymphoma (hypodiploid with a large number of rearrangements of all chromosomes except ch 10)

2.2. CULTURE CONDITIONS AND MAINTENANCE.

All leukemia and lymphoma cell lines were routinely grown in Dulbecco's modified Eagle's medium (DMEM)(Gibco) supplemented with 10% of fetal bovine serum (FBS, Gibco, Berlin, Germany), 50 U/ml penicillin and 50 □g/ml streptomycin (Gibco). Cells were maintained at 37ºC in a humidified atmosphere of 5% CO_2, remained free of *Mycoplasm* and were propagated in culture according to established protocols.

2.3. CRYOGENIC STORAGE AND RECOVERY OF CELLS.

Cells were cryogenically stored by resuspending the cells in DMEM medium containing 10% of dimethyl sulfoxide (DMSO) to a concentration of 10^6 cells per milliliter. The cell suspension was aliquoted into polypropylene cryotubes (Nunc, Denmark) and placed at -80ºC overnight in a cryogenic storage vessel containing 250 ml of isopropanol to slow the freezing process. Once frozen, cryotubes were stored indefinitely in the liquid nitrogen. Frozen cells were thawed to 37ºC in a water bath and immediately added to 5 ml of completed medium to dilute out the DMSO. Cells were then centrifuged at 1,000 rpm for 5 minutes and the cell pellet resuspended in full media and seeded in a 10 cm^2 flask.

2.4. CELL HARVESTING FOR DNA AND RNA EXTRACTION.

Suspension cells were pelleted by centrifugation at 1,000 rpm for 5 minutes and the media was removed. Cells were resuspended and washed in 5 ml of PBS and pelleted in a 1.5 ml Eppendorf tube by centrifugation at 2,500 rpm at 4ºC. Cell pellets for RNA were resuspended in 1 ml of TRIzol (Invitrogen) and stored at -80ºC. Cell pellets for DNA were snapped frozen and stored at -80ºC.

3. MOLECULAR BIOLOGY TECHNIQUES.

3.1. ISOLATION AND PREPARATION OF NUCLEIC ACIDS.

3.1.1. GENOMIC DNA EXTRACTION FROM FROZEN BLOOD.

45 ml of cold blood lysis buffer (0,64 M sucrose, 1 M $MgCl_2$, Triton X-100 and 1 M Tris-HCl pH=7.5) was added to 5 ml freshly thawed frozen blood and centrifuged at 10,000 rpm for 15 minutes at 4ºC to pellet the nuclei. The resulting pellets were stored at -20ºC for DNA extraction.

3.1.2. GENOMIC DNA AND RNA EXTRACTION FROM FRESH BLOOD.

Mononuclear cells were isolated from fresh peripheral blood by density centrifugation using Lymphoprep (Axis-Shield). Fresh blood samples were collected in a tube containing EDTA-anticoagulant. The blood sample was diluted in an equal volume of PBS and gentle layered over Lymphoprep solution. Samples were centrifuged at 2,200 rpm with no brake for 20 minutes at 4ºC. After centrifugation, mononuclear cells form a distinct layer at the Lymphoprep/medium interface, as shown in figure M.1. Cells were removed from the interface using a Pasteur pipette and washed in PBS. Following a final centrifugation at 1,500 rpm for 10 minutes, the resulting pellets were used for DNA, RNA or protein extraction and ChIP.

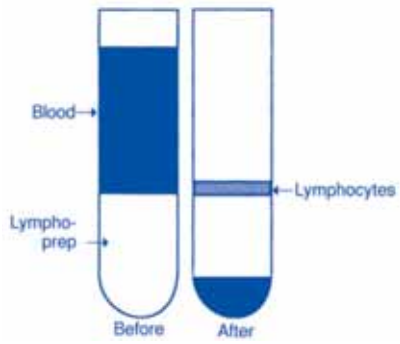


Figure M.1: Lymphoprep preparation, before and after centrifugation.

3.1.3. GENOMIC DNA EXTRACTION.

Genomic DNA was isolated by the standard phenol/chloroform extraction. Cell pellets were resuspended in 2 ml of lysis buffer (100 mM NaCl, 10 mM Tris-HCl pH=8 and 25 mM EDTA) supplemented with 25 µl of 20% SDS and 25 µl of Proteinase K (20 mg/mL) (Sigma-Aldrich) and incubated overnight at 55ºC. The following day, the cell lysates, were transferred to a Phase Lock gel tubes (Prime5) and mixed with an equal volume of phenol/chloroform. To separate organic layer from the aqueous phase containing the DNA, centrifugation was performed at 1,000 rpm for 5 minutes. The phenol/chloroform extraction was repeated a total of 3 times and was followed by 3 extractions with chloroform only. Genomic DNA was precipitated by the addition of 1/10 volume of 3M NaOAc and two volumes of 100% EtOH. After gentle inversion, the resulting DNA was transferred to a fresh tube and washed in 70% EtOH. Finally, DNA was isolated, briefly air-dried, and resuspended in TE or H_2O. The quantity and purity of the DNA was determined by measuring absorbance at 260nm (A260) and 280nm (A280) with a spectrophotometer (Nanodrop). A A260/280 ration of 1.8-2.0, indicating DNA free of contaminating phenol or protein, was obtained for all samples. Genomic DNA was stored at -20ºC until use.

3.1.4. SMALL-SCALE DNA EXTRACTION FROM BACTERIA.

Single bacteria colonies were picked from agar plates and grown overnight at 37ºC in 5 ml of LB media supplemented with 100 µg/ml of ampicillin (Sigma-Aldrich). Plasmids were extracted with NucleoSpin Plasmid kit (Macherey-Nagel) according to manufacturer's instructions. Briefly, bacteria were pelleted by centrifugation at 3,500 rpm for 10 minutes. The pellet was resuspended in 250 µl of buffer A1, to which 250 µl of buffer A2 and 250 µl of buffer A3 were added to lyse and neutralize the mix. The lysate was centrifuged at 12,000 rpm for 5 minutes, and the supernatant was transferred to a NucleoSpin Plasmid column. Wash steps were performed, and plasmid DNA was eluted in 50 µl of

buffer AE. On average, miniprep kits yielded approximately 15 µg of DNA.

3.1.5. MEDIUM-SCALE DNA EXTRACTION FROM BACTERIA.

When larger amounts of plasmid were required, purifications were conducted using a midiprep kit (Macherey-Nagel). From a single colony, a 5 ml starter culture was grown overnight at 37ºC. One ml of the starter culture was used to seed a 100 ml culture which was also grown overnight at 37ºC. The bacterial suspension was pelleted by centrifugation at 3,500 rpm for 10 minutes at 4ºC. The pellet was resuspended in 8 ml of buffer RES, then 8 ml of lyses buffer LYS was added and incubated for 5 minutes at room temperature. The supernatant was added to a NucleoBond Xtra Column that had been previously equilibrated with 12 ml of buffer EQU. Following a washing step, the DNA was eluted and precipitated with isopropanol. Then, DNA was pelleted by centrifugation and washed as described previously, before being resuspended in 250 µl of H_20.

3.1.6. RESTRICTION ENZYME DIGESTION.

To confirm the purity and quality of the mini & midipreps, restriction enzyme digestions were performed using 10 units of enzyme in a reaction volume of 25 µl containing 1X reaction buffer (New England Biolabs) and 0.5-1 µg of DNA. Digestion reactions were performed for 1 hour at 37ºC. The digestion was resolved by gel electrophoresis as described in section 3.4.

3.1.7. RNA EXTRACTION FROM TISSUES AND CELLS.

Total RNA was isolated using TRIzol reagent (Invitrogen). Cells were lysed in 1 ml of TRIzol, which is a commercial combination of guanidine isothiocyanate and phenol. Subsequently, 200 µl of chloroform were added to the sample. After 30 minutes of centrifugation at 12,000 rpm at 4ºC the sample segregated into an organic phase containing the DNA and proteins, leaving the RNA in the upper aqueous layer. RNA within the aqueous layer was precipitated in 0.8

volume of isopropanol and centrifuged for 1 hour at 12,000 rpm at 4ºC and washed with 70% EtOH. The RNA pellet was dissolved in 20-30 µl of RNase-free water. The amount and purity of the RNA was determined by measuring absorbance at 260nm (A260) and 280nm (A280) with a spectrophotometer (Nanodrop). A A260/A280 ratio of 1.8-2.0, indicating RNA free of contaminating phenol or protein, was obtained for all the RNA samples. RNA was stored at -80ºC until use.

3.2. cDNA SYNTHESIS.

All RNA samples were treated with DNaseI to remove any contaminating traces of DNA. One unit of DNaseI (Invitrogen) was added to 1 µg of RNA, 1 µl of 10X reaction buffer (Invitrogen) and made up to 10 µl with DEPC-treated H_2O (Invitrogen). After 30 minutes incubation at 37ºC the enzyme was inactivated by adding 1 µl of 25 mM EDTA stop solution (Invitrogen) followed by 10 minutes incubation at 75ºC. The DNA free RNA was subsequently used in a reverse transcription reaction to generate complementary DNA (cDNA). One µg of RNA was incubated at 70ºC to remove any RNA secondary structure, and a mix consisting of 2 µl DEPC-treated H_2O, 1 µl of random hexamer primers, 4 µl of reverse transcriptase buffer, 2 µl 100 nM dNTPs, 40 units (1 µl) of MMLV reverse transcriptase (Promega) and 1 U (0.25 µl) of RNase inhibitor (RNasin, Promega) was added to each sample once the temperature had cooled to 37ºC. The samples were then incubated at 37ºC for 90 minutes. The reaction was terminated by denaturation of the enzyme at 75ºC for 10 minutes. For each sample, duplicate sets of reactions were performed without MMLV to serve as an RT negative control to rule out amplification from traces of DNA. All resulting cDNAs were subjected to RT-PCR using both *β-ACTIN* and *GAPDH* housekeeping genes. cDNAs with prominent bands were deemed efficiently converted and were stored at -20ºC until required.

3.3. POLYMERASE CHAIN REACTION (PCR).

PCR was performed in a 25 µl reaction volume, with 5 U/µl of EcoTaq DNA polymerase (Bioline), 2.5 µl of 10X reaction buffer, 100 ng of each primer, 1.5 mM of $MgCl_2$, 0.25 mM of dNTPs (Promega), 7.5 µl of 5 M Betaine and ~50 ng of template (either genomic DNA or cDNA) in a thermal cycler (Applied Biosystems). The PCR cycling conditions were 95ºC for 5 minutes, followed by 35-45 cycles (95ºC for 1 minute, annealing temperature for 30 seconds, 72ºC for 30 seconds-1 minute) and a final extension step at 72ºC for 5 minutes. Alternative PCR conditions and reagents were used for amplification of bisulfite converted DNA or difficult templates using HotStar polymerase (Qiagen) in a 25 µl reaction volume, with 5 U/µl of HotStar Taq DNA polymerase (Qiagen), 10X HotStar PCR buffer, 5X Q solution, 0,25 mM of dNTPs (Promega), 100 ng of each primer and ~50 ng of template. The PCR cycling conditions were 96ºC for 16 minutes for denature and separate the polymerase of the inhibitory antibody, followed by 40-45 cycles (94ºC for 30 seconds, annealing temperature for 30 seconds, 72ºC for 30 seconds-1 minute) and a final extension step at 72ºC for 10 minutes.

Primes used throughout this project are listed in the appendix.

3.4. AGAROSE GEL ELECTROPHORESIS.

To visualize genomic DNA, RNA or PCR products, gel electrophoresis was performed in 0.8%-2% agarose gels, prepared in 0.5X TAE containing of SYBR SAFE (Invitrogen) using the HU15 standard SCIE-PLAS gel unit with removable casting trays. 0.5X TAE was used as a gel electrophoresis buffer. Samples were mixed with loading dye (Promega) and loaded into cast wells of the gel. A DNA ladder (either, 100 bp or 1.2 Kb, Promega markers) was run at the same time to confirm that products were of the expected size. Gel electrophoresis was carried out at 100-120V using a PowerPac basic power supply (Bio-Rad).

Samples were visualized using the Q-Box equipment and GeneSys automatic software.

3.5. PCR PRODUCT PURIFICATION FROM AGAROSE GELS.

Bands were cut out from the gel with a clean blade and placed into a 1.5 ml Eppendorf tube. DNA was extracted using the NucleoSpin Extract II kit (Macherey-Nagel) following the manufactures' protocol. Briefly, gel fragments were weighed, and an appropriate volume of buffer NT was added (100 mg agarose gel and 200 µl of Buffer NT) and incubated at 55ºC for 10 minutes to dissolve the gel. Samples were loaded into a NucleoSpin Extract II column and centrifuged at 10,000 rpm for one minute. Then columns were washed in buffer NT3 followed by 10,000 rpm centrifuged for two minutes to dry the membrane of the column. DNA was eluted in 20 µl of H_2O and used immediately for ligation or sequencing.

3.6. DNA SANGER SEQUENCING.

3.6.1. CLEAN-UP OF PCR PRODUCTS.

Sequencing templates were purified to remove unincorporated primers, dNTPs, salts and buffer. PCR products were precipitated by the addition of 2 volumes of precipitation mix (3 M sodium acetate pH=4.6 and 70% of EtOH) and centrifuged at 3,500 rpm for 40 minutes. The supernatant was removed and the pellets washed with 70% EtOH, before the supernatant was removed and the pellet air-dried. The pellets were then resuspended in H_20 to a final concentration of ~10-20 ng/µl.

3.6.2. FLUORESCENT-LABELLED CYCLE SEQUENCING.

Sequencing reactions were carried out using the ABI BigDye Terminator Cycle Sequencing Kit, according to the manufactures' protocols. Sequencing reactions were preformed in a final volume of 10 µl using between 5-100 ng DNA depending on template size (5 ng for PCR products less than 200 bp, up to 100 ng for plasmids), 4.7 µl of BigDye and 3.2 pmol of primer. Samples were placed in a thermocycler and subjected to an initial denaturation of 96ºC for one minute, followed by 28 cycles of 96ºC for 15 seconds, annealing at 55ºC for 15 seconds and extension for 4 minutes at 60ºC. Afterwards, sequence reactions were precipitated by adding 170 µl of precipitation solution (3 M sodium acetate pH=4.6 and 100% of EtOH) and centrifuged for 45 minutes at 3,200 rpm at 4ºC. The supernatant was removed and pellets were washed in 170 µl of 70% EtOH for 10 minutes at 3,200 rpm at 4ºC. The supernatant was again removed and the pellet air-dried. Sequencing reactions were resuspended in 10 µl of formamide and heat denatured at 96ºC for 2 minutes. Sequence reactions were run on an Electrophoresis ABI PRISM 3730 DNA analyzer (Applied Biosystems). The subsequent sequences electropherograms were then analyze using the Sequencher 4.8 program.

3.7. QUANTITATIVE REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (qRT-PCR).

Real-Time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems). All reactions were prepared in triplicate with 10 ng of cDNA in a final volume of 11 µl and preformed in a 7900HT Fast Real-Time PCR system (Applied Biosystems). The PCR conditions were 50ºC for 2 minutes, followed by 95ºC for 10 seconds and 40 cycles of 95ºC for 15 seconds and 60ºC for 1 minute. Data was analyzed using the RQ Manager 1.2 software (Applied Biosystems). All primers were subjected to temperature dissociation to ensure that the PCR primers amplified specific regions. As an internal control,

expression of *β-ACTIN* mRNA was monitored. Relative expression levels were quantified by the comparative Ct (fluorescence threshold value) method using *β-ACTIN* as a loading control, according to the Livack formula (Livack et al., 2001):

$$\Delta Ct\ (Sample) = Ct\ (Sample) - Ct\ (\beta\text{-}actin)$$

$$\Delta\Delta Ct = 2\text{^}(\Delta Ct\ (Reference) - \Delta Ct\ (Sample))$$

All samples were run in triplicate for each gene analyzed as well as a negative control reactions using H_2O. Only samples with 2 or more valid reading per triplicate were included. Optimized qRT-PCR primers are summarized in the appendix.

3.8. MicroRNAs QUANTITATIVE RT-PCR.

Expression levels of all microRNAs were analyzed using Taqman MicroRNA Assays (Applied Biosystems). This method allows real-time quantification of mature miRNAs in a two-step process (Figure M.2). First, cDNA is reverse transcribed from 5 µl of 10 ng of total RNA, using 3 µl miRNA-specific primers and TaqMan microRNA reverse transcription kit by adding a 7 µl volume of 0.15 µl of dNTP mix (100 mM), 1 µl of multi-scribe RT enzyme (50 U/µl), 1.5 µl of 10X RT buffer, 0.19 µl of RNase Inhibitor (20 U/µl) and 4.16 µl of nuclease-free water. The following reverse transcription program was utilized: 30 minutes at 16ºC, 30 minutes at 42ºC and 5 minutes at 85ºC. cDNA samples are then PCR-amplified and quantified. A 20 µl volume reaction consisted of 1.33 µl of the RT reaction, 1 µl of TaqMan small RNA assay (20X), 10 µl of TaqMan Universal PCR Master Mix II (2X) no UNG and 7.67 µL of nuclease-free water. Taqman probes contain the FAM reporter dye linked to the 5' end of each probe, a minor groove binder (MGB) at the 3' end of the probe and a non-fluororescent quencher (NFQ) at the 3' end of the probe. PCR reactions were performed in triplicate and run on a 700HT Fast Real-Time PCR system (Applied Biosystems). The small nucleolar RNA *RNU19B* was used as an endogenous

control. Relative expression was quantified by the comparative C_T ($\Delta\Delta$ C_T) method.

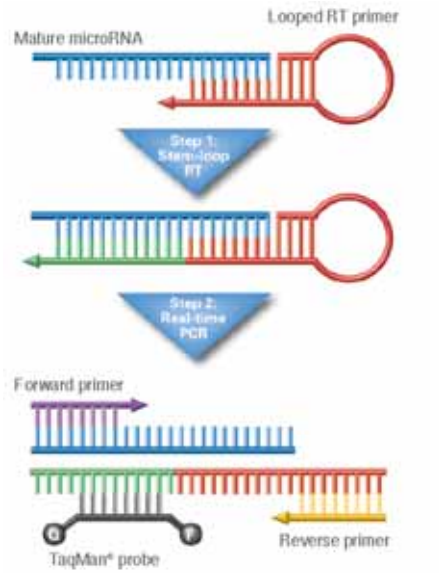


Figure M.2: MiRNAs are reverse transcribed individually by a using stem-loop-specific transcription primer. They are designed to have a short single-stranded region that is complementary to the known sequence on the 3' end of the miRNA, a double-stranded part (the stem) and the loop that contain the universal primer-binding sequence. The resulting reverse transcription product (cDNA) is then used as a template for qPCR with a miRNA-specific primer and a second universal primer.

3.9. WESTERN BLOT TECHNIQUE.

3.9.1. PROTEIN EXTRACTION AND QUANTIFICATION.

Nuclear and cytoplasm extracts were prepared for protein extraction to perform Western blot. Suspension cells were pelleted and washed twice with cold PBS, then 500 µl of buffer A or hypotonic solution (10 mM HEPES pH=7.9, 1,5 mM $MgCl_2$, 10 mM KCl, 0.1 mM PMSF, 0.5 mM of DDT and protease inhibitors) were added to the cell pellet. Samples were incubated for 10 minutes on ice, followed by thorough mixing by vortexing, and centrifuged for 30 seconds at 13,000 rpm. The supernatant was transferred to a 1.5 ml

microcentrifuge tube, and kept as cytoplasmatic extract. For the nuclear extracts, 500 µl of hypotonic solution or buffer C (10 mM HEPES pH=7.9, 1.5 mM $MgCl_2$, 0.42 mM NaCl, 25% Glycerol, 0.2% EDTA, 0.1 mM PMSF, 0.5 mM of DTT and protease inhibitors) were added to the pellet. Samples were then incubated for 20 minutes on ice, followed by vortexing, and centrifuged for 2 minutes at 13,000 rpm. This process was repeated twice. For whole cellular extracts, cytoplasmatic and nuclear extracts were combined. Samples were kept at -80ºC until use. Protein concentration was determined using BCA protein assay kit, according to manufacturer's instructions.

3.9.2. SDS-PAGE.

For 10% of SDS-PAGE gels, 2.5 ml of buffer B or lower gel buffer (1.5 mM Tris-HCl pH=8.8, 0.4%) were mixed with 3.3 ml of 30% acrylamide (Protogel), 60 µl of 10% APS, and 5 µl of TEMED. Approximately 10 ml were poured into a 1.5 mm cassette (Invitrogen). A lower percentage gel made was subsequently poured on top to produce a stacking gel. The 5% stacking gel was comprised of 2.5 ml of buffer C or upper gel buffer (0,5 M Tris-HCl pH=6.8, 0.4% SDS) and 1 ml of 30% acrylamide, which were polymerised with 30 µl of 10% APS and 12 µl of TEMED. Gel combs of the required number and size were added and the gel was allowed to set.

3.9.3. WESTERN BLOT.

Nuclear, cytoplasm or whole nuclear extracts, were mixed with 5X SDS loading buffer (1 M Tris-HCl pH=6.8, 87% glycerol, 10% SDS and 0.4% (w/v) of bromophenol blue), and samples were boiled for 5 minutes at 95ºC. Samples were loaded on gels with 10 µl of PageRuler pre-stained protein ladder (Fermentas) and the gel was run at 15mA. The gels were blotted with transfer buffer (Tris-HCl 0.25 M, Glycine 1.92 M and 1% SDS) on to nitrocellulose membrane in a blotting chamber (Biorad) for 45 minutes at 0.40A. After

transfer, the membranes containing the proteins were stained with Panceau (Sigma-Aldrich) to assure successful transfer. The membranes were blocked with 5% milk (Merck) for 1 hour at room temperature. Membranes were subsequently washed three times in PBS-0.01% Tween and incubated with the primary antibody in 5% BSA overnight at 4ºC with continuous agitation. All primary antibodies used are listed below in table M.5. Afterwards, membranes were washed three times for 5 minutes with PBS-tween, and the corresponding peroxidase-conjugated secondary antibody was applied in 5% milk dilution 1:5000 for 1 hour at room temperature. The membranes were washed with PBS-tween once more and placed in a developing film cassette with the chemiluminescence reagent luminol (0.1 M Tris-Cl pH 8.6, 50 mg sodium luminol, 30% H_2O_2), enhancer (11 mg acid p-hydroxy-coumaric, DMSO) and an X-ray film (Amersham Hyperfilm ECL, GE healthcare). Proteins bands were quantified using the Image J program.

Table M.5: List of antibodies used for Western blot.

TARGET PROTEIN	ANTIBODY	SPECIES
WT1	Santa Cruz, sc-192	Rabbit
WT1	Santa Cruz, sc-81617	Mouse
DNMT1	Abcam, Ab13537	Mouse
GAPDH	Sigma-Aldrich, G9545	Rabbit

3.10. CO-IMMUNOPRECIPITATION.

For co-immunoprecipitation (co-IP) assays, cells were harvested and washed twice with PBS. Cell lysate and protein extraction was performed as previously described. Two µg of the antibody were combined with the cell lysate and incubated for two hours on a rotating wheel at 4ºC. All primary antibodies are listed below in table M.6. In parallel, resin for the capture of the immunocomplex, was prepared, comprising of prewashed protein G

plus/protein A-agarose beads (Millipore). 2 µg of the secondary antibody were added to the protein G plus/protein A-agarose beads and incubated for two hours at room temperature on a rotating wheel. Afterwards, beads were washed twice in 10 volumes of 0,2 M of sodium tetraborate decahydrate (pH=9), and centrifuged for one minute at 4,000 rpm. The beads were resuspended in 10 volumes of 20 mM of dimethyl pimelimidate and incubated for thirty minutes at room temperature on a rotating wheel. Then, 10 volumes of 0.2 M of ethanolamine (pH=8) were added and incubated for two hours at room temperature on a rotating wheel. This was centrifuged and washed twice with PBS. After combining the cell lysate incubated/antibody with the protein G plus/protein A-agarose beads/antibody the mix was left overnight at 4ºC on a rotating wheel. Subsequently, the beads were washed twice with BC-100 (10 mM Tris-HCl pH=7.8, 0.5 mM EDTA, 0.1 mM PMSF, 0.1 mM DTT, 10% of glycerol and 100 mM KCl) and twice with BC-500 (10 mM Tris-HCl pH=7.8, 0.5 mM EDTA, 0.1 mM PMSF, 0.1 mM DTT, 10% of glycerol and 500 mM KCl). Immunoprecipitates were eluted by adding 100 µl of elution buffer (50 µl of 0.2M of glycine, 20 µl of loading buffer and 5 µl of Tris-HCl pH=8.7). Inputs and IPs were denatured at 95ºC for five minutes prior to SDS-PAGE gel electrophoresis.

Table M.6: List of antibodies used for co-immunoprecipitation.

TARGET PROTEIN	ANTIBODY	SPECIES
WT1	Santa Cruz, sc-192	Rabbit
DNMT1	Abcam, Ab13537	Mouse

4. EPIGENETIC TECHNIQUES.

4.1. SODIUM BISULFITE MODIFICATION OF GENOMIC DNA (Manual protocol).

One µg of genomic DNA was adjusted to a volume of 50 µl in H_2O and denatured by the addition of 5.7 µl 3 M of NaOH (final concentration of 0.3 M). The samples were incubated at 37ºC for 15 minutes. The denatured sample was then immediately mixed with 3.6 M of sodium bisulfite pH=5 (540 µL of 7.6 g of bisulfite (Sigma-Aldrich) in 20 ml of miliQ water, pH adjusted with 10 M of NaOH) and 33 µl of 20 mM Hydroquinone (0.09 g of hydroquinone (Sigma-Aldrich) in 50 ml of miliQ water), and incubated for 16 hours at 50ºC. Subsequently, the samples were desalted using a Wizard DNA clean-up column (Promega) following manufacturer's instructions. The DNA was eluted in 50 of µl H_2O. The converted DNA was desulphonated by the addition of 5.7 µl of 3 M NaOH for 15 minutes at 37ºC. DNA was then recuperated by precipitating with 100% of EtOH, using glycogen as a carrier. Following a centrifugation at 12,000 rpm at 4ºC for 30 minutes, the bisulfite converted DNA was washed with 70% EtOH. The DNA was finally resuspended in 30 µl of RNase/DNase-free water. Bisulfite modified DNA (Figure M.3) was stored at -20ºC until used for PCR.

4.2. SODIUM BISULFITE MODIFICATION OF GENOMIC DNA (Commercial kit).

The EZ-96 DNA Methylation Kit from ZYMO Research was used when preparing large numbers of samples. Briefly, 1 µg of genomic DNA was adjusted to a volume of 45 µl in H_2O. To the DNA 5 µl of M-Dilution Buffer were added and incubated for 15 minutes at 37ºC. Subsequently, 100 µl of CT conversion Reagent were added and the samples incubated at 50ºC for 12-16 hours after an initial denaturation of 95ºC for 5 minutes. Afterwards, converted samples were cleaned up, according to manufactures' protocol. Briefly, 400 µl of M-

Binding Buffer were mixed with the DNA and then transferred to a 96-well silicon-A binding plate. This was centrifuge at 4,000 rpm for 5 minutes. Next, sample was washed with 500 µl of M-Wash Buffer and centrifuged again. The samples were then desulphonated by the addition of 200 µl M-desulphonation Buffer and incubated at room temperature for 15 minutes. After the incubation the plate was centrifuged at 4,000 rpm for 5 minutes. The samples were washed and spun twice with M-Wash Buffer before the resin was allowed to dry. The silicon-A binding plate containing the converted DNA was placed in to a clean elution plate and 30 µl of M-Elution Buffer were directly added to the matrix. The DNA was finally collected after a 3 minutes centrifugation at 4000 rpm. The bisulfite modified DNA was stored at -20ºC until used for PCR.

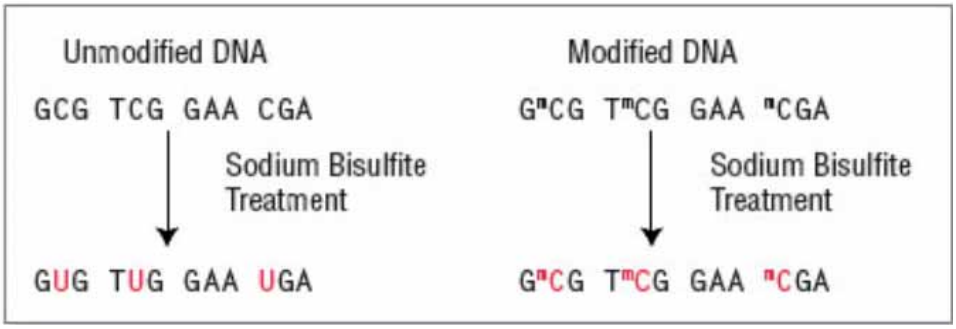


Figure M.3: Overview of bisulfite conversion DNA.

4.3. LIGATION OF PCR PRODUCTS INTO A pGEM-T EASY VECTOR.

3.5 µl of PCR product were added to 1 µl (50 ng) of pGEM-T Easy Vector, 5 µl of 2X rapid ligation buffer, 1 µl of T4 DNA ligase (3 units/µl), and the volume made up to 10 µl with nuclease-free H_2O. The ligation reaction was incubated overnight at 4ºC, and subsequently used for bacterial transformation.

4.4. PLASMID PREPARATION AND PURIFICATION.

4.4.1. BACTERIAL TRANSFORMATION.

For transformation of circular plasmids into bacteria, 5 µl of ligated plasmid were gently mixed with 50 µl of JM109 competent *Escherichia coli* cells (Promega) and incubated on ice for 30 minutes. Cells were heat shocked at 42ºC in a water bath for 45 seconds and chilled on ice for 2 minutes. Subsequently, 950 µl of warm LB or SOC media (free of antibiotics) was added to the cells and shaken at 250 rpm for 1 hour at 37ºC. Cells were centrifuged gently to form a pellet and resuspended in a 100 µl of LB which was evenly spread onto LB agar plates supplemented with 100 µg/ml of ampicillin (Sigma-Aldrich), IPTG 0.1 M (Sigma-Aldrich) and X-Gal 50 mg/ml (Promega).

4.4.2. PCR AMPLIFICATION OF CLONED DNA.

Individual white colonies were picked from LB culture plates and grown in 100 µl of LB media at 37ºC for 2 hours. The DNA from bacterial cultures was subjected to PCR, with 1 µl used as a template to enable the inserted sequences to be amplified using pGEM vector forward and reverse or T7 and SP6 primers present in the vector. The resulting amplicons were sequenced directly.

4.5. PYROSEQUENCING.

Quantitative methylation analyses of promoter regions were performed by pyrosequencing. Primers were designed using the PSQ assay designer software (Biotage). This technique requires the use of three primers, which one of them must be biotinylated. PCR reactions were performed using the pyromark PCR kit provided with a HotStar Taq polymerase, pyromark reaction buffer and 20 pmols of forward and reverse primers. PCR cycling conditions were 95ºC for 15 minutes and 45 cycles of 94ºC for 30 seconds, 65ºC for 30 seconds and 72ºC for 30 seconds, and 72ºC for 5 minutes final extension. 10 µl of the biotin labeled PCR product were made up to 40 µl with H_20, before 2 µl of streptavidin-coated sepharose beads (GE Healthcare) and 38 µl of binding buffer (Qiagen) were added to each sample. The PCR product was immobilized to the beads by shaking for 5 minutes at room temperature. The beads were captured on a Vacuum Prep Tool (Qiagen) and subjected to strand separation by treatment with 70% EtOH, denaturation solution (0.2 M NaOH) and wash buffer (Qiagen). The beads were released into a PSQ HS 96 plate containing 12 µl of 0.3 µM sequencing primer per well. Primer annealing was achieved by heating the plates to 80ºC for 2 minutes before cooling the samples to room temperature. Enzyme, substrate and nucleotides were mixed and dispensed into a cartridge according to the pre-run calculation from the PyroMark software (Qiagen). The pyrosequencing reaction was performed on a PyroMark MD sequencer (Qiagen). As illustrated in Figure M.4. A bisulfite conversion control was included in each assay. The methylation status of each CpG site was analyzed using the Pyro Q-CpG software (Qiagen), with average methylation values calculated for each PCR product.

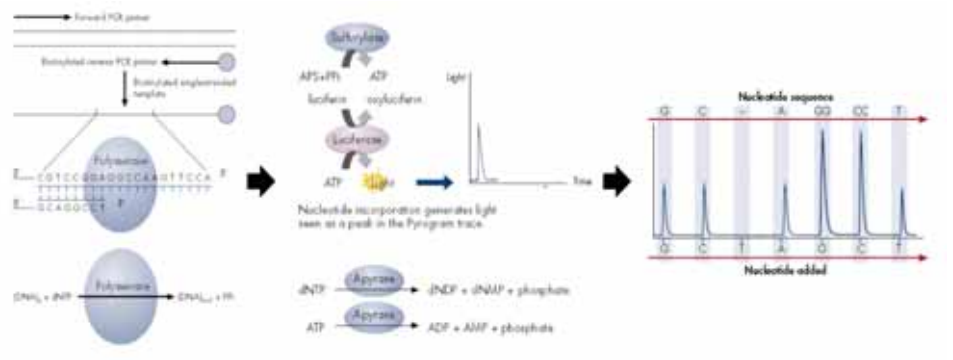


Figure M.4: Overview of the pyrosequencing reaction cascade.

4.6. DNA METHYLATION DETECTION.

Methylated DNA Immunoprecipitation (MeDIP) was used to estimate DNA methylation status using the 5-methylcytidine antibody. MeDIP (Diagenode) precipitation of 5mC tag DNA was preformed according to manufactures' protocol. Briefly, genomic DNA was extracted using the phenol/chloroform method as previously described, followed by DNA sonication to acquire fragments of 300-500 bp. 30 µl of IP incubation mix (24 µl of buffer A, 4.5 µl of buffer B and 1.5 µl of positive control), 45 µl of H_20 were added to the shredded DNA at a concentration of 0.1 µg/µl. Therefore, the total volume per IP was 75 µl. In parallel, an input sample was added. Samples were incubated at 95ºC for three minutes, and then chilled on ice. 20 µl of IP blocked beads were added to the single stranded DNA, as well as 5 µl of the diluted antibody to the IP samples (0.3 µl, 0.6 µl and 2.10 µl of H_20). IP samples were then placed on a rotating wheel over night at 4ºC. Then, the 5mC DNA/antibody complexes were washed with 1 ml of cold buffer 2, buffer 3 and twice with buffer 4. DNA was purified using the QIAquick PCR purification column according to the manufactures' instructions. Samples were analyzed by qPCR as described previously and the amount of 5mC was determined by comparing the amount of immunoprecipitated DNA after normalization to the input DNA:

%(5-mehtylDNA-IP/Input) =2^[(Ct (input)-Ct (5-methylDNA-IP)] x 100 with a positive commercially available control sample.

4.7. ChIP ASSAY.

4.7.1. CROSS-LINKING AND CHROMATIN SHEARING.

Chromatin immunoprecipitation was performed on cells that were split prior harvesting to ensure they were in the growth phase. Cells were harvested and chromatin was isolated from $1x10^7$ cells. For suspension cells, the appropriate number were resuspended in 10 ml of fresh media to which 37% of formaldehyde solution (Sigma-Aldrich) was subsequently added until a final concentration of 1%. This was incubated on a roller at room temperature for 8-10 minutes, depending on the cell line. To stop the cross-linking reaction, 2.5 M of glycine was added to a final concentration of 0.125 M and samples were incubated at room temperature for 5 minutes. Cells were pelleted by centrifugation at 2,500 rpm at 4ºC for 5 minutes. The supernatant was removed and the cells were washed twice in 5 ml of cold PBS. After the final wash, the cell pellet was resuspended in 5 ml of cell lysis buffer (HEPES 5 mM pH=8, KCl 85 mM, NP-40 0.5%, 1X protease inhibitor cocktail (Roche). To facilitate the cell membrane breakage, the cell lysate was transfer into a 7ml Douncer. After 3 stokes of the Douncer, the cell lysate was incubated for 15 minutes at 4ºC. Samples were centrifuged at 4,000 rpm at 4ºC for 5 minutes. The supernatant was discharged and the cell pellet resuspended in 900 µl of nuclear lysis buffer (EDTA 10 mM, Tris-HCl pH=8.1, SDS 1%). This nuclear lysate volume was split into three Eppendorf tubes and sonicated on ice using a Bioruptor (Diagenoide) for 20 to 30 minutes, depending on the cell line, at the highest power with 30 seconds breaks between each pulse. Samples were centrifuged at 12,000 rpm at 4ºC for 10 minutes and the supernatant containing the chromatin was quantified using a Nanodrop at A260/280. The

chromatin was subsequently de-crosslinked in 300 µl of TE which was incubated overnight at 65ºC. The following day 1 µl of 50 mM Proteinase K (Qiagen) was added and incubated for 1 hour at 37ºC. The sheared DNA was extracted using the phenol-chloroform protocol as described above. The eluted DNA was incubated in 1 µl of RNase (Qiagen) for 30 minutes at 37ºC and run on a 1.8% agarose gel to determine the degree of fragmentation. The optimal size of the sheared DNA for ChIP is between 200-1000 bp (Figure M.5). If the sheared chromatin was of appropriate size, the remainder was either used immediately for immunoprecipitation or frozen at -80ºC.

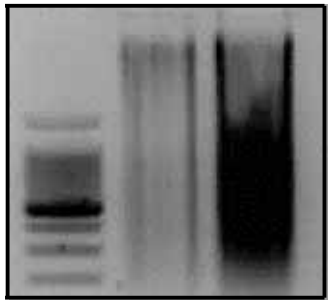


Figure M.5: Sheared chromatin analyzed on a 1.8% agarose gel. Chromatin efficiency is analyzed alongside the DNA molecular weight marker on the left lane.

4.7.2. PREPARATION OF PROTEIN G DYNABEADS.

For each immunoprecipitation reaction, 70 µl of Protein G Dynabeads (Invitrogen) were transferred to 1.5 ml siliconized microcentrifuge tubes (Sigma-Aldrich) and washed twice with 600 µl of PBS/BSA 5%. Washing was facilitated using a magnet (DynaMag, Invitrogen). After the last wash, the beads were resuspended in 70 µl of PBS/BSA 5%.

4.7.3. CHROMATIN IMMUNOPRECIPITATION.

45 µl of the pre-washed Dynabeads were incubated with 1 µg of a polyclonal antibody overnight at 4ºC with rotation. Antibodies used for ChIP are listed in the following table M.7.

Table M.7: List of antibodies used for ChiP.

Target protein	Antibody	Species
H3K4me2	Millipore 07-030	Rabbit
H3K4me3	Millipore 07-473	Rabbit
H3K9Ac	Cell Signaling 9671	Rabbit
H3K9me2	Millipore 07-441	Rabbit
H3K9me3	Abcam 8898	Rabbit
H4K20me3	Millipore 07-463	Rabbit
H3K27me3	Millipore 07-449	Rabbit
WT1	Santa Cruz, sc-192	Rabbit
DNMT1	Abcam, Ab13537	Mouse

After incubation, the antibody bound beads were isolated using the magnet and the supernatant was removed. In parallel, 3.5 U (150 µg) of sonicated chromatin was diluted in dilution buffer (SDS 0.1%, Triton X-100 1.1%, EDTA 1.2 mM, NaCl 165 mM, Tris-HCl 16.7 mM pH=8.1) with 25 µl of beads for each antibody and incubated overnight at 4ºC with rotation. The immune-Dynabeads complexes were isolated using the magnet (DynaMag, Invitrogen) and combined with the sonicated chromatin incubated at 4ºC in a rotating wheel for two hours. Samples were washed twice with 1 ml of low salt buffer (Tris-HCl 50 mM pH=8, NaCl 150 mM, SDS 0.1%, NP-40 1%, EDTA 1 mM, Sodium deoxycholate 0.5%), twice in 1 ml of high salt buffer (Tris-HCl 50 mM pH=8, NaCl 500 mM, SDS 0.1%, NP-40 1%, EDTA 1 mM, Sodium deoxycholate

0.5%), twice with 1 ml of LiCl buffer (Tris-HCl 50 mM pH=8, LiCl 250 mM, SDS 0.1%, NP-40 1%, EDTA 1 mM, Sodium deoxycholate 0.5%) and finally twice with TE (Tris-HCl 10 mM pH=8, 0.25 mM EDTA). The samples were eluted in a 100 µl of elution buffer and incubated at 65ºC in a thermomixer to de-crosslinking.

4.7.4. DNA ISOLATION AND PURIFICATION.

Input and IPs DNA were purified using the QIAquick PCR purification Kit (Qiagen). Briefly, binding buffer was added to each sample, mixed and transferred into a spin column. Samples were centrifuged at 13,000 rpm for 30 seconds and the flow-through was discarded. Samples were washed in buffer and centrifuged at 13,000 rpm for 30 seconds. This step was repeated twice. Columns were transferred into a 1.5 ml microcentrifuge tube and 60 µl of elution buffer were added. Columns were centrifuged at 13,000 rpm for 1 minute and elution buffer containing the DNA was recuperated and used immediately for PCR or kept at -20ºC.

4.7.5. qPCR TO AMPLIFY THE X-CHIP IMMUNOPRECIPITATES.

qPCR was performed using SYBR green (Applied Biosystems) to detect DNA in chromatin IPs and input samples. The primers used are listed in the appendix. qPCR reactions were performed in a 7900HT Fast Real time PCR system Applied Biosystems. Reactions were run in triplicate, in a total volume of 11 µl with 1 µl of DNA, 3.5 µl of H_20, 5.5 µl of SYBR green master mix, and 0,5 µl of 50 ng of each primer. Cycling conditions were a denaturation of 95ºC for 10 seconds followed by 40 cycles of 95ºC for 15 seconds and 60ºC for 1 minute. The amount of DNA present in the IP fractions was determined as % of input and compared to negative controls.

4.8. DNA 5-HYDROXYMETHYLATION DETECTION.

DNA 5'-hydroxymethylation (5hmC) detection was performed by two different techniques: meDIP with a specific 5-hydroxymethylcytosine antibody and an enzymatic based reaction utilising a highly specific 5hmC Glucosyltransferase enzyme that tags 5hmC with a glucose moiety but not 5mC.

4.8.1. MeDIP ANALYSIS.

MeDIP (Diagenode) precipitation of 5hmC tag DNA was preformed according to manufactures' protocol. Briefly, genomic DNA was extracted using the phenol/chloroform method as previously described, followed by DNA sonication to acquire fragments of 300-500 bp. 30 µl of IP incubation mix (24 µl of buffer A, 4.5 µl of buffer B and 1.5 µl of positive control), and 45 µl of H_20 were added to the shredded DNA at a concentration of 0.1 µg/µl. Therefore, the total volume per IP was 75 µl. In parallel, an input sample was added. Samples were incubated at 95ºC for three minutes, and then chilled on ice. Twenty µl of IP blocked beads were added to the single stranded DNA, as well as 5 µl of the diluted antibody to the IP samples (0.3 µl, 0.6 µl and 2.10 µl of H_20). IP samples were then placed on a rotating wheel over night at 4ºC. Then, the 5hmC DNA/antibody complexes were washed with 1ml of cold buffer 2, buffer 3 and twice with buffer 4. DNA was purified using the QIAquick PCR purification column according to the manufactures' instructions. Samples were analyzed by qPCR as described previously and the amount of 5hmC was determined by comparing the amount of immunoprecipitated DNA after normalization to the input DNA: %(5-hydroxyDNA-IP/Input) =2^ [(Ct (input)-Ct (5-HydroxyDNA-IP)] x 100 with a positive commercially available control sample.

4.8.2. ENZYMATIC GLUCOSYLATION REACTION.

In order to determine the presence of 5hmC, three reactions were setup in parallel using the Quest 5hmC Detection Kit™ (Zymo Research) (Figure M.6). First, a positive glucosylation reaction which consist in 5 µl of the Quest 5hmC positive control DNA [50 ng] and 400 ng of the test sample being mixed with 5 µl of 10X 5hmC GT reaction buffer, 5 µl 10X UDPG [1 mM], 2 µl of 5hmC GT Enzyme (2 U/µl) to a final volume of 50 µl. The negative glucosylation reaction using 50 ng of negative control DNA was incubated with the above mix without the 5hmC GT enzyme in a volume of 50 µl. All reactions were incubated at 37ºC for two hours. Secondly 30 U of *Msp*I enzyme were added directly to each of the reactions and incubated for four hours at 37ºC. And last, after digestion, DNA was cleaned-up using the supplied spin columns. Following elution, DNA samples were analyzed using qPCR.

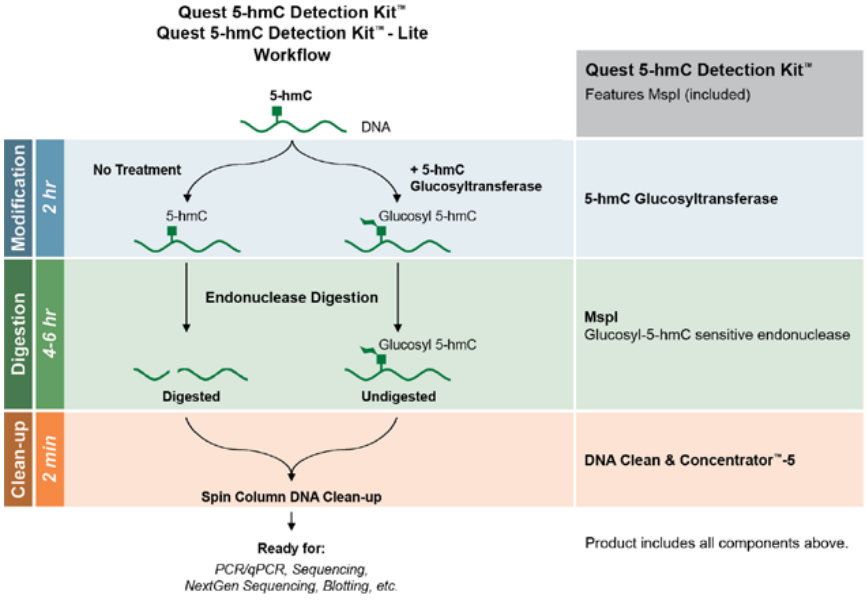


Figure M.6: Overview of the 5hmC detection workflow.

4.9. DRUG TREATMENT WITH DEMETHYLATING AGENTS.

4.9.1. DECITABINE TREATMENT ON CELL LINES.

10,000 cells were seeded in 96 well plates. After being left to settle for 24 hours, cells were treated for 72 hours with various concentrations of Decitabine (Sigma-Aldrich) ranging from 0 μM to 5 μM. Drug sensitivity was determined using the CellTiter 96 Aqueous One Solution Cell Proliferation assay (Promega). This assay is performed by the addition of a premixed dye solution to culture for 4 hours of incubation, in which living cells convert the tetrazolium component of the dye solution into a colored formazan product. Data is then collected reading the plate at 450nm in a multi-well plate reader (Glomax multi detection system, Promega). The IC_{50} value (half maximal inhibitory concentration) represents the concentration of a compound where 50% of the maximal inhibition is observed. This value was calculated by extrapolation from the obtained curves. Once IC_{50} was established, drug treatment was then repeated and cells were harvested for DNA extraction.

5. STATISTICAL ANALYSIS.

Data are expressed as mean ± SEM. Graphpad Prism software was used to calculate dose-response curves to determine the IC_{50} value, two-sided test was performed to compare methylation levels before and after Decitabine treatment, Spearman rho coefficient to correlate methylation values of bisulfite sequencing and pyrosequencing, and the Mann-Whitney test to evaluate the methylation values on the primary tumor samples. A value of $p<0.05$ was considered statistically significant (* $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$).

OBJECTIVES AND HYPOTHESIS

OBJECTIVES.

The main objective of this project was to fully characterize the epigenetic deregulation of the *WT1* and *AWT1* transcripts in myeloid origin malignancies, acute myeloid leukemia and chronic myeloid leukemia, and to determine whether aberrant epigenetic profiles could be markers for disease diagnosis and prognosis.

The objectives of the work presented here were to:

1- Assess the imprinting state of the *WT1* gene in normal tissues.
2- Determine the epigenetic status of *WT1* and *AWT1* in a large cohort of hematological malignancies.
3- Decipher the mechanism resulting in aberrant *WT1* and *AWT1* expression in hematological malignancies.

HYPOTHESIS.

In hematological malignancies very little is known about the deregulation of imprinted transcripts. The *WT1* gene has been reported to be imprinted in blood and kidney. We hypothesize that the overexpression of *WT1* transcripts, that is routinely used as a molecular marker for minimal residue of disease, could result from biallelic expression due to LOI. Furthermore, we hypothesize that abnormal epigenetics profiles, including DNA methylation and histone modifications, could contribute to the deregulation of the *WT1* in leukemia, and if this occurs frequently, may be used as a molecular marker for various disease states and outcomes.

RESULTS

1.*WT1* AND THE REPORTED IMPRINTED TRANSCRIPTS *AWT1* AND *WT1-AS.*

Genomic imprinting is the monoallelic parent-of-origin specific expression orchestrated by allelic methylation inherited from the gametes. To date, over 64 human genes are subjected to genomic imprinting, including the *WT1* gene (igc.otago.ac.nz/). The genomic organization of the *WT1* gene on chromosome 11p13 has been previously shown to contain various promoters resulting in transcripts with alternative first exon (Figure R.1.1). The promoter region for *WT1* is embedded within a defined CpG island of 2.4Kb, from which a full-length and non-imprinted transcript arises. Approximately 4kb downstream in intron 1, there is a second alternative promoter region embedded within a CpG island of 500 bp, giving rise to the *AWT1* imprinted isoform (Dallosso et al., 2004). The *WT1* loci, also contains a non-coding antisense transcript, *WT1-AS* that originates from the opposite DNA strand, with a promoter within intron 1 that also incorporates the *WT1* exon 1 and continues upstream (Malik et al., 1995). Adjacent to this promoter is the region of proposed allele specific DNA methylation, the *WT1-ARR* (Malik et al., 2000). Previous characterization of the methylation of the *WT1-ARR* is restricted to studies of adult kidney, in which the maternally methylated allele acts as a methylation-dependent bidirectional silencer, co-regulating the paternal expression of the *WT1-AS* and *AWT1* transcripts. In Wilms' tumor, a nephroblastoma, disruption to normal *WT1* expression has been associated to the initiation of the disease. Malik and colleagues over a decade ago (Malik et al., 2000), reported the loss of methylation of the *WT1-ARR*, which they proposed, leads to the concomitant biallelic expression of *AWT1* and *WT1-AS* in Wilms' tumor. These reports of imprinted epigenetic regulation of the *WT1* gene in normal kidney, and the subsequent LOI in Wilms' tumor, lead us to hypothesize that similar mechanisms could also occurred in normal leukocytes, and that LOI resulting in biallelic expression could explain the overexpression observed in leukemia.

Figure R.1.1: Genomic organization of the *WT1* gene. The green boxes represent the CpG islands of *WT1* (CpG 188) and *AWT1* (CpG 102). Adjacent to the *WT1* CpG island is the described allele specific DNA methylation region, *WT1-ARR.* In blue are the paternal transcripts, *AWT1* and the non-coding *WT1-AS*, which are described to be co-regulated by the DMR (*WT1-ARR)*.

1.1. METHYLATION ANALYSIS OF THE CpG ISLAND PROMOTERS OF *WT1* AND *AWT1,* AND THE *WT1-ARR* IN NORMAL TISSUES.

The methylation profiles of the *WT1* and *AWT1* CpG island promoters, and the *WT1-ARR* described by Malik et al., 2000, were determined using PCR on bisulfite converted DNA and sequencing. Using this technique, eight CpGs dinucleotides were evaluated within the *WT1* promoter, five CpGs dinucleotides in the *AWT1* promoter and eight CpGs in the differential methylated *WT1-ARR* region. Of the eight CpGs targeting the *WT1-ARR*, one corresponds to the *Hpa*II restriction enzyme site (CCGG) utilized in the original Southern hybridization approach used to identify this potential regulatory region (Malik et al., 2000). The methylation status of both promoters and the *WT1-ARR* were assessed in various normal tissues: fetal kidney, adult kidney, podocytes, brain, placenta, leukocytes and various immunosorted hematopoietic cells (CD15+ neutrophils, CD19+ B-cells and CD3+ T-cells). The first tissues evaluated were fetal and adult kidney, which revealed that both CpG islands are completely unmethylated at both developmental ages (Figure R.1.2). Interestingly, when performing allelic bisulfite analysis on the *WT1-ARR*

region in fetal kidney, both alleles in rs11031781 heterozygous samples were lowly methylated. This is similar to the previously published results, reflecting the temporal and tissue-specific nature of the *WT1-ARR* methylation (Malik et al., 2000). In adult kidney, *the WT1-ARR* region showed an increase of methylation, but subsequent allelic bisulfite analysis revealed that this methylation was mosaic and equally present on both alleles. To ensure we had not missed allelic methylation, we also evaluated this region in podocytes cells, a specific cell-type found in the Bowman's capsule in the kidney, which *WT1* has been reported to be expressed (Morrison et al., 2008). This region was fully methylated, indicating a lack of an allele specific methylation region in this specific cell-type. Therefore, using a more targeted and informative technique, we could not verify that the region described by Malik and colleagues over a decade ago was indeed an allelic DMR in kidney samples. Therefore the DMR profile originally reported in adult kidney probably reflexes the temporal mosaic methylation state we observed (20% increasing to 40%).

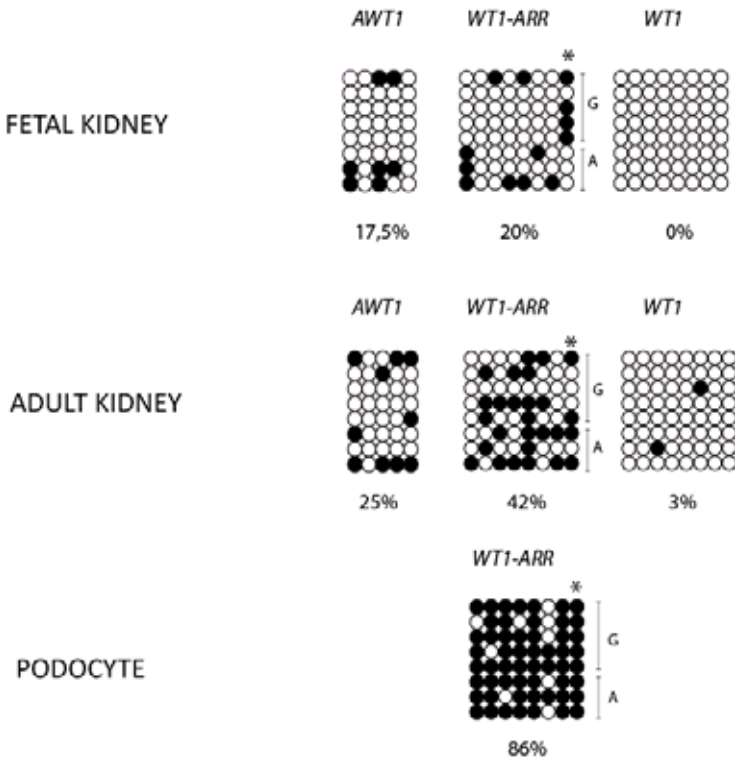


Figure R.1.2: Bisulfite sequencing of *AWT1*, *WT1-ARR* and *WT1* in fetal and adult kidney. In podocyte cell line bisulfite sequencing only on the *WT1-ARR.* The G and A refer to the SNP rs11031781 from different alleles. In asterisk is the CpG analyzed by Southern blot (Malik et al.,2000).

As outlined in the introduction, once a DMR is established at fertilization by uniting gametes, it is maintained on opposing alleles throughout somatic tissues withstanding the preimplantation developmental reprogramming, and is independent of expression levels (Woodfine et al., 2011). To confirm that the *WT1-ARR* is not differentially methylated in additional embryonic and placental tissues, we performed bisulfite PCR analysis on DNA samples heterozygous for the SNP rs11031781 that would allow us to distinguish alleles. As observed in kidney, we did not detect the presence of differential methylation in any tissue, including fetal and adult brain, placenta, leukocytes or in immunosorted cells (neutrophils, B-cells and T-cells). Consistent with the majority of CpG

islands in the genome, the *WT1* and *AWT1* CpG island promoters were hypomethylated in all tissues analyzed (Figure R.1.3).

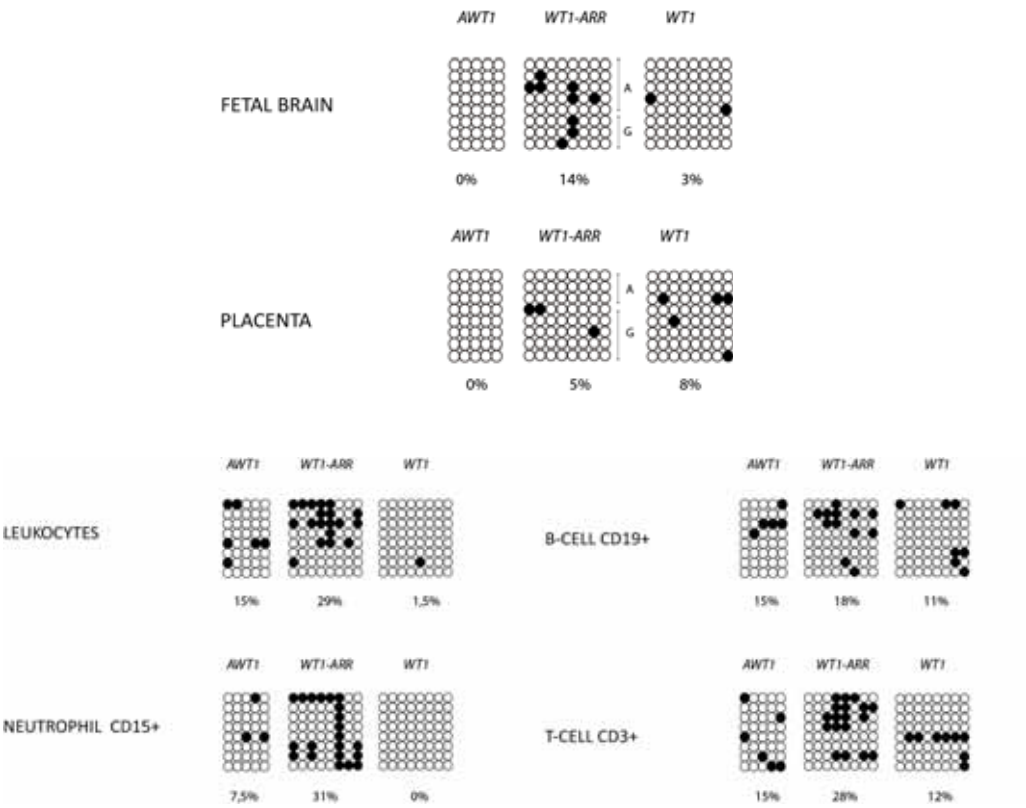


Figure R.1.3: Bisulfite sequencing of *AWT1*, *WT1-ARR* and *WT1* in various human tissues.

1.2. METHYLATION ANALYSIS OF THE CpG ISLAND PROMOTERS OF *WT1* AND *AWT1,* AND THE *WT1-ARR* IN NORMAL TISSUES USING THE ILLUMINA INFINIUM HUMAN METHYALTION 450K ARRAY.

Genome-wide methylation analysis is now possible at high-resolution using the Infinium Human Methylation 450K BeadChip array, which is a high-throughput quantitative technique that covers over more than 450,000 methylation sites throughout the genome. The targeted sites are equally distributed across promoters, gene bodies and intragenic regions (Sandoval et al., 2011). A

genome-wide methylation analysis was performed on a wide range of normal tissues, including fetal and adult kidney in our laboratory, with special emphasis on the *WT1* locus. A total of 43 probes spanned the ~50 kb domain, with 19 probes concentrated in the promoter regions. This analysis corroborated our bisulfite PCR findings, with the *WT1* and *AWT1* CpG islands being uniformly hypomethylated in all tissues, and that the *WT1-ARR* is a mosaically methylated interval. A subsequent comparison of normal adult leukocytes with reciprocal genome-wide UPD (uniparental disomy) samples failed to detect allelic methylation (Figure R.1.4), despite accurately identifying the differentially methylated region associated with the *H19* gene also on chromosome 11 (data not shown).

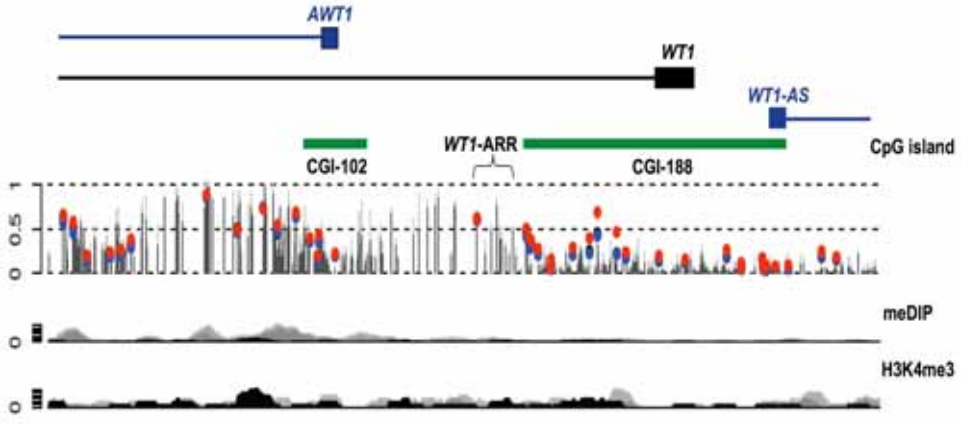


Figure R.1.4: Red dots represent the methylation of Illumina Infinium probes in the genome mUPD (maternal UPD), blue dots represent the pUPD (paternal UPD) and black dots are normal leukocyte sample (most data points are hidden by overlapping mUPD and pUPD samples). The bars reflect the methylation profile throughout the interval as determined by whole genome bisulfite sequencing in normal leukocytes. The lower panels show the meDIP-seq and H3K4me3 ChIP-seq data for leukocytes.

1.3. TISSUE-SPECIFIC EXPRESSION OF *WT1*, *AWT1* AND *WT1-AS* IN FETAL TISSUES.

qRT-PCR was used to evaluate transcription levels of *WT1*, the alternative transcript *AWT1* and the antisense *WT1-AS* in human tissues. The transcription levels were assessed in a panel of normal fetal tissue RNA (Agilent technologies), which contained bone marrow, brain, kidney, heart, liver, lung, lymphocyte and placenta. The qRT-PCR primers were specifically design in regions capable of discriminating between the *WT1* and *AWT1* transcripts, since the *AWT1* transcript has an alternative first exon not included in the full length *WT1* transcript. Moreover, qRT-PCR primers were designed to cross exons to assure that no amplification could occur from low level contaminating DNA (Figure R.1.5). The *WT1* gene presents many different isoforms, with alternative splicing of exons 5 and 9. By specifically targeting the initial exons of each transcript the resulting amplifications will reflect all splicing events, since our interest was not to quantify each single splice form but rather the promoter output.

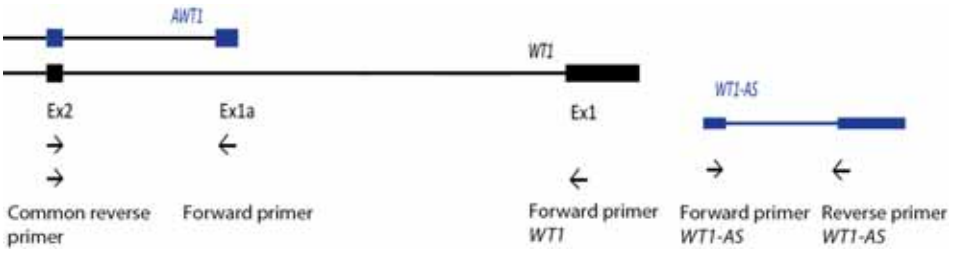


Figure R.1.5: Schematic representation of the primer sets for the transcripts *WT1*, *AWT1* and the *WT1-AS*.

The optimized qRT-PCRs generated a 125bp *WT1* product, a 109bp product for *AWT1* and a 123bp product specific for the antisense transcript. Expression values were based on the amplified product reaching a gene expression threshold following a number of PCR cycles, normalized to the expression of an

endogenous control (*β-ACTIN*). The ΔCT value was then used as a measure of relative expression. All experiments were preformed in triplicate with the mean ± SEM values. From the panel of fetal tissues evaluated, *WT1, AWT1* and *WT1-AS* transcripts were only present in kidney and not detectable in any other fetal tissue (Figure R.1.6). These results are consistent with those obtained by other groups, who assessed a different fetal tissue panel, and concluded that *WT1* and *AWT1* are predominantly expressed in kidney (Hossain et al., 2006). Unfortunately all kidney samples in our collection were not informative for polymorphisms that would allow us to determine allelic expression status.

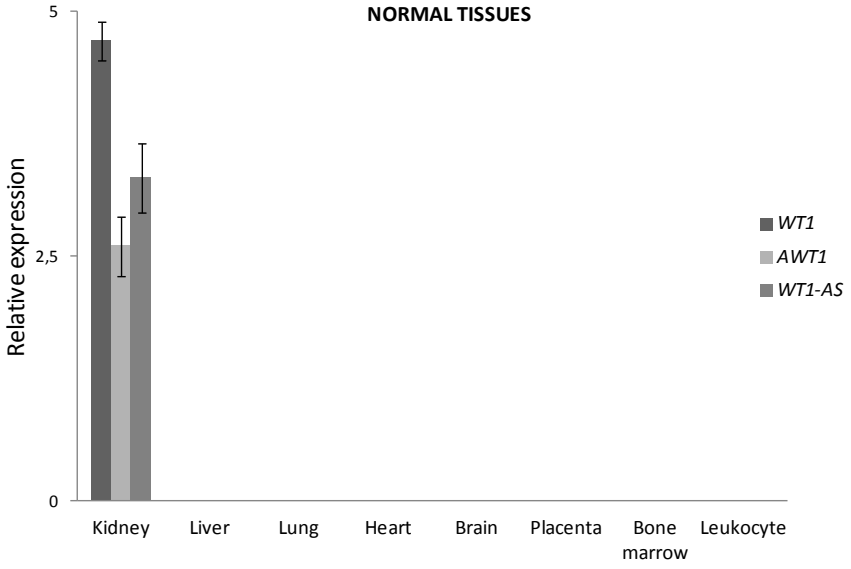


Figure R.1.6: Transcription levels assessed on a panel of eight different normal fetal tissues.

1.4. EXPRESSION LEVELS OF *WT1*, *AWT1* AND *WT1-AS* IN HUMAN BLOOD SAMPLES.

qRT-PCR was also performed on bone morrow, immunosorted cells CD19+, CD3+ and leukocytes to evaluate transcription levels of *WT1*, *AWT1* and *WT1-AS.* No expression was detected in any of these normal human blood samples. Previous studies also show the identical results, with the exception that *WT1* is detectable in CD34+ cells, both uncommitted dormant CD34+CD38-, and lineage-committed proliferative CD34+CD38+ of the bone marrow (Hosen et al., 2002). Unfortunately we were unable to confirm the expression in CD34+ as these cells are present at very low levels in bone marrow (0.5-3% of cells) and cord blood (0.1-0.5% of cells).

2. DNA METHYLATION PROFILING AND *WT1* RE-EXPRESSION IN LEUKEMIA CELL LINES.

WT1 has been reported to be expressed in various leukemia types (Inoue et al., 1997) as well as contributing to the carcinogenesis of the disease. The expression of this gene is also used as a molecular marker for minimal residual disease since it is not expressed in normal peripheral blood (Inoue et al., 1994). The mechanisms giving rise to the transcription of this gene is still elusive. At first, since this gene was described to be imprinted and regulated by the *WT1-ARR* region, we postulated that re-expression of this gene could in part be attributed to loss-of-imprinting. However a detailed analysis of the methylation throughout the *WT1* domain failed to identify any regions of allelic methylation, making it highly unlikely that this gene is in fact imprinted. Therefore, other epigenetic mechanisms could account for aberrant expression observed in leukemia. Herein, we first evaluated levels of expression of various transcripts in a panel of well-characterized leukemia and lymphoma cell lines, and subsequently methylation status and histone tail modification profile of the promoter regions.

2.1. EXPRESSION LEVELS OF *WT1*, *AWT1* AND *WT1-AS* IN LEUKEMIA AND LYMPHOMA CELL LINES.

qRT-PCR was performed on a wide range of leukemia and lymphoma cell lines to evaluate the transcription levels of the *WT1*, *AWT1* and the non-coding antisense transcript *WT1-AS*. For the evaluation of these transcripts in the cell lines, the same quantification method and PCR primer pairs were used as described in section 1.3. The leukemia cell lines were classified into three different categories (myeloid, B-cell and T-cell) according to the cell origin. It is very important to take into consideration that even though cell lines are separated into different categories, each single line has its own genetic background as well as its own non-random chromosomal translocations. The

myeloid origin cell lines evaluated included MV4;11, SKNO-I, MOLM 13, KASUMI, KG1a, HL-60, NB4, K562, HEL-R and UOCMII. In all of these cell lines the *WT1* and *AWT1* transcripts were readily detectable, with an expression value similar to kindey, in which *WT1* is known to be functional. These two transcripts seem to be co-expressed, with a correlation of R^2= 0.90, indicating that they are likely to be co-regulated. The non-coding antisense transcript *WT1-AS* was also expressed in most myeloid cell lines. Transcription levels are shown in figure R.2.1.

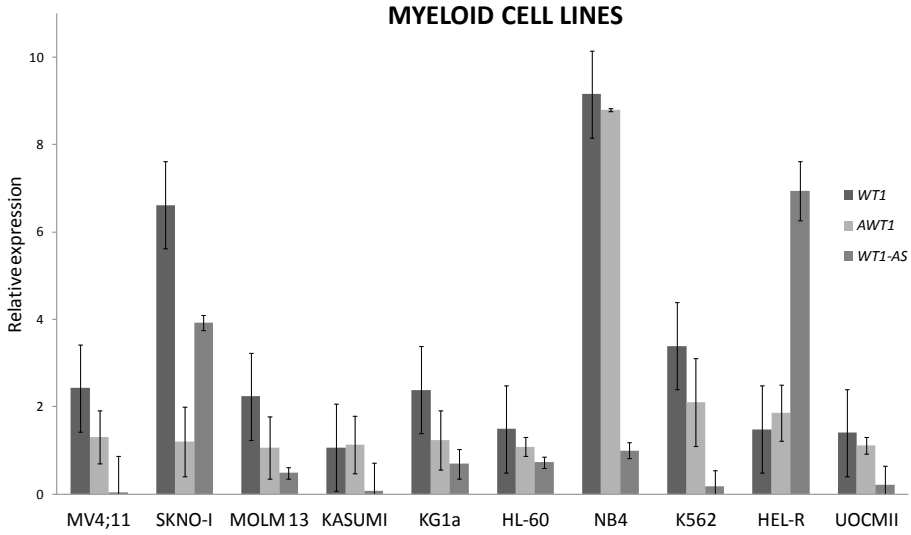


Figure R.2.1: Transcription levels of *WT1, AWT1* and *WT1-AS* in a panel of myeloid cell lines.

We also evaluated the expression levels in 9 leukemia cell lines of B-cell origin, including KOPN-8, REH, SD-1, SEM, RS4;11, TOM-I, NALM-20, TANOUE and JVM2 (Figure R.2.2). The expression levels of the *WT1* and *AWT1* transcript were not uniformly present in all of these with REH, SEM, RS4;11 showing low expression, while NALM-20, a cell line with a t(9;22) translocation and biphenotypic B-cell and myeloid cell characteristics (Matsuo et al., 1991) had transcript levels similar to the myeloid cell lines.

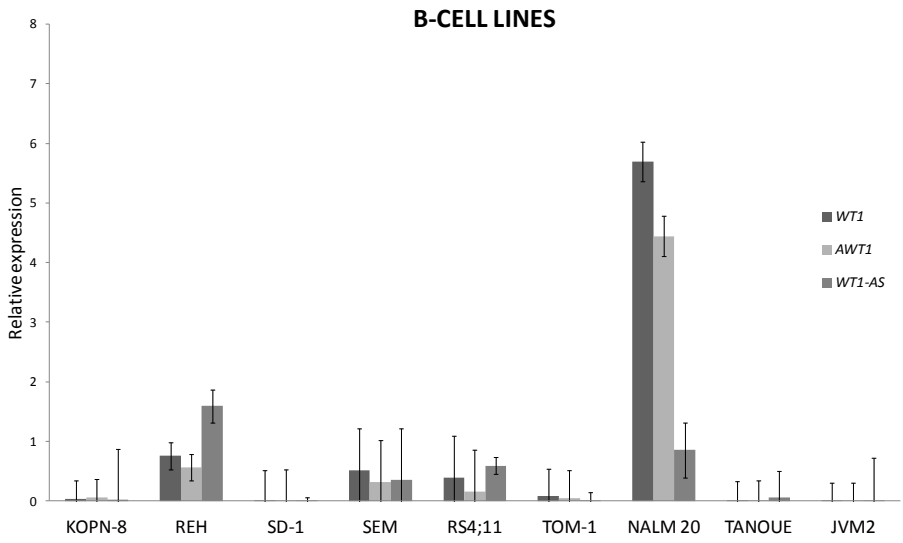


Figure R.2.2: Transcription levels of *WT1, AWT1* and *WT1-AS* in a panel of B-cell lines.

The T-cell origin leukemia cell lines evaluated in this study included MOLT 16, MOLT 4 and JURKAT (Figure R.2.3). Of the three cell lines, which showed varying degrees of expression, MOLT 4 consistently showed the highest levels of *WT1*, *AWT1* and *WT1-AS*.

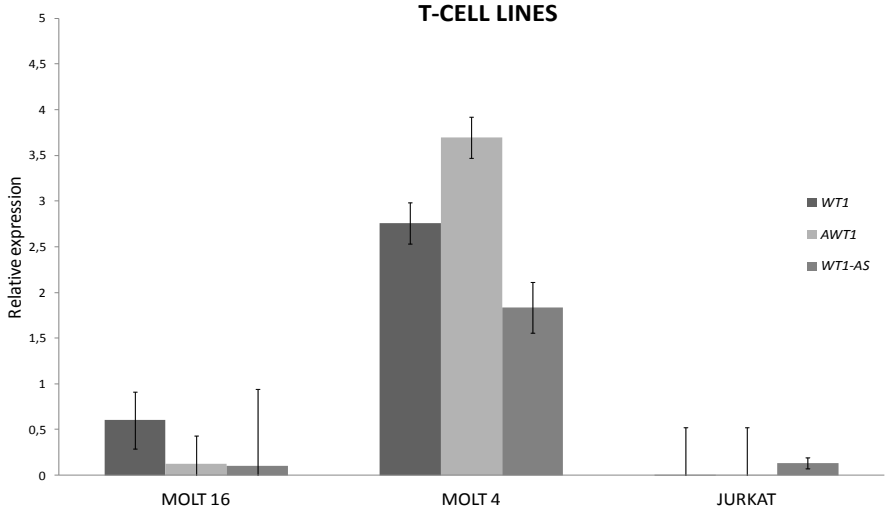


Figure R.2.3: Transcription levels of *WT1, AWT1* and *WT1-AS* in a panel of T-cell lines.

Finally we assessed the expression levels in a panel of lymphoma cell lines, including 3 Hodgkin's lymphomas lines L1236, KMH2 and HDLM2, one Burkitt's lymphoma line, RAJI, the Mantle cells GRANTA-519 and the histiocytic lymphoma (rare non-Hodgkin lymphoma) U937 cell line. None of these cell lines showed expression for any of the three transcripts.

2.2. DNA METHYLATION ANALYSIS OF CpG ISLAND PROMOTERS OF *WT1* AND *AWT1* AND THE *WT1-ARR* IN LEUKEMIA AND LYMPHOMA CELL LINES.

Methylation analysis of the entire panel of leukemia and lymphoma cell lines was performed on both CpG island promoters, *WT1* and *AWT1,* and on the intervening *WT1-ARR* region using the previously optimized bisulfite PCR with sequencing of individually cloned DNA molecules. In the myeloid cell lines (Figure R.2.4) the *WT1* CpG island promoter was unmethylated, except for the HL-60 cell line that was completely hypermethylated. On the contrary, the *AWT1* CpG island promoter was aberrantly hypermethylated in all of the myeloid cell lines evaluated, despite being highly expressed. The *WT1-ARR* region showed varying amounts of methylation, being completely unmethylated in NB4 and K562, partially methylated in MOLM 13 and HEL-R, and hypermethylated in the remainder of cell lines.

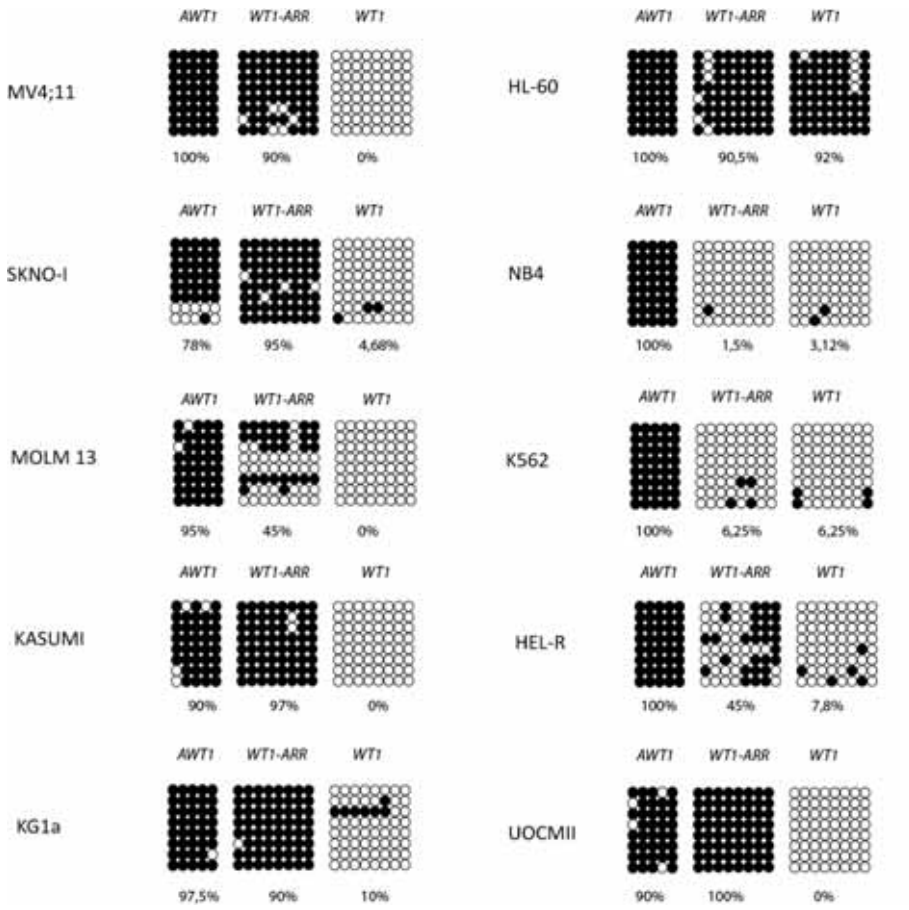


Figure R.2.4: Methylation status of the *WT1-ARR* and the CpG island promoters *WT1* and *AWT1* in myeloid cell lines.

The methylation status of the 3 regions was also evaluated in the 9 B-cell leukemia cell lines: KOPN-8, REH, SD-1, SEM, RS4;11, TOM-I, NALM-20, TANOUE and JVM2 (Figure R.2.5). The methylation status of the *WT1* CpG island promoter in most cell lines was completely unmethylated (less than 20% methylated), except TOM-I, KOPN-8 and TANOUE. The *AWT1* CpG island promoter was completely hypermethylated in all samples, except JVM2 that was mosaically hypermethylated to 60%. The methylation at the *WT1-ARR* region was very variable among the different cell lines evaluated, from hardly no methylation, 4% in NALM-20, to more than 80% methylated in REH and RS4;11.

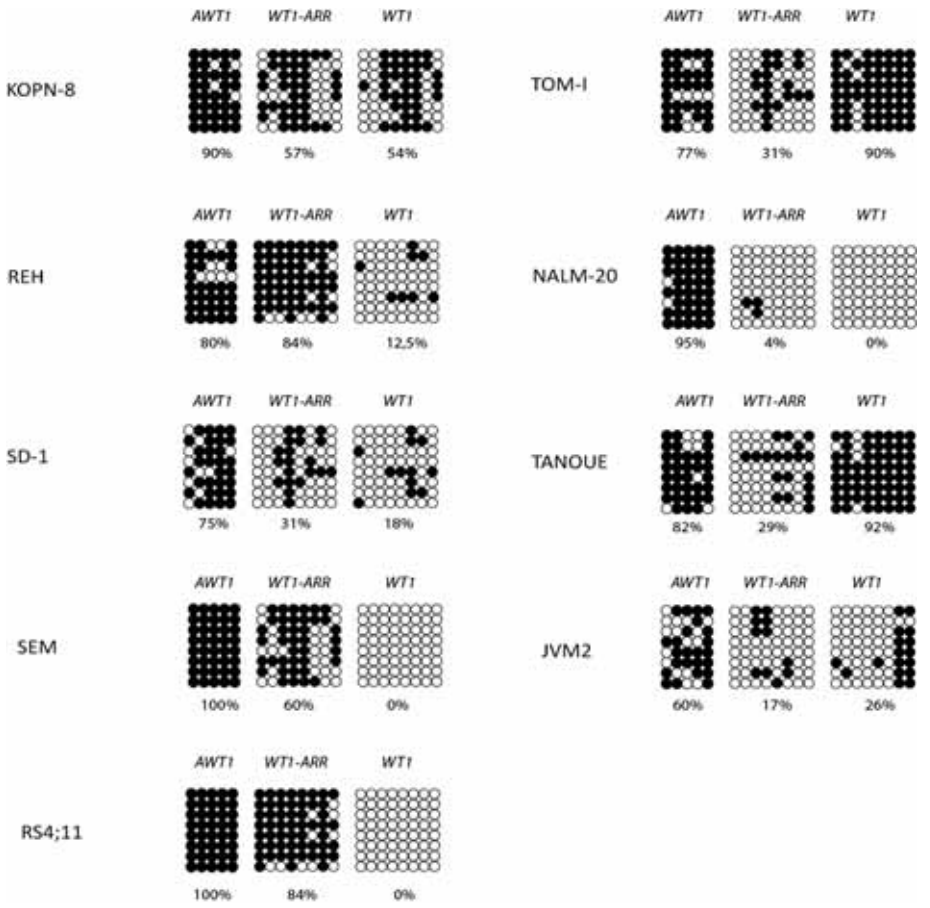


Figure R.2.5: Methylation status of the *WT1-ARR* and the CpG island promoters *WT1* and *AWT1* in B-cell lines.

The T-cell leukemia cell lines MOLT 16, MOLT 4 and JURKAT were more variable (Figure R.2.6). The methylation status of the *WT1* CpG island promoter was erratic in all of these three cell lines, from completely unmethylated in MOLT 16, to 26% hemimethylation in JURKAT to completely methylated in MOLT 4. As for the *AWT1* CpG island promoter, all 3 cell lines were from 45% in JURKAT cells to completely hypermethylated in MOLT 16 cells and MOLT 4. The *WT1-ARR* region, was hypomethylated in the 3 cell lines. In figure R.2.6 is the representation of the methylation values of the T-cell origin cell lines.

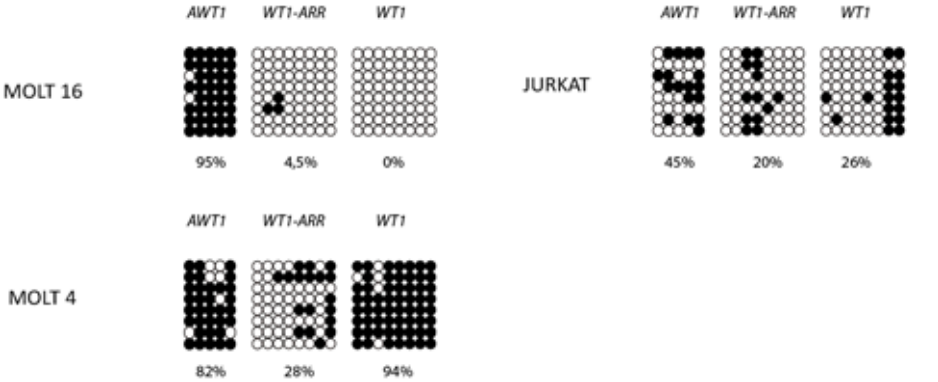


Figure R.2.6: Methylation status of the *WT1-ARR* and the CpG island promoters *WT1* and *AWT1* in T-cell lines.

In all the lymphoma cell lines both promoters were hypermethylated, with all cell lines presenting with a mosaic methylation profile at the *WT1-ARR* (Figure R.2.7).

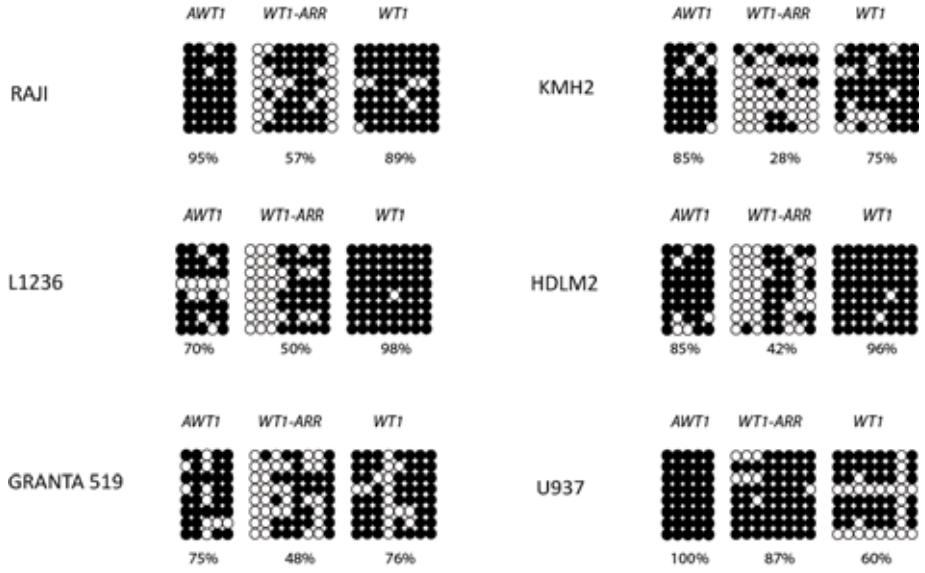


Figure R.2.7: Methylation status of the *WT1-ARR* and the CpG island promoters *WT1* and *AWT1* in lymphoma cell lines.

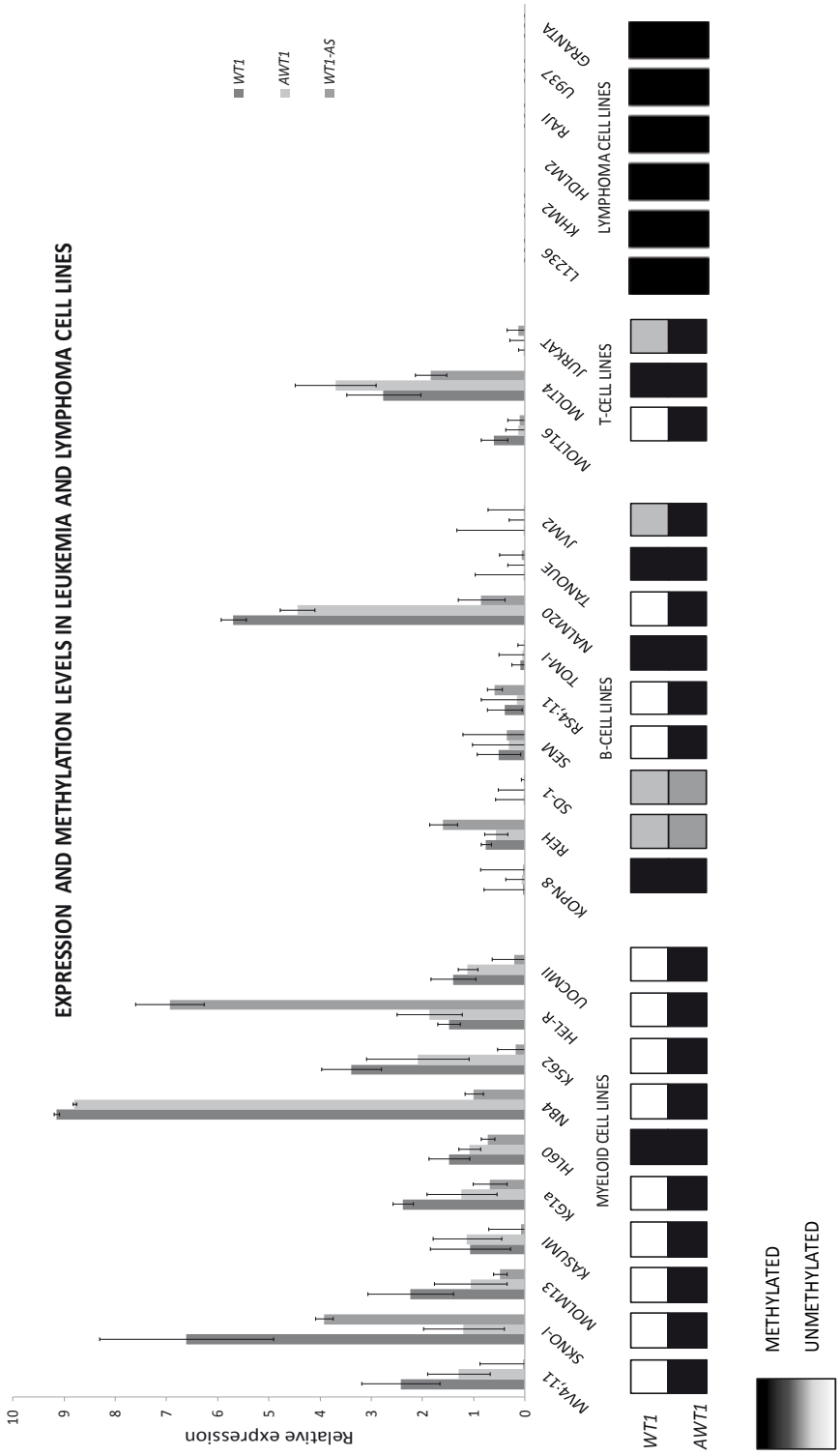
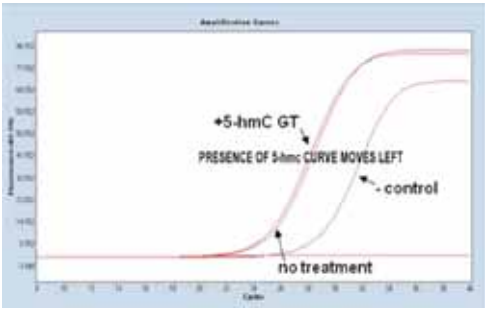


Figure R.2.8: Summary of expression and methylation data for the 28 leukemia and lymphoma cell lines

2.3. 5-HYDROXYMETHYLCYTOSINE (5hmC) IN MYELOID ORIGIN CANCER CELL LINES.

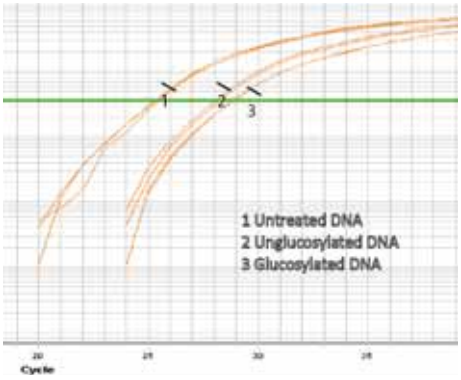
5-hydroxymethylation (5hmC) is a new discovered epigenetic modification, known as the 6th base of the DNA. Its precise role is still elusive, but it has been suggested to control gene expression since it is enriched within moderately CpG rich promoters and actively transcribed gene bodies (Wu et al., 2011). Unfortunately the deamination of unmethylated cytosines that occurs during conventional bisulfite reactions does not distinguish between 5mC and 5hmC. Therefore, in order to detect the enrichment of 5hmC specific techniques are needed. To determine if the methylation we observed at the *AWT1* promoter actually reflects 5hmC rather than conventional 5mC, we selected the myeloid cell lines, K562, KG1a and NB4 for further analysis due to their high transcription levels and *AWT1* hypermethylation in relation to the unmethylated adjacent *WT1* promoter. Herein, we used two different approaches: a 5hmC glucosyltransferase enzyme method and meDIP (methylated DNA immunoprecipitation) utilizing a specific antibody raised against 5hmC. The first technique relies on the highly specific 5hmC glucosyltransferase activity that tags a glucose moiety to 5hmC but not to 5mC. After glucosylation of 5hmC the DNA is refractory to digestion with a $^{Glucosyl\text{-}5hm}$C Sensitive Restriction Endonucleases (GSRE) enzyme (*Msp*I-CCGG), which allows for the differentiation between 5hmC and 5mC. Subsequently, the treated DNA is analyzed by qPCR, where a CT value similar to undigested DNA represents high 5hmC and a lowering in CT values reflect the lack of 5hmC. The theoretical amplification expected from the Quest 5hmC Detection Kit, Zymo research, is shown below (Figure R.2.9).



*Msp*I Digestion qPCR	
Sample	**CT Value**
No Treatment	25,1
Negative control (unglucosylated)	28,5
Positive 5hmC glucosyltransferase	24,9

Figure R.2.9: Theoretical amplification of the detection of 5hmC by qPCR.

In the three AML cell lines, K562, KG1a and NB4, no 5hmC enrichment was detected using a qPCR designed to incorporate five *Msp*I sites that could distinguish the glucosylation status of the CpGs dinucleotides. All *AWT1* amplification curves had a CT value equal to the negative digested control. As expected the *WT1* CpG island was also free of 5hmC consistent with the bisulfite analysis (Figure R.2.10).



*Msp*I Digestion qPCR	
Sample	**CT Value**
No Treatment	25,5
Negative control (unglucosylated)	28,4
Positive 5hmC Glucosyltransferase (actual sample)	29,2

Figure R.2.10: Example of a qPCR results obtained for K562 cell line.
The untreated sample represents a 5hmC control DNA. The unglucosylated DNA represents the undigested sample and the glucosylated DNA represents the interrogation site. In this case the glucosylated DNA curve is not between the untreated and the unglucosylated DNA indicating the lack of 5hmC.

Despite the use of a highly-specific T4 β-glucosyltransferase that adds a glucose to the hydroxyl group of 5hmC, the technique is limited to the number and location of GSRE enzyme restriction sites, of which *Msp*I was the most abundant within the *AWT1* region (there were only 1 *Csp6*I, 2 *Taq*I and no *Mbo*I sites present). To ensure that we had not missed any 5hmC within this interval, we used a second technique. 5hmC can be detected using meDIP with a 5hmC specific antibody. Aliquots of the same DNA extracted from K562, KG1a and NB4 were subjected to meDIP for both 5mC and 5hmC. As we see in figure R.2.11, *AWT1* is enriched in 5mC. After performing both techniques we can conclude that 5hmC is not present at the *AWT1* nor *WT1* promoters. These results indicate that *AWT1* is unique in that the transcript is highly expressed despite the presence of promoter hypermethylation.

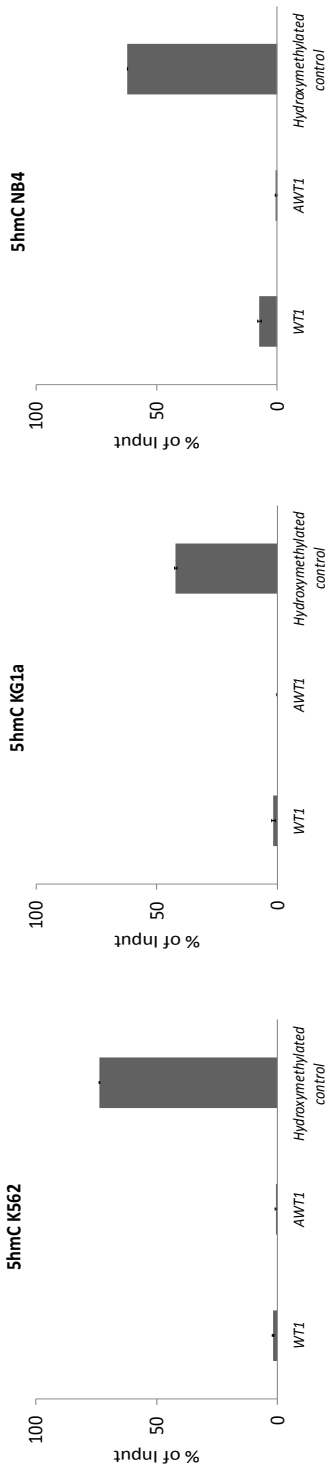


Figure R.2.11: The upper graphs represent the meDIP using 5hmC specific antibody. The lower graphs represent meDIP using 5mC antibody. Results show conclusively the lack of 5hmC in the *WT1* and *AWT1* CpG islands, and the presence of unmethylated *WT1* CpG island and a DNA.

2.4. TOTAL PROTEIN EXPRESSION IN LEUKEMIA AND LYMPHOMA CELL LINES. WT1 AND AWT1 LOCALIZATION : NUCLEAR OR CYTOPLASMATIC?

Western blot analysis was carried out on the same panel of leukemia and lymphoma cell lines to correlate transcription and protein levels, since transcription does not always necessarily imply protein production. Cell lines were immunobloted with an antibody directed against the C-terminal of the WT1 protein. The antibody specifically recognized the 52kDa, full length of the WT1 protein and the 32kDa of the truncated protein, the AWT1. Both isoforms were detected in the all the myeloid leukemia cell lines evaluated. In the B-cell line NALM-20 and in the T-cell MOLT 4, both isoforms were present. In the lymphoid cell lines that presented no transcription levels no protein was detected, as expected. Following is table R.1 with the protein values of WT1 and AWT1 in ratio to β-ACTIN. In all cases the WT1 protein was more readily detectable that the shorter AWT1 form.

Table R.1: WT1 and AWT1 protein quantification using ImageJ. Data represents protein levels as a ratio to the to β-ACTIN loading control.

CELL LINE	WT1	AWT1
MV4;11	1,65	0,5
MOLM 13	2,22	0,7
KG1a	5,2	1,25
HL-60	7,8	0,75
NB4	13	4,10
K562	7,15	5,02
HEL-R	1,5	1,05
NALM-20	5,52	3,2
MOLT 4	3,15	1,75

Afterwards, specific western blot separating the nucleus and cytoplasmatic compartment was performed, using an antibody that immunobloted the N-terminal of the WT1 protein. This experiment was carried out to determine the cellular localization of the cancer associated WT1 proteins. The cell lines evaluated were three myeloid cell lines (K562, NB4 and KG1a) and one T-cell line (MOLT 4) which all had abundant RNA and cellular WT1/AWT1 protein levels. The Western blot revealed that the protein is nuclear retained. We only detected one band of approximately 52kDa, presumably we did not detected the AWT1 protein due to the lack of antibody epitope recognitizion, as the AWT1 protein has a different N-terminus (Figure R.2.12).

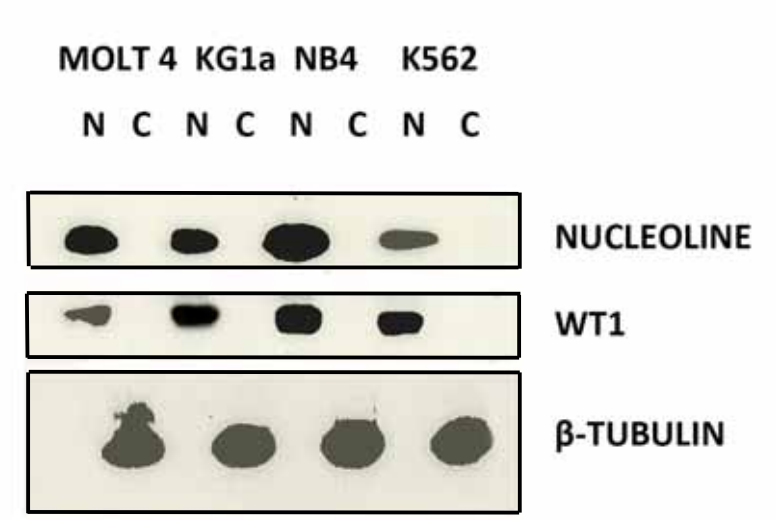


Figure R.2.12: Western blot on separated nuclear (N) and cytoplasmatic (C) cellular compartments. Nucleoline (100kDa) is a nuclear retained protein, whereas β-tubulin (55kDa) is cytoplasmatic. The western blots are deliberately over-exposed to ensure purity of fractionation.

2.5. ALLELIC EXPRESSION IN LEUKEMIA CELL LINES.

The overexpression of *WT1* transcripts in the leukemia cell lines allows for the allelic expression to be determined. Using primer pairs designed to amplify various SNPs identified in the UCSC sequence browser (GRC27/hg19- dbSNP 135) all cell lines expressing *WT1, AWT1* and *WT1-AS* were genotyped. Using RT-PCR primers that could distinguish each transcript, *WT1, AWT1* and *WT1-AS* were biallelically expressed in all heterozygous cell lines (Figure R.2.13).

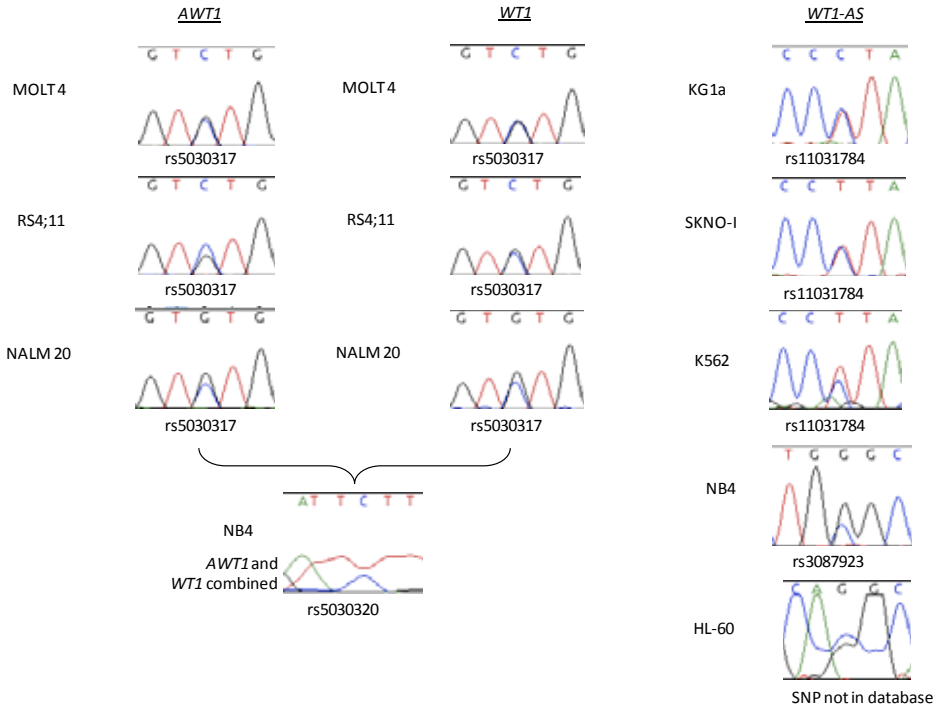


Figure R.2.13: Allelic expression of *WT1, AWT1* and *WT1-AS* in different leukemia cell lines.

2.6. *WT1* MUTATION SCREENING.

Mutation screening was performed on all exons of the *WT1* gene of all the leukemia and lymphoma cell lines. Specific primers were used to amplify and sequence these regions to screen for mutations. No mutations were detected in myeloid, B-cell or T-cell leukemia cell lines. However, in the lymphoma cell lines one mutation was detected. In the U937 cell line in exon 7 a non sense mutation G>A, p.R369*. This mutation was also corroborated in the Cancer Genome Project/Sanger database.

3. EVALUATION OF HISTONE TAIL MODIFICATIONS AT THE *WT1* AND *AWT1* PROMOTER REGIONS IN LEUKEMIA CELL LINES.

In order to determine the specific chromatin landscape associated with *WT1*/*AWT1* expression despite extensive DNA hypermethylation, we evaluated the histone tail modifications at both promoters using chromatin immunoprecipitation (ChIP) with high-specific antibodies against 7 histone tail modifications in 3 myeloid origin cancer cell lines. Both, active and repressive histone marks were evaluated. The transcriptionally permissive histone marks studies included H3K4me2, H3K4me3 and H3K9ac, and the repressive marks H3K9me2, H3K9me3, H3K27me3 and H3K20me3. Primers for the qPCR targeting *AWT1* and *WT1* were designed to overlap the precise regions that were evaluated by bisulfite PCR. For the control regions, we designed amplicons across the *GAPDH* promoter which is enriched for permissive/active histone marks, and the centromeric alpha satellite repeat region on chromosome 1, which is enriched for repressive histone modifications. All immunoprecipitated DNA samples were analyzed in triplicate by qPCR and the data expressed as percentage of precipitation relative to input chromatin.

3.1. THE HISTONE LANDSCAPE AT THE *WT1* LOCI IN NORMAL LEUKOCYTES.

Before performing the experiment on the leukemia cell lines, the normal epigenetic situation was determined on the buffy coat fraction isolated from peripheral blood. All IPs were assessed at the 2 control regions. Consistent with public data available from NIH-Epigenomes project, the *GAPDH* promoter is highly enriched for H3K4me3 with no enrichment for any repressive marks. Similarly, the control region incorporating the alpha satellite repeats was highly enriched for H3K9me3, which is functionally linked to DNA hypermethylation.

In fitting with the DNA methylation profiles in leukocytes obtained in section 1, the *WT1* CpG island was enriched for the permissive histone mark H3K4me2 and did not show enrichment for any repressive mark. Similarly, the unmethylated *AWT1* CpG island region was also enriched H3K4me2 and slight enriched in H3K9me2 (Figure R.3.1). The precipitation of this repressive mark is likely due to the small size of the *AWT1* CpG island in relation to the size of the sonicated chromatin used for the IP. These results are in good agreement with those published by Weber and colleagues (Weber et al., 2007), who described that a hypomethylated CpG islands promoters show elevated levels of H3K4me2 in the absence of transcription.

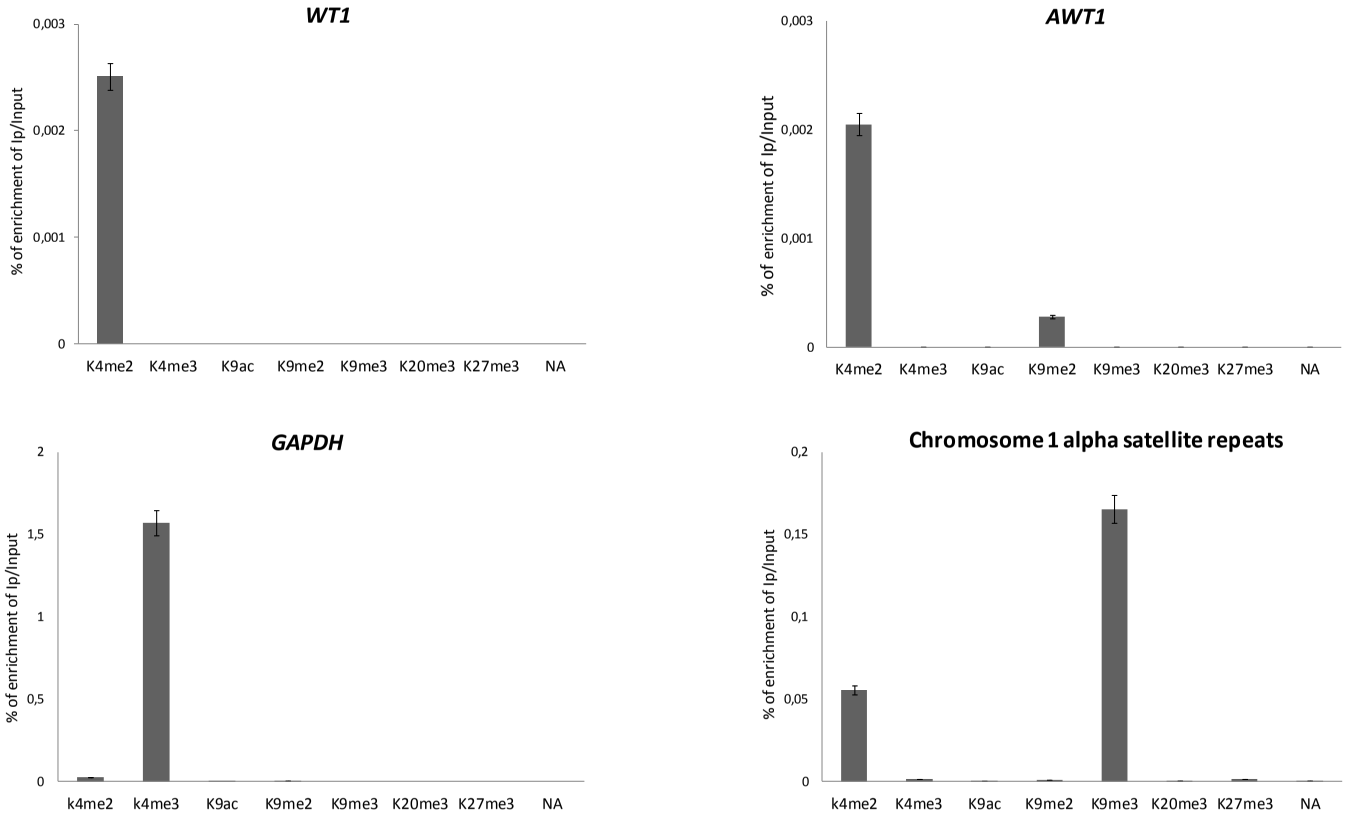


Figure R.3.1: ChIP on leukocytes. Graphs represented as percent of precipitation relative to input chromatin (mean values ± SEM).

IP with no antibody (NA) was added as a negative control

3.2. ALTERED HISTONE MODIFICATION PROFILES AT *AWT1* PROMOTER IN MYELOID CANCER CELL LINES.

Chromatin immunoprecipitation was subsequently performed on 3 cell lines that had hypermethylation at *AWT1*, unmethylated at the *WT1* CpG island promoter and with high expression levels of both transcripts. The profiles obtained for K562 and NB4 have histone profiles resembling each other, reflecting the similarity in expression and DNA methylation. For both cells lines, the *GAPDH* and alpha satellite repeat regions were normal and comparable to control leukocytes. Interestingly, hypomethylation of repeat elements such as LINE, SINE, and centromeric repeats are a hallmark of cancer (Yang et al., 2004), but the maintenance of high H3K9me3 suggests that the heterochromatic profile at the chromosome 1 alpha satellite repeat region is normal (see section 5.2 of section 5 for pyrosequencing confirmation). In the CML cell line K562 (Figure R.3.2) the *WT1* CpG island promoter was enriched for the active histone mark H3K4me3. This switch from H3K4me2 may directly reflect the high levels of active transcription from this promoter. ChIP for the promyelocytic leukemia cell line, NB4 (Figure R.3.3) displayed both H3K4 di- and trimethylation enrichment, being the cell line with highest *WT1*/*AWT1* expression. The *AWT1* CpG island promoter in both, K562 and NB4, had epigenetically switched from an H3K4me2 enriched unmethylated domain to having high levels of H3K9me3, which is functionally associated with DNA hypermethylation (Ohm et al., 2007). In the AML cell line KG1a (Figure R.3.4) the *WT1* CpG island promoter was enriched in both active and repressive marks in the same region. This surprise coexistence may reflect heterochromatic spreading, since this cell line is the only one of the 3 assessed to have hypermethylation of the *WT1-ARR* that maps to the CpG island shore of *WT1*. The *AWT1* CpG island promoter region was heavily enriched for H3K9me3, consistent with the hypermethylation of the region. The same controls were also run in parallel, with an appropriate profile observed at the

alpha satellite repeats, with co-enrichment of H3K4me3 and H3K9me3 at the *GAPDH* promoter. This result may reflect wider epigenetic deregulation in the genome of this cell line.

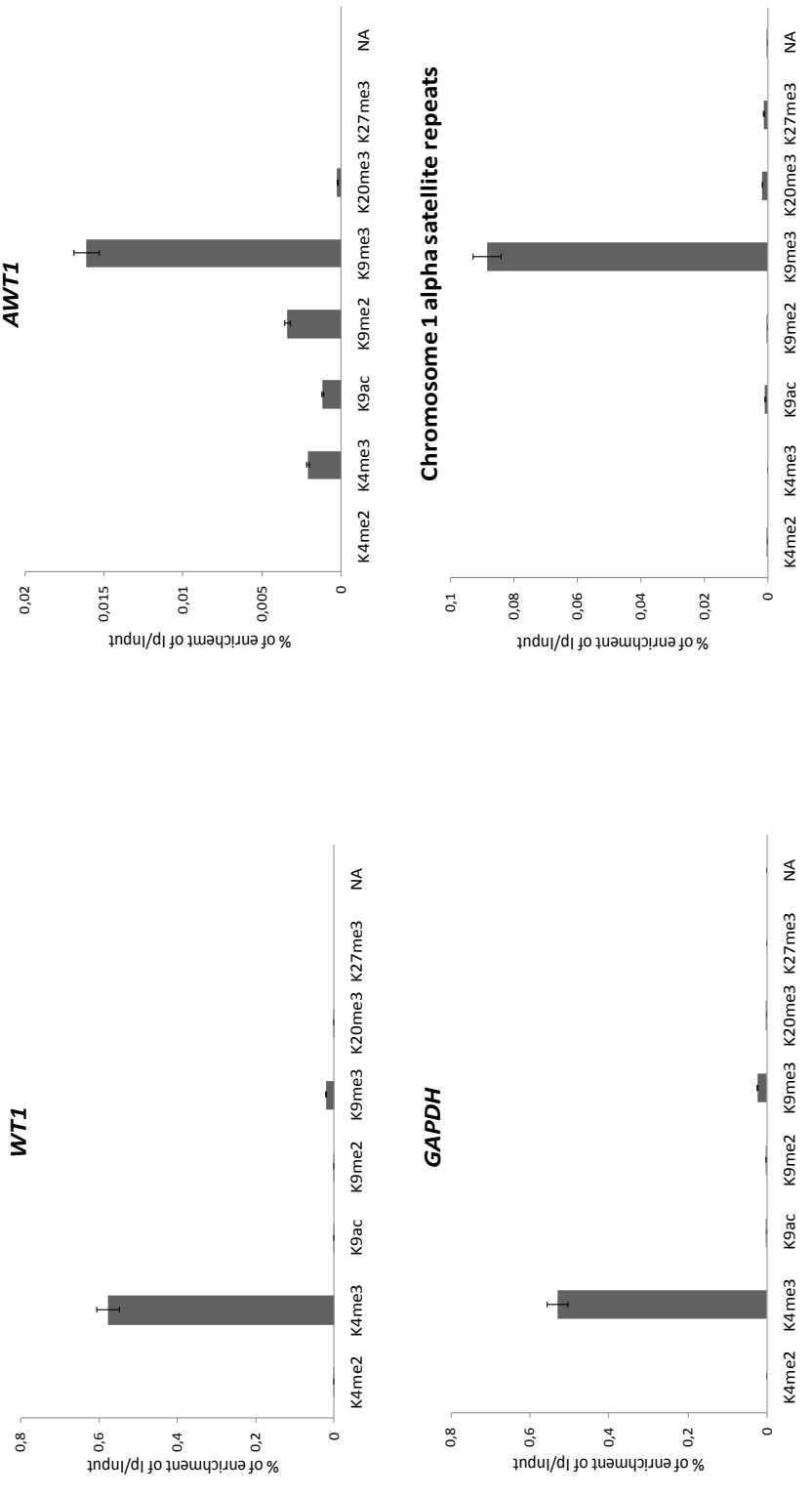


Figure R.3.2: ChIP on K562 cell line. Graphs are represented as percent of precipitation relative to input chromatin (mean values ± SEM).
IP with no antibody (NA) was added as a negative control.

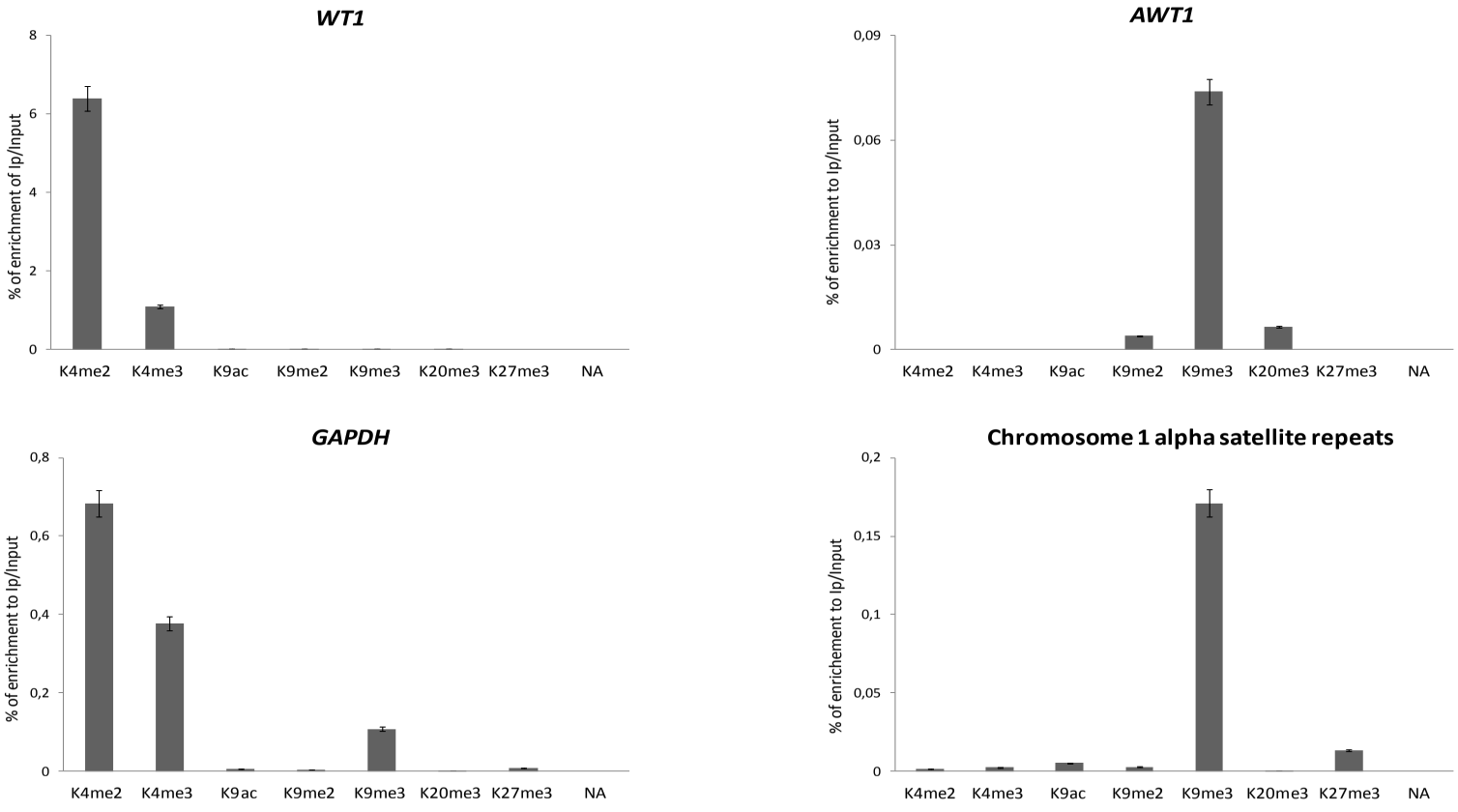
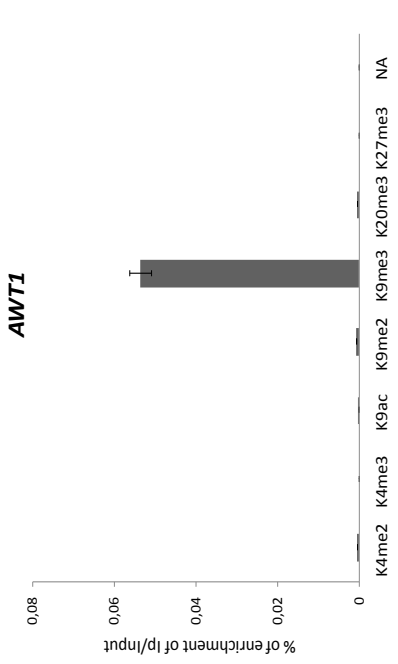
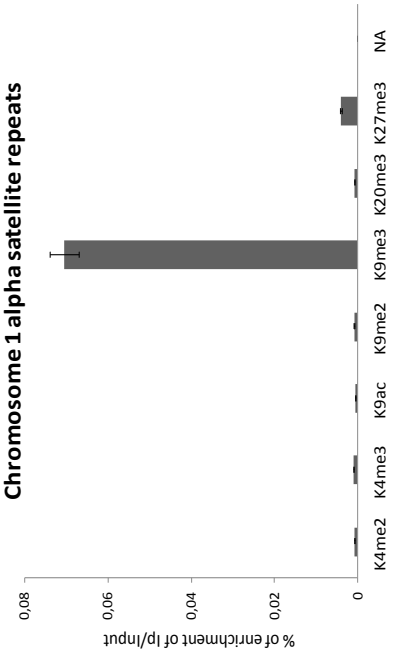
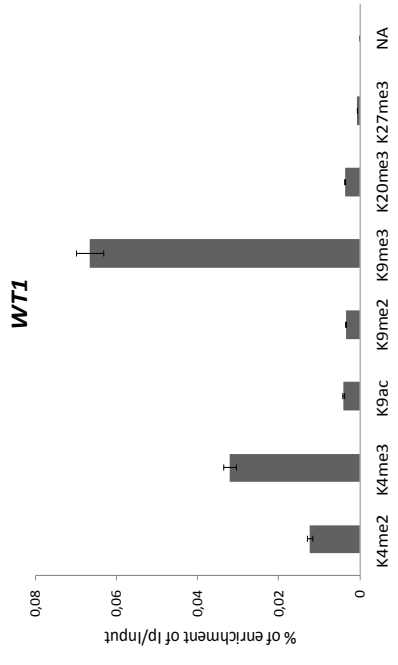


Figure R.3.3: ChIP on NB4 cell line. Graphs are represented as percent of precipitation relative to input chromatin (mean values ± SEM). IP with no antibody (NA) was added as a negative control.







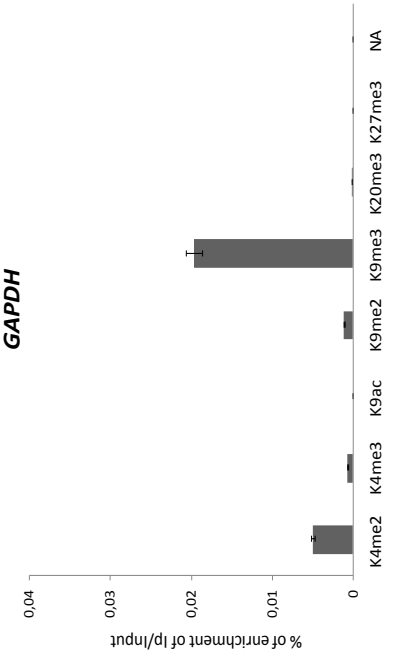


Figure R.3.4: ChIP on KG1a cell line. Graphs are represented as percent of precipitation relative to input chromatin (mean values ± SEM).
IP with no antibody (NA) was added as a negative control.

4. METHYLATION ANALYSIS OF THE *WT1* AND *AWT1* PROMOTERS IN PRIMARY SAMPLES.

Hypermethylation of the *AWT1* promoter of varying degrees was observed in the cancer cell lines analyzed, irrespectively of the cells origin. It has previously been reported that cancer cell lines show an exaggerated methylation profile compared to primary cancer samples. This may reflect clonal selection during *in vitro* propagation and the accumulation of epimutations acquired during cell culture (Smiraglia et al., 2001) or the fact that primary cancers are generally a mixture of normal and tumor cells types. To determine if *AWT1* promoter hypermethylation was also prevalent in primary samples, methylation profiling was performed on a large cohort of well-characterized hematological malignancies of both myeloid and lymphoid origins. The methylation status of the promoter regions was assessed using the pyrosequencing technique, a high-throughput and quantitative method that analyses levels of DNA methylation of CpGs dinucleotides within bisulfite PCR products. To ensure that this assay reflects the underlying methylation pattern, the pyrosequencing profile of a cell line was compared to that obtained using standard bisulfite PCR and sequencing. This analysis revealed excellent reproducibility between techniques for the CpGs analyzed (Rho Spearman coefficient=0.92, $p<0.0001$). This high correlation was also observed for the cancer cell lines (Rho Spearman coefficient=0.90 $p<0.0001$). Indicating that these CpGs analyzed showed similar DNA methylation percentages indicating the presence of a homogeneous methylation pattern. Figure R.4.1 illustrates the pyrosequencing trace obtained for the myeloid cancer cell line Kasumi.

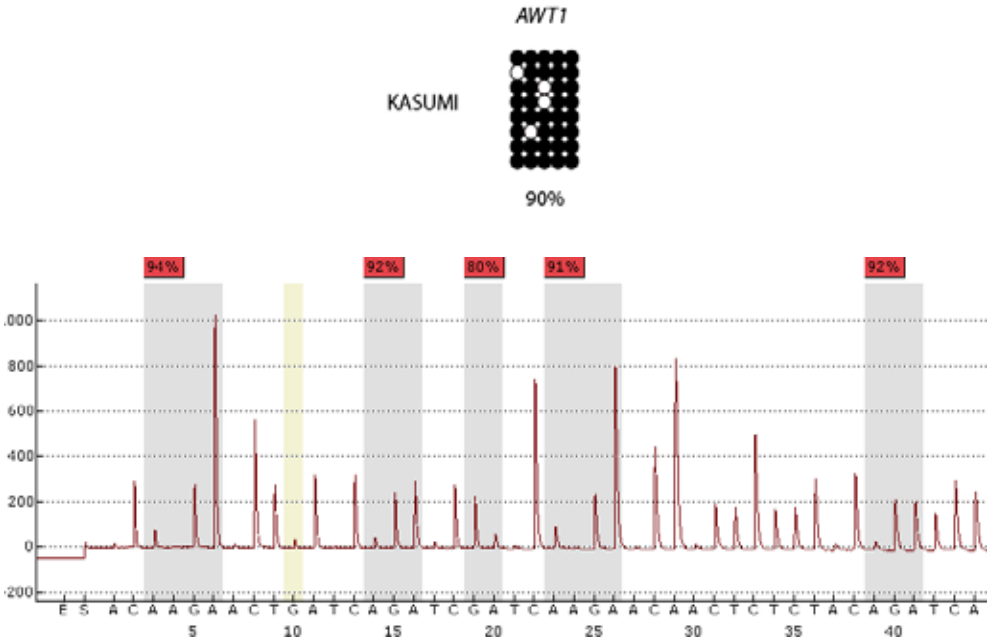


Figure R.4.1: The upper image represents five CpGs of the CpG island promoter of *AWT1* analyzed by bisulfite sequencing. In the lower graph is a typical pyrogram of the same CpG dinucleotides.

4.1. THE HYPOMETHYLATION AT THE *WT1* AND *AWT1* PROMOTERS IS STABLE DURING AGING.

To determine if the methylation state of these promoters shifts throughout life, the methylation status of the *AWT1* and *WT1* CpG island promoters were evaluated in leukocytes of new born (DNA isolated from cord bloods) (n=9), adults aged between 21-40 years (n=8) and centenarians (n=8). This experiment is deemed important as the age of the leukemia/lymphoma patients may vary, and a methylation base-line is needed to be determined. As observed in figure R.4.2, the methylation of the two regions of interest were extremely stable during life, with the extreme ages showing similar hypomethylated profiles.

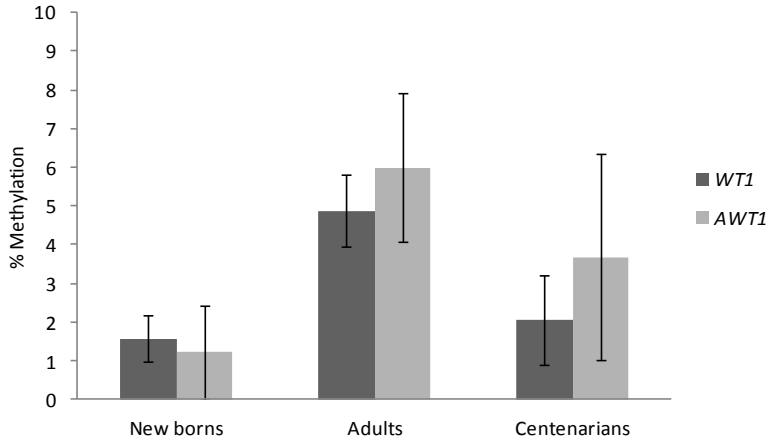


Figure R.4.2: Methylation values of the *WT1* and *AWT1* promoters in leukocytes of newborn, adults and centenarians. Bars represent mean of methylation value and error bars is the ± SEM.

4.2. DNA METHYLATION ANALYSIS OF *WT1* AND *AWT1* PROMOTERS IN A LARGE COHORT OF HEMATOLOGICAL MALIGNANCIES.

Methylation profiling was performed in a large cohort (327 samples) of cytogenetically characterize hematological malignancies from the Institute of Human Genetics of the Christian-Albrechts University Kiel, that were classified according to the WHO classification. Methylation profiling was also performed on a second smaller leukemia cohort of 41 samples of myeloid origin from the Division of Oncology, Center of Applied Medical Research from the University of Navarra, classified according to the FAB. We determined the levels of methylation of the *WT1* and *AWT1* promoters, and combined results from both cohorts (Figure R.4.3, R.4.4 and R.4.5).

General classification:

A- Myeloid neoplasm, including:

1. Myeloproliferative neoplasms
2. Myelodysplastic/Myeloproliferative neoplasms
3. Myelodysplastic syndromes
4. Acute myeloid leukemia
5. Acute leukemia of ambiguous lineage

B- Lymphoid neoplasms, including:

1. Precursor lymphoid neoplasms
2. Mature B-cell neoplasms
3. Mature T-and NK-cell neoplasms
4. Hodgkin lymphoma

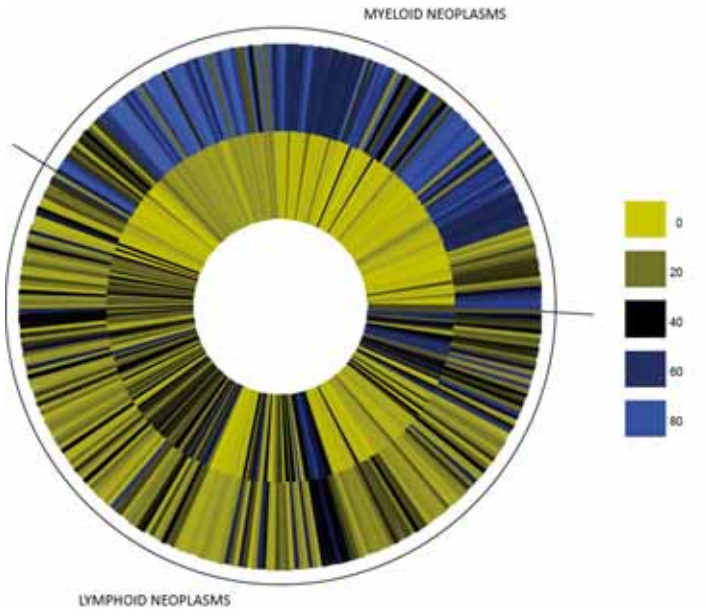


Figure R.4.3: Heat map of all the myeloid and lymphoid neoplasms analyzed. The inner circle represents the methylation values of the *WT1* CpG promoter and the outer circle represents the *AWT1* CpG promoter. The methylation values are represented in a colour code, indicated on the right size of the figure.

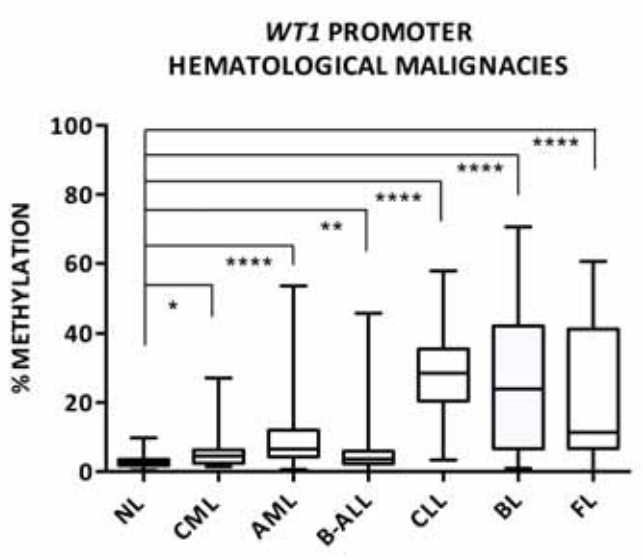


Figure R.4.4: The box-blot represents the values of the methylation levels of the *WT1* promoter in normal leukocytes (NL) and in hematological malignancies. The differences between groups were evaluated using the Mann-Whitney test (*p<0.05, ** p<0.01, *** p<0.001****p<0.0001).

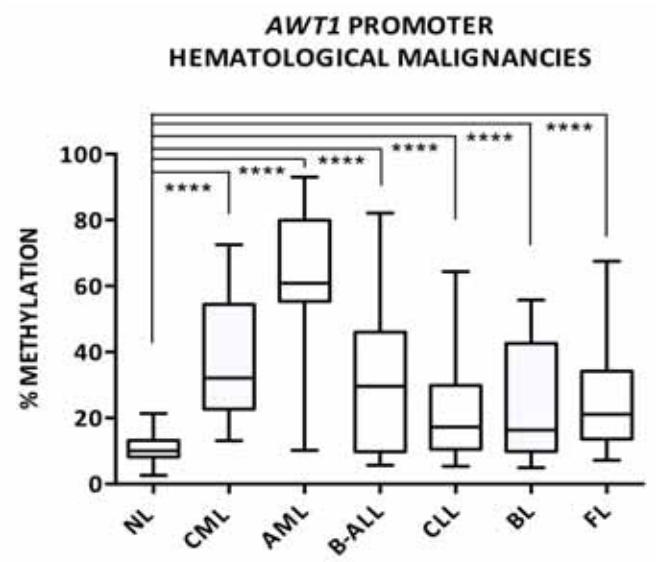


Figure R.4.5: The box-blot represents the values of the methylation levels of the *AWT1* promoter in normal leukocytes (NL) and in hematological malignancies. The differences between groups were evaluated using the Mann-Whitney test (****p<0.0001).

4.3. MYELOID NEOPLASMS.

4.3.1. METHYLATION STATUS OF THE *WT1* AND *AWT1* PROMOTERS IN MYELOPROLIFERATIVE NEOPLASMS.

The myeloproliferative neoplasms (MPN) are clonal hematopoietic stem cell disorders characterized by proliferation of one or more of the myeloid lineages (granulocytic, erythroid, megakaryocytic and/or mast cell). The myeloproliferative neoplasms include up to eight different types of diseases, but we have only analyzed cases of CML with the BCR-ABL1 translocations (Philadelphia chromosome), polycythaemia vera (PV) and essential thrombocythaemia (ET). In samples of chronic myelogenous leukemia positive for the BCR-ABL1 fusion gene, the levels of methylation at the *WT1* promoter are indistinguishable from control samples; however, the *AWT1* CpG island was variably methylated, ranging from 25 to 70%. Approximately 20% of our chronic myeloproferative disorders are found to have *JAK2* mutations, and pathalogical variants are extremely common in PV. These acquiered mutations result in a constitutively active cytoplasmatic forms of the protien that stimulates activator of transcription (STAT), mitogen activated protein kinase (MAPK) and phosphotidlinositol 3-kinase (PI3K) signaling pathways to promote transformation and proliferation of hematopoietic progenitors. Only one sample of PV, and ET were analyzed, with normal methylation values. These two samples corroborate observations by other groups who have reported low levels of aberrant methylation in PV and ET (Barrio et al., 2011) (Figure R.4.6).

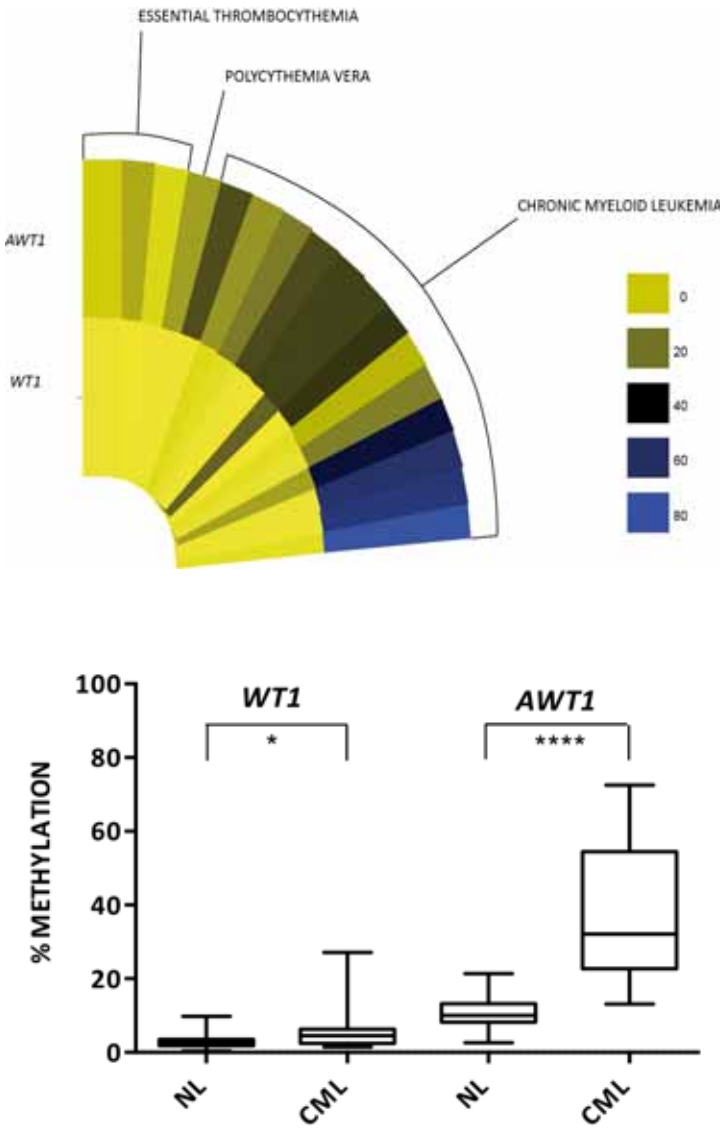


Figure R.4.6: Heat map of myeloproliferative neoplasms. The inner part of the half semi-circle represents the methylation values of the *WT1* CpG island promoter and the outer part represents the *AWT1* CpG island promoter. Color code of the methylation values is represented on the right side of the figure, yellow indicating low levels of methylation and blue indicating high levels of methylation. Box-blots represent values of methylation of *WT1* and *AWT1* promoter in normal leukocytes (NL) and CML. Differences among groups were evaluated using the Mann-Whitney test (*$p<0.05$,****$p<0.0001$).

4.3.2. METHYLATION STATUS OF THE *WT1* AND *AWT1* PROMOTERS IN ACUTE MYELOID LEUKEMIA.

Acute myeloid leukemia is a disease resulting from the clonal expansion of the myeloid blast in the peripheral blood, bone marrow, or other tissues. It is a heterogeneous disease clinically, morphologically and genetically, and may involve one or all-myeloid lineages. We analyzed samples that were categorized into 6 of the WHO classification including samples with the recurrent genetic abnormalities Inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*, t(15;17)(q22;q12); *PML-RARA*, t(9;11)(p22;q23); *MLLT3-MLL*, t(11; 10), Inv(3), mutated *NPM1*, AML with myelodysplasia related changes and unspecified AML (Figure R.4.4). In all cases except one Inv(3), one t(10;11) and a complex AML sample, the *AWT1* promoter was hypermethylated, with all but 3 of the hypermethylated samples having >40% methylation. The exceptions, all complex AML samples did not have corresponding blast count information that may account for the lower methylation values. In contrast, only 3 samples had hypermethylation of the *WT1* promoter, 2 with Inv(3) and a complex AML sample. 41 samples shorted according to the FAB classification, were indistinguishable from the cytogenetic well-classified AML samples, with all samples showing *AWT1* hypermethylation and normal *WT1* levels (Figure R.4.7). These observation perfect recapitulate the methylation profiles observed in the cancer cell lines of myeloid origin.

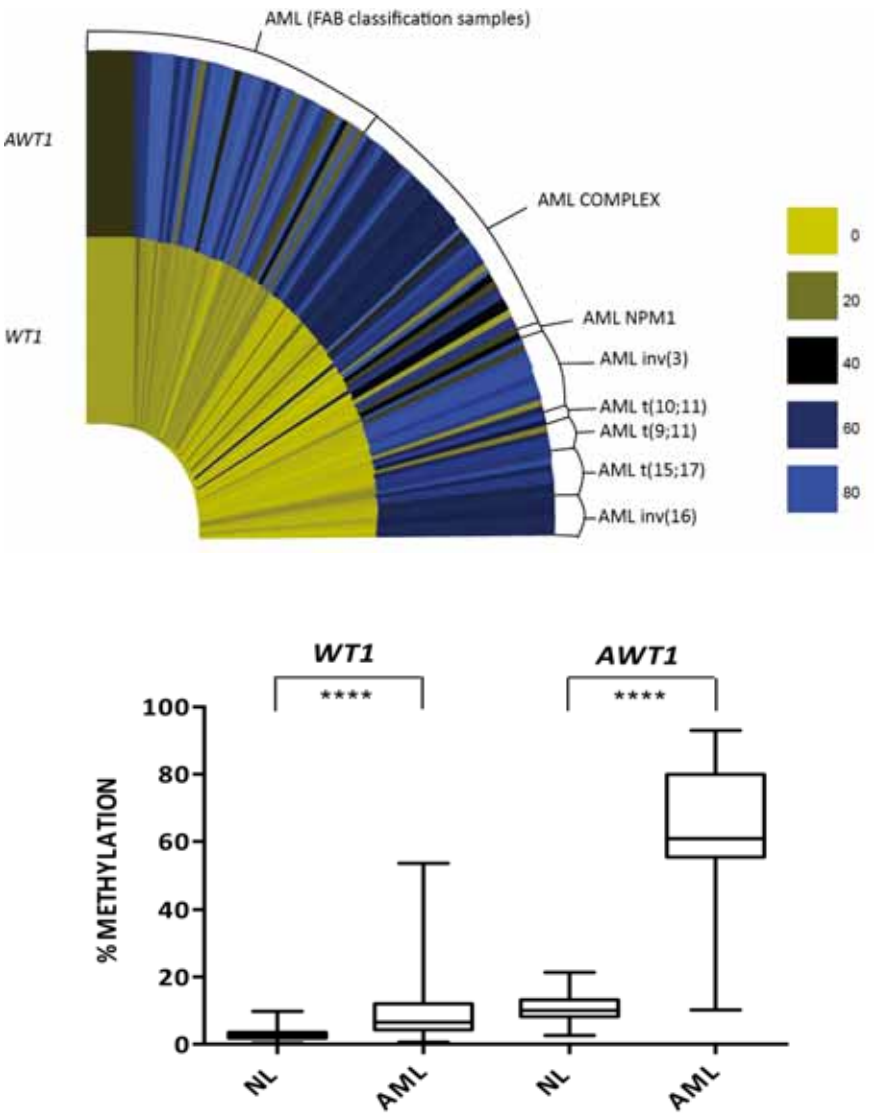


Figure R.4.7: Heat map of AML. The inner part of the half semi-circle represents the methylation values of the *WT1* CpG island promoter and the outer part represents the *AWT1* CpG island promoter. Color code of the methylation values is represented on the right side of the figure, yellow indicating low levels of methylation and blue indicating high levels of methylation. Box-blots represent values of methylation of *WT1* and *AWT1* promoter in normal leukocytes (NL) and in AML. Differences among groups were evaluated using the Mann-Whitney test (****p<0.0001).

4.4. LYMPHOID NEOPLASMS.

Lymphoid neoplasms are essentially a group of cancer that can affect the B-, T- or NK cells and can be chronic or acute in nature. Leukemia subtypes are generally distinguish from lymphomas by the origin of the disease, with leukemia starting in the bone marrow and spreading to other tissues, while lymphomas start in lymph nodes or other organs and then can spread to the bone marrow. Like myeloid neoplasms, each type of lymphoid neoplasm has characteristic genetic mutations and oncogenic fusion proteins. Herein, we have assessed the *WT1/AWT1* promoter methylation in two major groups of lymphoid neoplasms: the precursor lymphoid and the mature B-cell neoplasms.

4.4.1. METHYLATION STATUS OF THE *WT1* AND *AWT1* PROMOTERS IN PRECURSOR LYMPHOID NEOPLASMS (B-CELL AND T-CELL LYMPHOBLASTIC LEUKEMIA/LYMPHOMA).

In this group of precursor lymphoid neoplasms, for which we have assessed the *WT1/AWT1* promoters methylation in the 4 most common B-cell groups including: t(9;22)(q32;q11.2) *BCR-ABL1;* t(4;11q23) *MLL* rearranged, t(12;21)(p13;q22) *TEL-AML1* and hyperploidy, as well as T-cell neoplasms. The methylation profile identified in myeloid leukemias, *WT1* hypomethylation and *AWT1* hypermethylation, was not present in this group (Figure R.4.8).

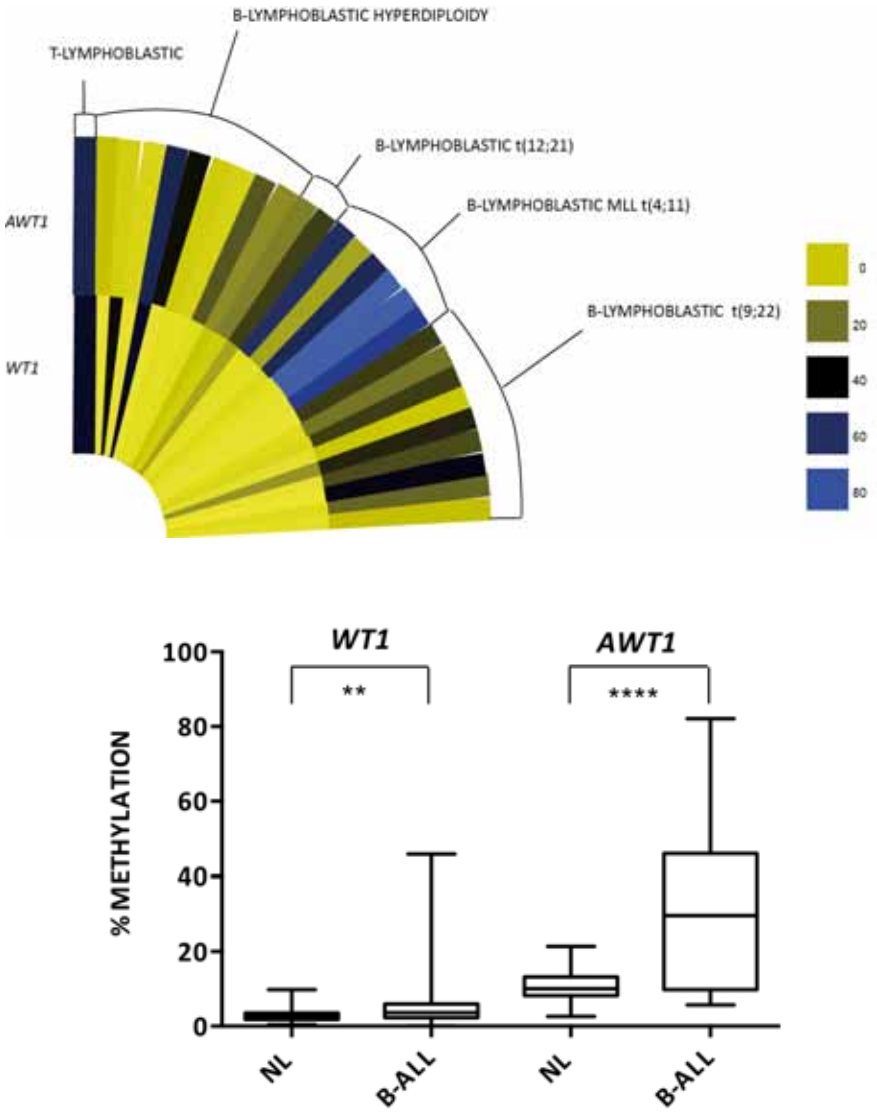


Figure R.4.8: Heat map of B-cell and T-cell lymphoblastic leukemia samples. The inner part of the half semi-circle represents the methylation values of the *WT1* CpG island promoter and the outer part represents the *AWT1* CpG island promoter. Color code of the methylation values is represented on the right side of the figure, yellow indicating low levels of methylation and blue indicating high levels of methylation. Box-blots represent values of methylation of *WT1* and *AWT1* promoter in normal leukocytes (NL) and B-ALL. Differences among groups were evaluated using the Mann-Whitney test (**p<0.01,****p<0.0001).

4.4.2. METHYLATION STATUS OF THE *WT1* AND *AWT1* PROMOTERS IN MATURE B-CELL NEOPLASMS.

According to the WHO over 26 different diseases fall into this category. Herein, we have only analyzed three of them: Follicular lymphoma (FL) (n=69), Burkitt lymphoma (BL) (n=30) and chronic lymphocytic leukemia (CLL) (n=79). In all of the mature B-cell neoplasms evaluated, we did not observe a distinct pattern of hypermethylation, except in a small subset of Burkitt lymphoma samples, which had both promoters hypermethylated. Therefore, the values for the *WT1/AWT1* promoter were very variable (Figure R.4.9 and Figure R.4.10).

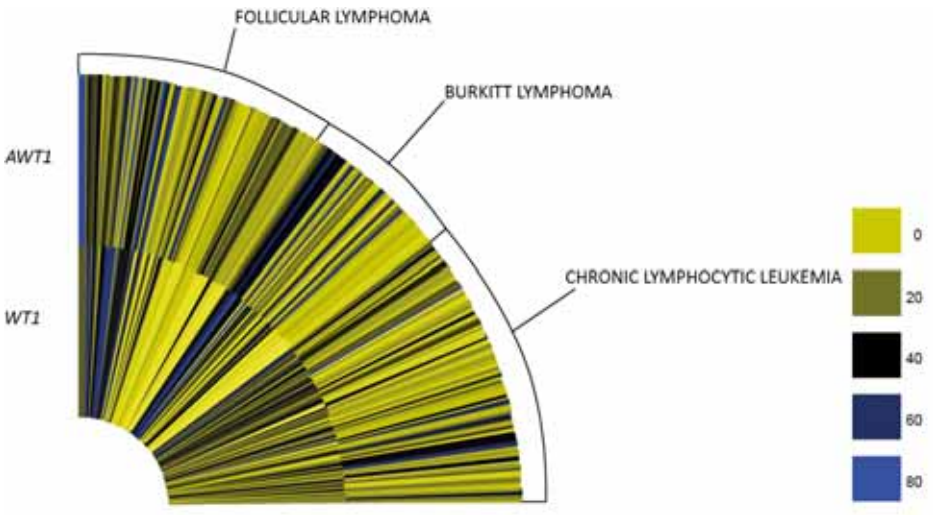


Figure R.4.9: Heat map of mature B-cell neoplasms. The inner part of the half semi-circle represents the methylation values of the *WT1* CpG island promoter and the outer part represents the *AWT1* CpG island promoter. Color code of the methylation values is represented on the right side of the figure, yellow indicating low levels of methylation and blue indicating high levels of methylation.

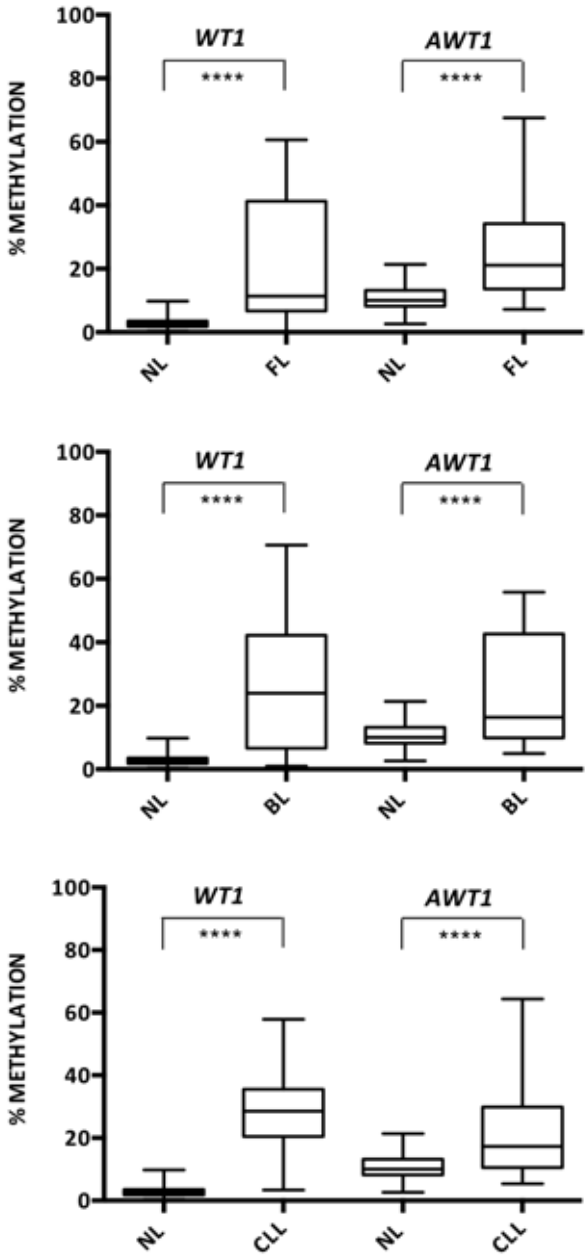


Figure R.4.10: Box-blots represent values of methylation of *WT1* and *AWT1* promoter in normal leukocytes (NL) and in FL, BL and CLL. Differences among groups were evaluated using the Mann-Whitney test (****p<0.0001).

5. METHYLATION ANALYSIS OF THE *WT1* AND *AWT1* PROMOTERS IN CELL LINES AFTER DECITABINE TREATMENT. A POTENTIAL MARKER FOR TREATMENT RESPONSE?.

Decitabine or 5-Aza-2'-deoxycytidine is a potent DNA hypomethylating agent that incorporates into DNA and traps DNA methyltransferase in the form of a covalent-DNA adduct. As a result, cellular DNA methyltransferase is rapidly depleted, and concomitantly genomic DNA is hypomethylated in a replication dependent fashion. Both Decitabine and 5-azacytidine have been approved by the FDA as antitumor treatments for MDS, and several clinical trials are ongoing for the treatment of AML and CML. To determine the effect of Decitabine on *AWT1* hypermethylation, 3 myeloid derived cancer cell lines (K562, KG1a and NB4) and one lymphoma cell line (RAJI) were treated with this epigenetic agent to evaluate if the promoter demethylation could be used as a marker for treatment response to this drug.

5.1. ESTABLISHING THE IC_{50} FOR EACH CELL LINE.

The selected cells lines were treated with various concentrations of Decitabine to evaluate the dose-response curves and to determine the IC_{50} values. All four lines were sensitive to the drug treatment. The IC_{50} values were determined from the dose-response curves using Graphpad Prism software.

After determing the IC_{50} for each cell line, which were approximately 0,5□M, cells were treated with this concentration, as well as concentrations ten times lower and ten times higher, corresponding to 0,05□M, 0,5□M and 5□M.

5.2. DEMETHYLATION OF REPEAT ELEMENTS AFTER DECITABINE EXPOSURE.

To evaluate genome-wide methylation changes after drug treatment, we evaluated methylation changes at the DNA repetitive elements, SINE (ALU-Yb8) elements, LINE-1 elements and α-satellite elements using pyrosequencing-based assays, since they are common used as a surrogate marker for assessing global hypomethylation (Yang et al., 2004). The myeloid cell lines KG1a and NB4 had high methylation levels at α-satellites and ALU-Yb8 (> 80%) and 50-60% methylation at LINE-1 prior to treatment. Unexpectedly, K562 cells were relatively hypomethylated, with α-satellite and LINE-1 methylation below 20% and ALU-Yb8 below 50% and these cell were refractory to Decitabine treatment. The methylation levels in both KG1a and NB4 cells decreased by ~20% after drug treatment at these control regions. At higher doses, methylation levels subtly increased, a phenomenon previously reported for both Decitabine and 5-azacytidine in KG1a and THP-1 cells as well patients (Hollenbach et al., 2010; Yang et al., 2006). In the Burkitt lymphoma cell line, RAJI, methylation levels decreased in a dose-dependent manner for all concentrations. Methylation values of the repeat elements are shown in figure R.5.1.

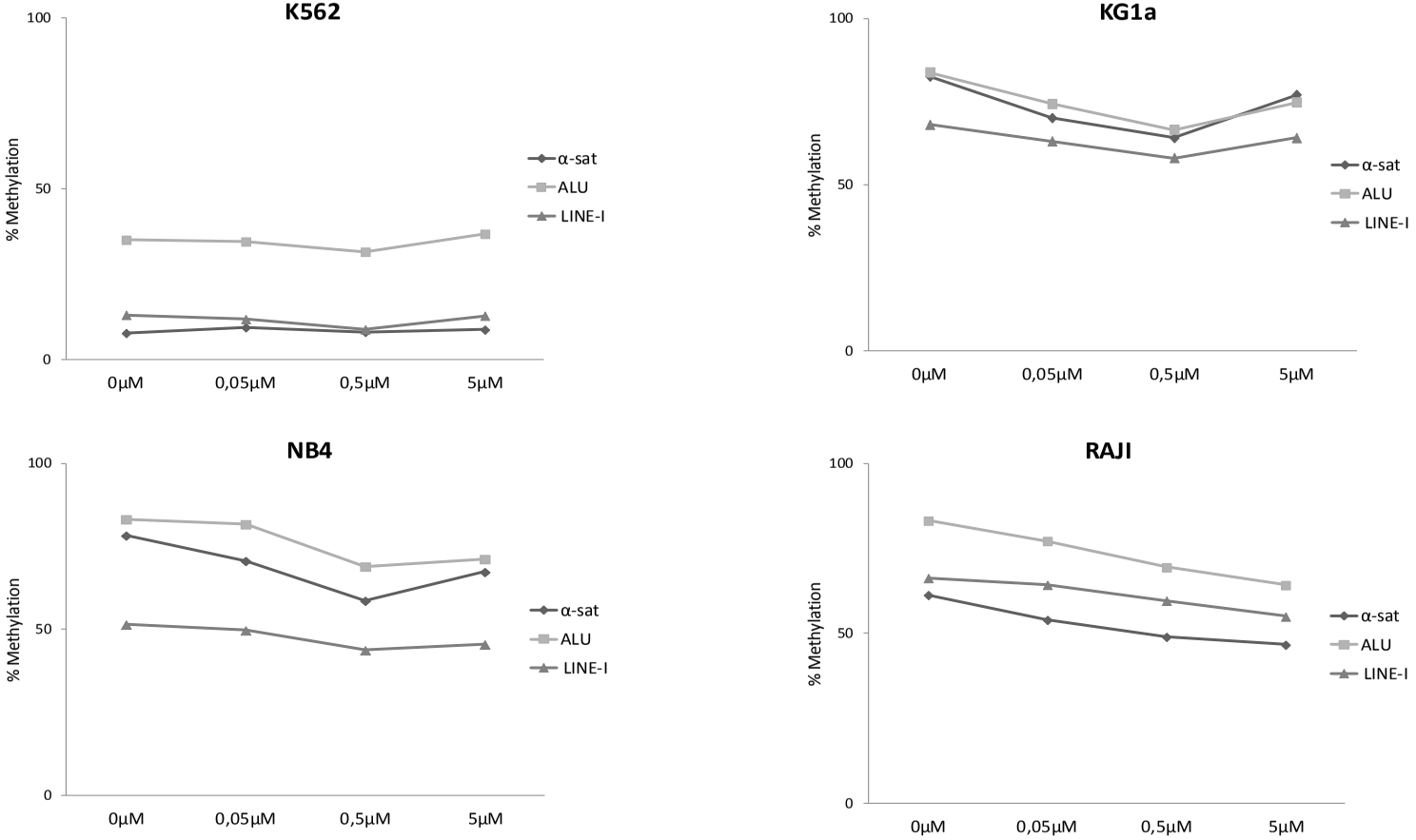


Figure R.5.1: Methylation values of the repeat elements evaluated in the leukemia and lymphoma cell lines treated at different concentrations of Decitabine.

5.3. METHYLATION ANALYSIS OF THE *WT1* AND *AWT1* PROMOTERS IN TREATED CELL LINES.

After confirmation that Decitabine induces global demethylation after five days of exposure, we assessed if the aberrant methylation we identified at the *AWT1* promoter also responds to treatment. At drug doses at the IC_{50} concentration and below, the *AWT1* promoter showed significant demethylation, by more than 20-30% in KG1a and NB4 cells (Figure R.5.2 and R.5.3). The same region remained stable at all drug concentrations in K562 cells (Figure R.5.4). Since the *WT1* promoter is normal in these cell lines no decrease of methylation levels were detected.

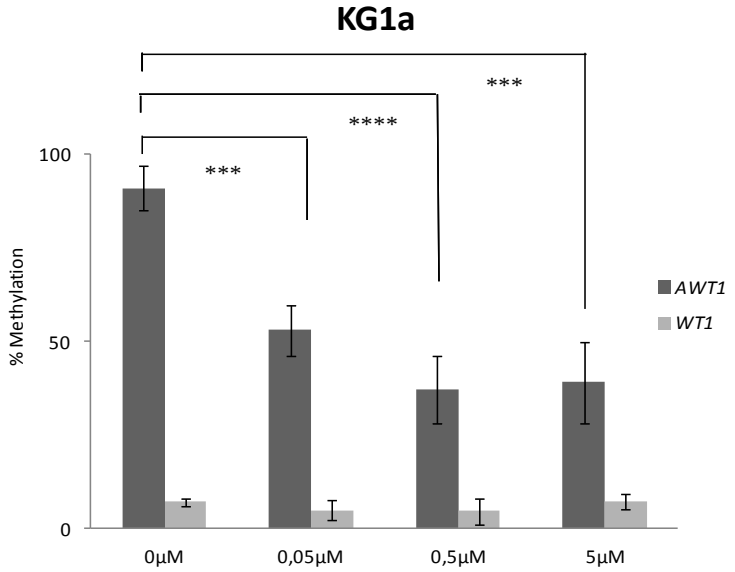


Figure R.5.2: KG1a cell line treated with Decitabine at various concentrations. A two side T-test was used to compare methylation levels before and after treatment (***p<0.001, **** p<0.0001).

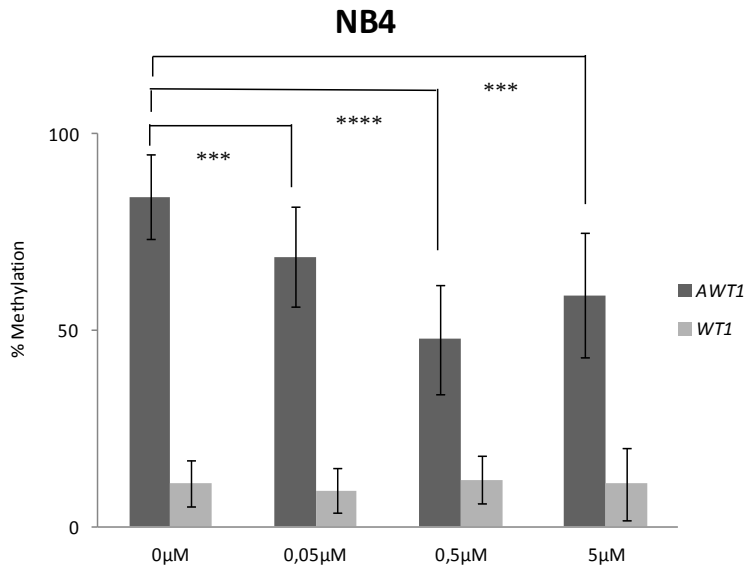


Figure R.5.3: NB4 cell line treated with Decitabine at various concentrations. A two side T-test was used to compare methylation levels before and after treatment (***p<0.001, **** p<0.0001).

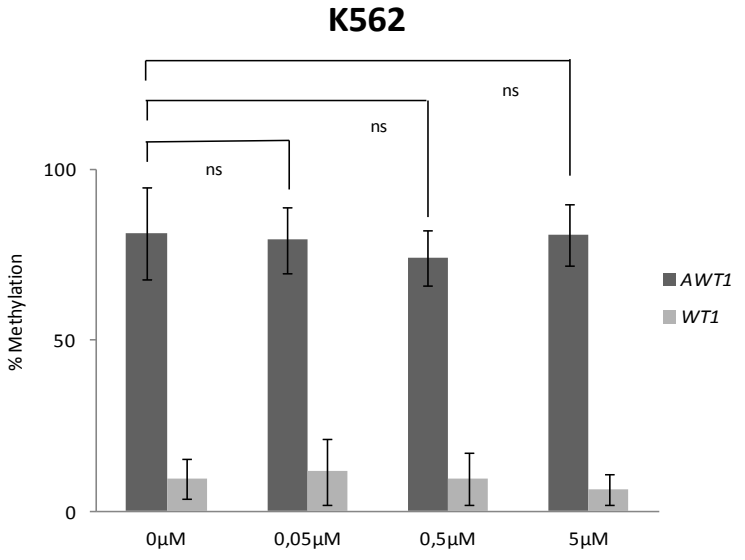


Figure R.5.4: K562 cell line treatment with Decitabine at various concentrations. A two side T-test was used to compare methylation levels before and after treatment (ns, not statistically significant).

The lymphoma cell line RAJI was selected, as we previously revealed both *WT1* and *AWT1* promoters are hypermethylated. After drug treatment the levels of methylation decrease significantly at the *AWT1* promoter without showing a coupled effect at the *WT1* promoter ~4 kb away (Figure R.5.5).

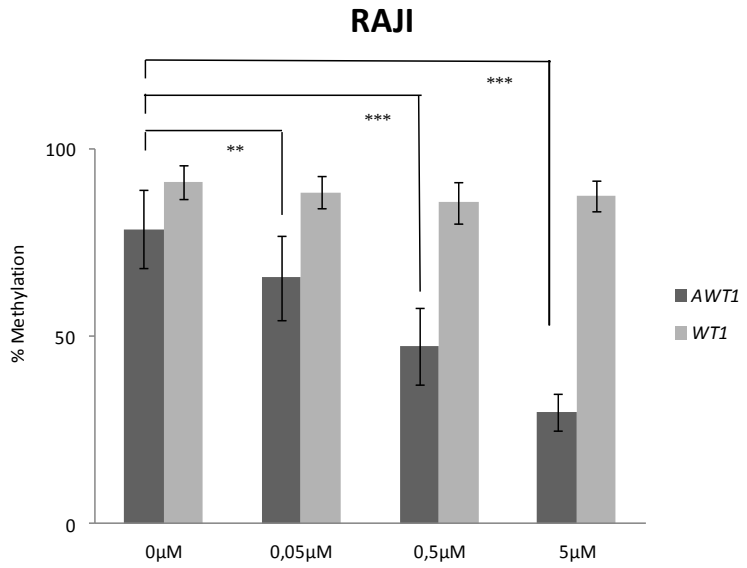


Figure R.5.5: RAJI cell line treatment with Decitabine at various concentrations. A two side T-test was used to compare methylation levels before and after treatment (**p<0.01, ***p<0.001).

6. MicroRNAs DEREGULATION AND OVEREXPRESSION OF *WT1/AWT1.*

MicroRNAs are a 19-24 nucleotides non-coding RNAs that targets the 3'UTRs of the mRNAs for degradation or inhibition of translation, therefore decreasing the expression of the resulting protein. It is estimated that 20-30% of human genes could be controlled by miRNAs (Bartel et al., 2004). MicroRNAs are deregulated in cancer by a variety of mechanisms including amplification, deletion, coding mutations and epigenetic silencing. Since we were unable to conclusively show a promoter associated epigenetic mechanism, including DNA methylation or histone modifications account for the re-expression of the *WT1*

and *AWT1* in leukemia, we hypothesize that a deregulation of a miRNA may be responsible, since both of these transcripts seem to be co-expressed, and share the same 3'UTR sequence. In previous studies, Calin and co-workers demonstrated that WT1 protein levels are modulated by the miRNAs miR-15a and 16-1 when transfected into the megakaryocytic cell line MEG-01 (Calin et al., 2008). During the course of our experiments, a complementary study was published by Goa and co-workers demonstrating that miR-15a and 16-1 can also inhibit growth of the leukemia cells lines HL-60 and K562, by down-regulating WT1 protein levels (Gao et al., 2011). Therefore, we wanted to determine if the deregulation of these tumor suppressing miRNAs, which are located within a intron of *DLEU2* (Figure R.6.1), by deletion or promoter epigenetic silencing, could down-regulate the miRNAs resulting in a concomitant increase of *WT1/AWT1* in leukemia cell lines.

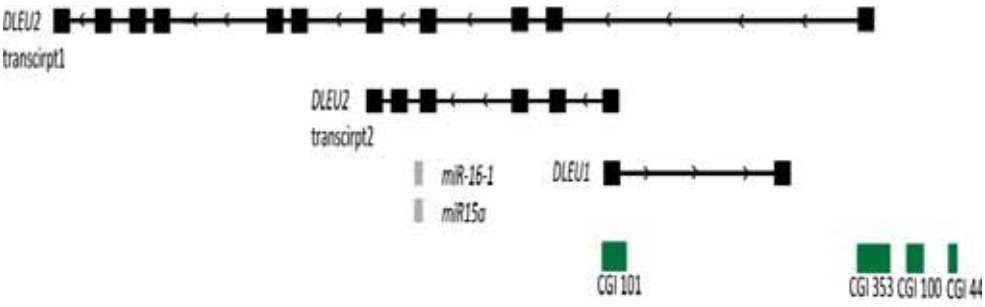


Figure R.6.1: Illustration of the of the 13q14 chromosome region. Presence of both microRNAs, miR-15a and 16-1, which are located in intron of the noncoding RNA, *DLEU2*. Transcript 2 is the prominent isoform.

6.1. DELETION ANALYSIS OF MiR-15A AND 16-1 IN LEUKEMIA CELL LINES.

The miR15a/16-1 cluster resides at chromosome 13q14.3, a genomic interval frequently deleted in chronic lymphocytic leukemia (Calin et al., 2002). To determine if this genomic region is also deleted in our leukemia cell lines we interrogated the CGH SNP-array database to check for previously reported LOH and copy number variations. No deletions were found at the resolution of the SNP-arrays in the 9 cell lines for which information was available (http://www.sanger.ac.uk/cgi-bin/genetics/CGP/cghviewer/CghViewer.cgi). To confirm that the miR-15a/16-1 region is not subject to homozygous microdeletions, we performed a PCR encompassing the highly informative SNP rs7984907 within intron five of the *DLEU2* transcript. In all cases a prominent PCR product was observed, and subsequent sequence analysis revealed that 4 out of 9 were heterozygous at this loci, indicative of a normal haploid genotype.

6.2. METHYLATION STATUS OF THE *DLEU2* PROMOTER.

The 2 miRNAs of interest are located within a intron of *DLEU2* (Lagos-Quintana et al., 2001) and the unspliced primary transcript is reported to be the pri-miRNA. The *DLEU2* promoter is embedded in a CpG rich region comprised of 3 closely spaced CpG islands. To evaluate the methylation status of the promoter region in leukocytes, immunosorted cells and the myeloid leukemia cell lines, we optimized a pyrosequencing assay that would quantify four CpG dinucleotides (CpG island 101) within the bisulfite PCR product (Figure R.6.2). An initial methylation profile was determined for adult leukocytes using standard bisulfite PCR and sequencing to ensure that the pyrosequencing targeted CpGs reflected the overall methylation status of the entire amplicon. This analysis revealed that the *DLEU2* promoter region is completely unmethylated in leukocytes. Subsequent pyrosequencing revealed that this

was also the case in immunosorted B-, T- and myeloid cells and for all leukemia cell lines (methylation range 1-4%).

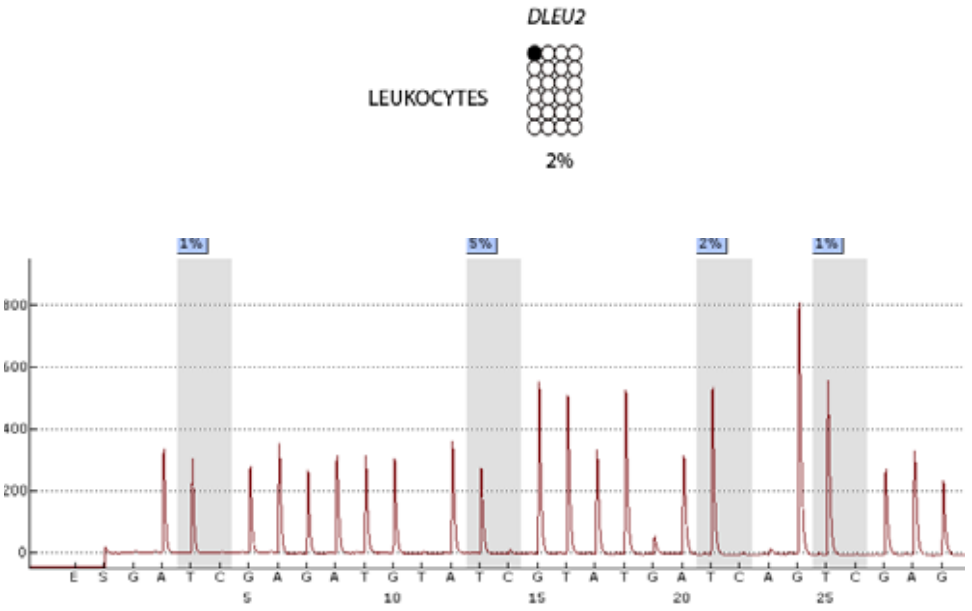


Figure R.6.2: The upper image represents four CpGs of the CpG island promoter of *DLEU2* analyzed by bisulfite sequencing. In the lower graph there is a typical pyrogram graph analyzing the same CpGs positions.

6.3. MicroRNAs EXPRESSION.

After establishing that the *DLEU2* promoter methylation was normal in leukemia cell lines, and that the mature miRNA sequences were not deleted, we confirmed that the miRNA seed sequence in the 3'UTR of *WT1/AWT1* was not mutated. All cell lines had the predicted sequence free of any variations. The expression levels of miR-15a and 16-1 were subsequently evaluated in the myeloid origin cancer cell lines and normalized to *RNU19B* (Figure R.6.3). The mature miRNAs were analyzed using Taqman microRNA assays.

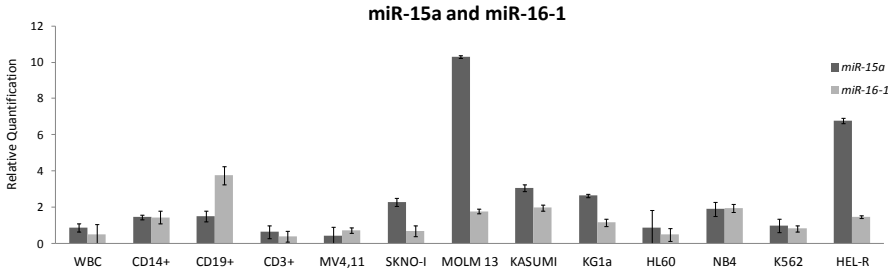


Figure R.6.3: Relative expression levels of miR-15a and 16-1 in leukocytes, immunosorted cells, and in nine myeloid cell lines was examined by qRT-PCR and normalized to *RNU19B.* Values represent the mean ± SEM.

MicroRNA 15a/16-1 expression was readily detectable in normal leukocytes and in the immunosorted cells. All 9 cell lines showed expression of both miRNAs, indicating that these myeloid leukemia cell are not aberrantly silencing these miRNAs, and are therefore unlikely to be directly regulating the *WT1/AWT1* mRNAs. In addition to the miR-15a/16-1, we also determined the expression of the miR-212 (Figure R.6.4), as this miRNA is also often deregulated in cancer and a TargetScan analysis revealed a predicted seed sequence in the 3'UTR of *WT1/AWT1*. Like miR-15a/16-1, this miRNA was expressed in both normal blood lineages and selected cancer cell lines making a direct involvement in *WT1-AWT1* re-expression unlikely.

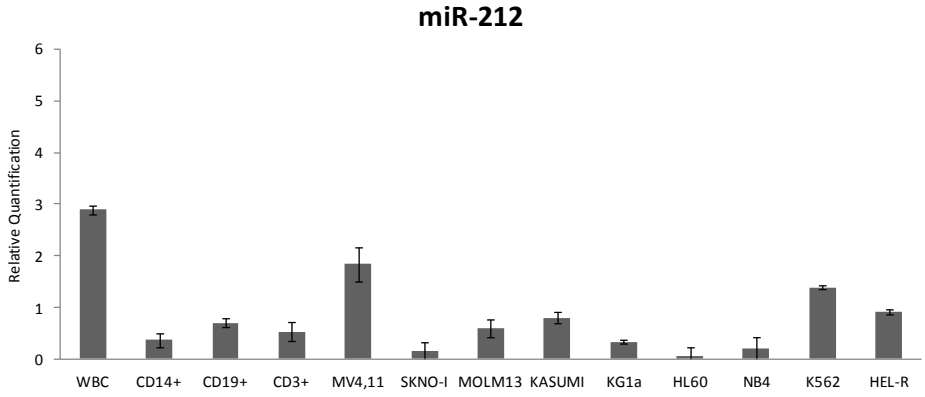


Figure R.6.4: Relative expression levels of miR-212 in leukocytes, immunosorted cells, and in nine myeloid cell lines was determined by qRT-PCR and normalized to *RNU19B.* Values represent the mean ± SEM.

7. *GATA-2* CO-EXPRESSION AND REGULATION OF *WT1* EXPRESSION IN MYELOID CANCER CELL LINES AND IN ACUTE MYELOID LEUKEMIA SAMPLES.

Up to this point of the project, we had no correlative link between the transcriptional activation of *WT1*/*AWT1* observed in AML and an aberrant epigenetic mechanism, except the hypermethylation of the *AWT1* promoter. Continuing with the idea that deregulation of a shared regulatory element could be responsible for the *WT1* and *AWT1* up-regulation we next focused on the interplay between the GATA transcription factors and *WT1* expression. Both *GATA-1* and *GATA-2* are zinc finger transcription factors that have emerged as key regulators of lineage genes in haematopoiesis (Weiss et al., 1995). These factors bind to an enhancer located in ~1 Kb past the *WT1/AWT1* 3'UTR inducing their expression (Furuhata et al., 2009). To confirm that *GATA*-2 is expressed in our collection of leukemia cells, we performed qRT-PCR (Figure R.7.1).

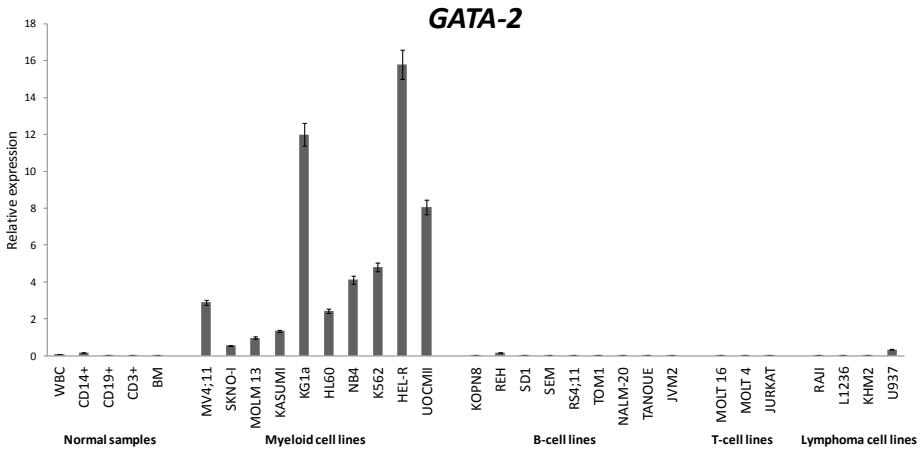


Figure R.7.1: qRT-PCR analysis of *GATA-2* in normal samples and in a panel of 26 leukemia and lymphoma cell lines. Expression values were calculated using the Livack method, and normalized to the reference gene *β-ACTIN.* Values represent the mean ± SEM.

RESULTS

Consistent with previous published results, *GATA*-2 is expressed at very low levels in bone marrow and in RNA from leukocytes, and was not detectable in immunosorted cells B-, T- and monocyte cells (Vicente et al., 2011). In the leukemia cell lines, we find the transcription levels were readily detectable in all cell lines of myeloid origin, with a wide range of expression , but absent in all other hematological malignancies. Therefore a correlative co-expression exists in the myeloid cell lines between *GATA-2* and *WT1*/*AWT1*. Despite expression of *WT1/AWT1* in the B-cell REH and NALM-20 cells lines, and MOLT 16 and MOLT 4 of T-cell origin, we failed to detect *GATA*-2 expression, suggesting that the *GATA*-2 activation is myeloid neoplasia specific. In Figure 7.2 there is a detailed graph with the expression levels of the three transcripts.

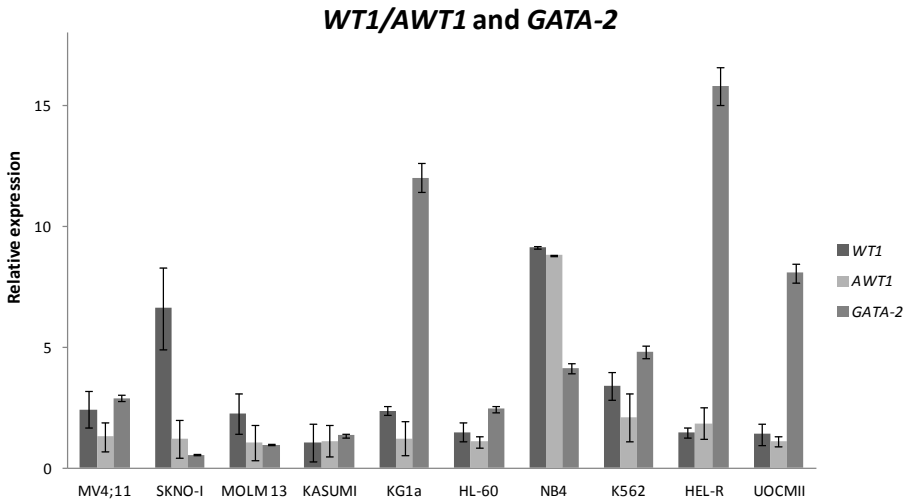


Figure R.7.2: qRT-PCR analysis of *WT1, AWT1* and *GATA-2* in a panel of myeloid cell lines. Expression values were calculated using the Livack method, and normalized to the reference gene *β-ACTIN.* Values represent the mean ± SEM.

Of the 41 primary leukemia samples analyzed for *WT1/AWT1* promoter hypermethylation, 39 AML samples had data on *GATA-2* and *WT1* abundance (the cohort from the Division of Oncology, Center of Applied Medical Research from the University of Navarra, Pamplona). Within this sample subset, all but 2

samples co-expressed both proteins (data not shown; personal communication from Dr Odero) (Vicente et al., 2012). Interrogation of the *WT1/AWT1* pyrosequencing data for these samples revealed that 38/41 of the samples had the methylation signature characteristic for AML, with hypermethylation of *AWT1* and normal *WT1*. Interestingly the *GATA-2* promoter was unmethylated in all samples (data not shown).

8. WT1 AUTOREPRESSION BY RECRUITING DNMT1.

The WT1 protein is a zinc finger transcription factor that shows a high degree of homology with the EGR family of transcription factors and these 2 factors have similar consensus DNA binding sites and can regulate the same genes. Both EGR and WT1 bind to GC-rich sequences (EGR- GCGGGGGCG; WT1-GCGTGGGCG(T/G(G/A/T)(T/G))(Cao et al., 1993; Hamilton et al., 1995). The CpG islands in the promoters of *WT1* and *AWT1* have several predicted binding sequences. The binding sites in the *WT1* promoter are assumed functional, as almost 20 years ago, Malik and co-workers identified sequences flanking exon 1 that mediate *WT1* auto-repression in a luciferase based promoter assay in WT1-KTS inducible cells (Malik et al., 1994). Recent work in mouse has suggested that the WT1 protein may be involved in chromatin switching, and recruitment of repressive epigenetic machinery, including DNMT1, to gene promoters resulting in hypermethylation (Essafi et al., 2011; Xu et al., 2011). If this occurs in humans, it is plausible that GATA-2 binding to the *WT1* enhancer results in *WT1/AWT1* expression, with the translated WT1 protein mediating DNMT1 recruitment and hypermethylation of the *AWT1* promoter in AML. To decipher if this mechanism takes place in AML, 3 cell lines were selected for endogenous co-immunoprecipitation experiments to confirm interactions between WT1 and DNMT1 proteins. We selected the cell lines K562, NB4 and KG1a as they express high levels of *WT1, GATA*-2 and we confirmed that they have high levels of *DNMT1* expression (Figure R.8.1).

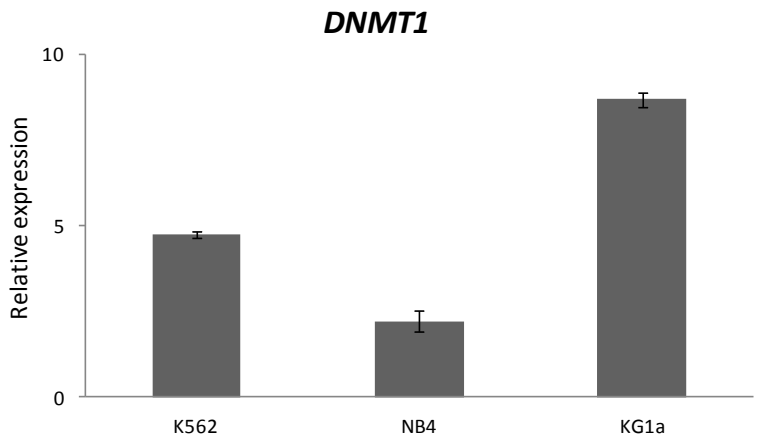


Figure R.8.1: qRT-PCR analysis of *DNMT1* in K562, NB4 and KG1a cell lines. Expression values were calculated using the Livack method, normalized to the reference gene *β-ACTIN.* Values represent the mean ± SEM.

In the co-immunoprecipitation experiments on whole extracts, the three cell lines show the same pattern, with low, but reproducible interaction between WT1 and DNMT1 (Figure R.8.2). As a control, IgG failed to pull down these proteins. The WT1 antibody used for the Co-IP experiments recognises the C-terminus of the protein, therefore it is not possible to differentiate if WT1 or AWT1 interact with DNMT1.

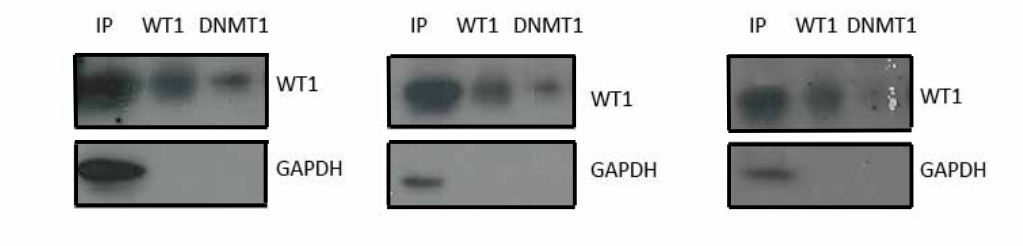


Figure R.8.2: Co-IP of DNMT1 and WT1 in K562, NB4 and KG1a cell lines.

After detecting an interaction between WT1 and DNMT1 through the endogenous co-immunoprecipitation, ChIP experiments were performed to determine if this protein complex is found at the *WT1 /AWT1* promoters. We performed ChIP for the KG1a and NB4 cell lines and confirmed that the WT1 protein was enriched within the promoter regions (Figures R.8.3 and R.8.4). As a positive control to evaluate the quality of the ChIP precipitated material, qPCRs targeting the *PAX2* promoter were used. This gene is not expressed in blood cells (GenCard eNorthern) and is believed to be a target of transcriptional suppression by WT1 (Ryan et al., 1995). Both KG1a and NB4 show high levels of *PAX2* precipitation with antisera against *WT1*, with no precipitation in the IgG negative control. In addition, lower, but reproducible levels of *WT1* were also observed at the *WT1* and *AWT1* promoter regions.

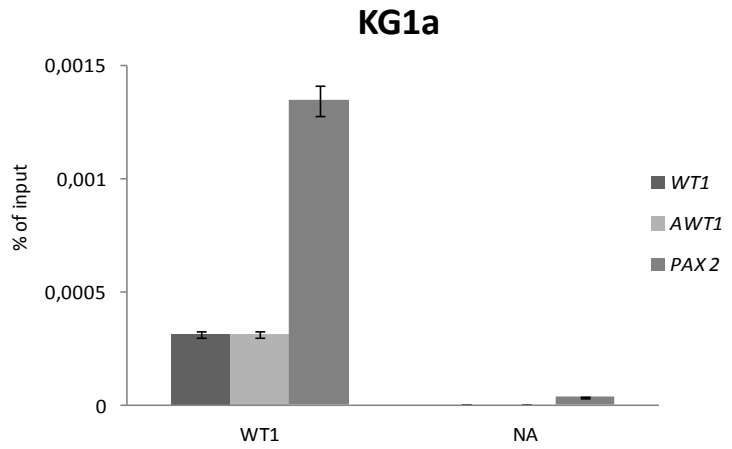


Figure R.8.3: *WT1* and *AWT1* promoters in the KG1a cell line were analyzed by chromatin immunoprecipitation (ChIP) with the antibody against the C-terminal of WT1 protein. *PAX2* promoter was used as a control. IP with no antibody (NA) was added as a negative control. Data are percentage of input chromatin amount, analyzed by qPCR (mean ± SEM).

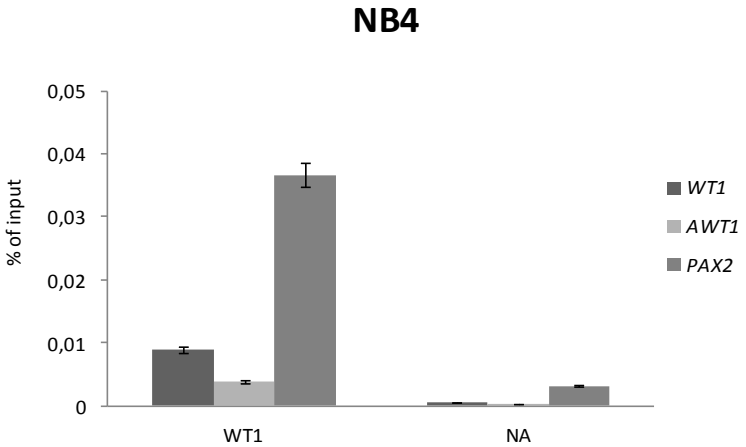


Figure R.8.4: *WT1* and *AWT1* promoters in the NB4 cell line were analyzed by chromatin immunoprecipitation (ChIP) with the antibody against the C-terminal of WT1 protein. *PAX2* promoter was used as a control. IP with no antibody (NA) was added as a negative control. Data are percentage of input chromatin amount, analyzed by qPCR (mean ± SEM).

To determine whether DNMT1 also occupies the *WT1* and *AWT1* promoter regions, ChIP was performed on identical chromatin preparations from the 2 cell lines. As a positive control for immunoprecitation, we used a PCR targeting the centromeric alpha satellite repeat on chromosome 1 that were confirmed to be > 70% methylated in these cells by pyrosequencing (see section 5.2 of section 5). In the KG1a cell line, DNMT1 was highly enriched in the *AWT1* promoter, at approximately twice the level of the control region, and more than 5 times that of *WT1* (Figure R.8.5). This profile fits perfectly with the methylation pattern observed at the *WT1* interval, with the promoter of *WT1* being unmethylated and *AWT1* hypermethylated. The low level of DNMT1 precipitation at the *WT1* promoter may reflect the hypermethylation at the *WT1-ARR*, which is in the *WT1* CpG shore and will be picked up in some of the longer fragments of sheared chromatin.

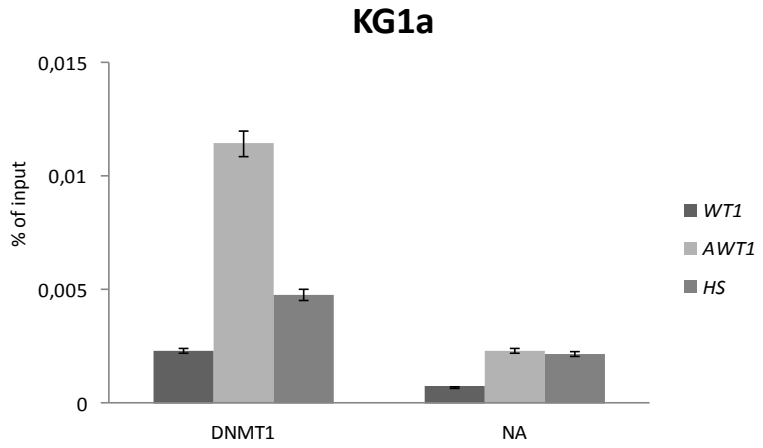


Figure R.8.5: *WT1* and *AWT1* promoters in the KG1a cell line were analyzed by chromatin immunoprecipitation (ChIP) with the antibody against DNMT1 protein. Centromeric alpha satellite repeat on chromosome 1 was used as a control. IP with no antibody (NA) was added as a negative control. Data are percentage of input chromatin amount, analyzed by qPCR (mean ± SEM).

In the NB4 cell line, we obtained enrichment at all regions, with more DNMT1 precipitation at the *AWT1* promoter than the *WT1* region (Figure R.8.6), again, consistent with the methylation profile of this cell line.

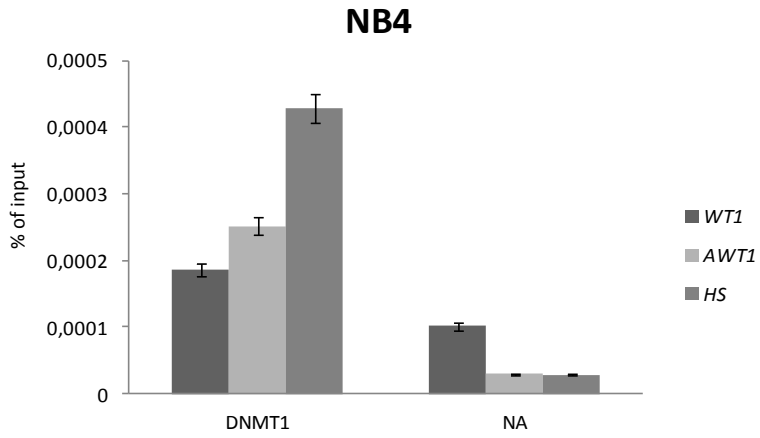


Figure R.8.6: *WT1* and *AWT1* promoters in the NB4 cell line were analyzed by chromatin immunoprecipitation (ChIP) with the antibody against DNMT1 protein. Centromeric alpha satellite repeat on chromosome 1 was used as a control. IP with no antibody (NA) was added as a negative control. Data are percentage of input chromatin amount, analyzed by qPCR (mean ± SEM)

DISCUSSION

The main goal of this thesis was to provide a more detailed characterization of the deregulation of the *WT1* locus during the development of acute and chronic myeloid leukemias. The evolution from a normal cell into a leukemic one is a multistep process, progressing from a disturbed gene to altered protein function within the cell. At the moment, there is a large but yet an incomplete knowledge of the changes involved in leukemia, such as chromosomal abnormalities, translocations, mutations, and deletions, as well as epigenetic changes. The *WT1* gene is deregulated in leukemia since it is aberrantly re-expressed. Therefore, our initial objective was to elucidate the epigenetic events that lead to *WT1/AWT1* expression and their contribution to leukemia.

THE *WT1* TRANSCRIPTS ARE NOT IMPRINTED.

As outlined previously, genomic imprinting is the parent-of-origin specific monoallelic transcriptional silencing observed in placental mammals (Verona et al., 2003). The allelic differences in transcriptional activity originate from the distinct patterns of DNA methylation established in the gametes, these patterns of methylation are maintained throughout somatic development (Bourc'his et al., 2001). In cancer, a misregulation of genomic imprinting undergoes what is known as the LOI, and is one of the most common alterations in cancer, causing either biallelic expression or complete silencing of both alleles (Jelinic et al., 2006; Monk, 2010). At present, 64 human genes are subject to genomic imprinting (igc.otago.ac.nz/) with many of them located within clusters, presumably as a consequence of co-regulation by DMRs (Edwards et al., 2007). Importantly, imprinted genes can have tissue restricted expression patterns; however, DMRs maintain their allele-specific methylation in all adult somatic tissues irrespectively of levels of expression. This is highlighted by a recent study in peripheral blood, which revealed that despite only 6 imprinted being monoallelically expressed in leukocytes, all 15 DMRs

assessed were differentially methylated, confirming that imprinted methylation is maintained irrespectively of transcription levels (Frost et al., 2010).

The *WT1* transcript was initially reported to be partially or completely maternally expressed in 5 of 9 placentas and 2 fetal brains (Jinno et al., 1994). However conflicting data was subsequently published suggesting that *WT1* is paternally expressed in fibroblast and lymphocytes from 2 of 7 individuals (Mitsuya et al., 1997). Since these initial observations, transcripts originating from the *AWT1* promoter and a non-coding RNA *WT1-AS* were reported to be paternally expressed in adult kidney (Malik et al., 2000; Dallosso et al., 2004). This imprinted expression was proposed to be co-regulated by a region of allele-specific methylation (*WT1-ARR)* that is methylated on the maternal allele and acts as a methylation-dependent bidirectional silencer. These methylation studies were performed in adult kidney where *AWT1* and *WT1-AS* were monoallelically expressed compared to the biallelically expression in Wilms' tumor or nephroblastoma without LOH, due to reduced methylation at the *WT1-ARR* region (Dallosso et al., 2004). We therefore hypothesized that DNA methylation defects at the *WT1-ARR* could account for aberrant re-expression of *WT1/AWT1* in leukemia. To characterize the DNA methylation at base pair resolution in an allelic manner, we performed PCR and sequencing of bisulfite converted DNA in a panel of normal fetal and adult tissues. In all of the tissues evaluated, including the fetal/adult kidney and a podocyte cell line, we did not observe any evidence of allele-specific methylation at the *WT1-ARR* or methylation at the *WT1* and *AWT1* CpG island promoters. This genomic interval was also analyzed using the latest high-throughput Infinium Human Methylation 450K BeadChip array, in which a total of 43 probes span the ~50 kb of the *WT1* domain. In this analysis, kidney, leukocytes and a genome-wide UPD samples were used to evaluate the methylation at the *WT1* locus (personal communication D. Monk and F. Court). This analysis corroborated

the bisulfite PCR results, confirming that both promoter CpG islands are hypomethylated, and that the *WT1-ARR* region is mosaically methylated, despite identifying the differential methylated region associated with the *H19* gene also on chromosome 11. These genome-wide UPD samples are a powerful tool in identifying imprinted methylation with all known imprinted-DMRs, and other novel imprinted domains have been identified by our laboratory using these rare samples and genome-wide methylation techniques (Nakabayashi et al., 2011, D. Monk personal communication). Therefore our results conclusively show that the *WT1-ARR* is not an imprinted-DMR, since we were unable to detect allelic methylation in multiple tissues. However, a recent work in mouse suggests that some imprinted-DMRs maybe transiently regulated. The *Cdh15* gene contains an intergenic DMR that inherits allelic methylation from oocytes. This maternally methylated region results in the paternal expression of *Cdh15* during embryogenesis. However in adult mice, the paternal allele of this DMR is *de novo* methylated in the cortex and cerebellum, despite all other tissues maintaining the differential methylation status (Proudhon et al., 2012). This work suggests that a limited number of imprinted-DMRs are dynamically regulated during development. However this scenario is unlikely to account for the lack of allelic methylation at *WT1-ARR*, since we fail to find any evidence of maternal methylation at early developmental time points. Interestingly, a work by Kim and colleagues have shown that the various transcripts within the *WT1* locus are biallelically expressed in numerous human embryonic stem cell lines, and that the *WT1-ARR* is unmethylated, confirming that even in every early stages of development, imprinting is undetectable (Kim et al., 2007; personal communication J. Frost). One possible explanation for the discrepancy between the identified methylation profiles is the techniques used to study DNA methylation. The initial studies were performed using the Southern blot, a technique that is quantitative at the designated restriction enzyme recognition

sequences, but fails to give information at base pair resolution or DNA strand information. Therefore regions of hemimethylation can be misinterpreted and be considered an imprinted-DMR. The fact that *WT1* has been reported to be an imprinted gene with monoallelic expression only in one normal kidney sample, is a clear example, of how it is still difficult to confirm the imprinted states for genes with restricted tissue expression with few informative polymorphisms, and emphasizes that any allelic expression should always be interpreted with quantitative expression data, since cells with very few transcripts may give skewed allelic expression after high PCR cycling.

BREAKING THE DOGMA: EXPRESSION OF *WT1/AWT1* FROM HYPERMETHYLATED PROMOTERS IN MYELOID LEUKEMIAS.

Despite the lack of evidence for a role of genomic imprinting at the *WT1* locus, we continued with the analysis to decipher the mechanism resulting in aberrant expression of the *WT1* transcripts in specific types of leukemia. In hematological malignancies of lymphoid origin, with the exception of two B-cell lines, REH and NALM-20, and the T-cell line, MOLT 4, no expression of *WT1* was observed. This is in agreement with the varying degrees of hypermethylation at the locus. In the myeloid leukemia cell lines, however, with the exception in the promyelocytic cell line, HL-60, the *WT1* promoter was hypomethylated and with high transcriptional levels. The *AWT1* promoter was hypermethylated in the entire myeloid malignancy collection, despite the high transcriptional levels. Consequently, the *WT1* and *AWT1* transcripts seem to be co-expressed and co-regulated in all of the myeloid cell lines with *AWT1* transcript being expressed at approximately 2/3 the level of the full length *WT1* isoform. This expression profile was also evident for most cell lines at the protein level as identified by western blotting using an antibody targeting the C-terminus, with nuclear and cytoplasmatic fractioning confirming that this protein is nuclear retained, consistent with it being a transcription factor. Over

a decade ago, Loeb and colleagues observed a similar phenomenon in primary breast tumor samples, where they detected expression of *WT1* despite promoter hypermethylation (Loeb et al., 2001). This could indicate that the epigenetic status of these promoter regions has limited transcriptional control, and that regulation by additional in *cis* enhancers dictate the transcription levels.

Published mutation screens of AML and T-ALL primary samples has revealed a trend for cells harboring *WT1* mutations to be more prominently expressed (King-Underwood et al., 1998; Heesch et al., 2010). We therefore performed a mutation screen on all *WT1* exons in the 28 cell lines. No mutations were detected in any myeloid, B-cell and T-cell cell lines. However, in the lymphoma cell line U937, we identified a non sense mutation G>A, p.R369* in exon 7. The *WT1/AWT1* transcripts are not expressed in this cell line. In 9 of the 28 cell lines we identified heterozygous SNPs that confirmed that the locus was not subject to cancer-associated copy number variation, which was confirmed by copy number analysis using Cancer Genome Project/Sanger database. Also, the identification of exonic heterozygous SNPs allowed us to perform allelic expression analyses in those cell lines with abundant transcription. In 7 AML cell line, and the MOLT 4 and NALM-20 cells, we readily detected equal expression from both parental alleles for *WT1*, *AWT1* and the anti-sense transcript. This suggests that the gene is either not subject to imprinting, or that in all cases there is LOI. This would represent an abnormally high frequency of LOI, since this type of epigenetic deregulation is less prevalent for *bona fide* imprinted genes in other cancer types, for example, 9/17 *KCNQ1OT1* in colorectal cancer (Nakano et al., 2006); 5/8 *MEST* in head and neck cancer (Kataoka et al., 2009); 9/21 *MEST* in lung cancer (Nakanishi et al., 2004); and 12/24 *IGF2* in AML (Wu et al., 1997).

5-HYDROXYMETHYLATION IN MYELOID CANCER CELL LINES?.

Recently 5hmC has been described to participate in the epigenetic regulation of gene expression during embryogenesis and cancer (Ito et al., 2010; Zhang et al., 2010; Ko et al., 2010). The function of 5hmC is currently under debate, but it could be an intermediate state of TET-1 mediated demethylation, a process that ensures unmethylated states of CpG islands. Alternatively 5hmC maybe an epigenetic signal itself, potentially influencing transcription. In line with these potential mechanisms, 5hmC is enriched at CpG rich promoters and actively transcribed gene bodies (Wu et al., 2011). Since both 5hmC and 5mC are converted to uracils during bisulfite DNA treatment and to thymidines in the subsequent PCRs (Jin et al., 2010), we wanted to determine if the *AWT1* hypermethylation we detected in the myeloid cell lines reflects the presence of 5hmC, since this CpG island is within the first intron of the full length *WT1*, a location enriched for 5hmC at other loci (Stroud et al., 2011). We utilized 2 different techniques that can readily distinguish 5mC from 5hmC; meDIP with specific monoclonal antibodies against 5mC and 5hmC and a glucosyltransferase assay. Three different myeloid cell lines, K562 (chronic myeloid leukemia), NB4 (promyelocytic leukemia) and KG1a (acute myelogenous leukemia) were assessed for 5hmC content, as each line had abundant *WT1* transcripts, and the *AWT1* promoter was hypermethylated in relation to the unmethylated adjacent *WT1* CpG island. Neither the 5hmC glucosyltransferase experiment nor the meDIP against 5hmC showed enrichment at the *AWT1* or *WT1* promoter regions. As a control, meDIP using the 5mC antibody revealed high levels of precipitation of methylated DNA at the *AWT1* promoter, conclusively indicating the absence of 5hmC.

HISTONE TAIL MODIFICATIONS AT THE CpG ISLAND PROMOTERS OF *WT1* AND *AWT1*.

In order to gain further insights into *WT1/AWT1* expression, we determined the chromatin landscape of the promoters by evaluating the presence of various histone modifications associated with gene expression and repression. To assess the abundance of histone tail modifications at the *WT1* and *AWT1* promoter regions we utilized ChIP using antibodies specific for permissive modifications and repressive modifications. In normal leukocytes, where the *WT1* and *AWT1* promoter CpG islands are unmethylated, we observed enrichment for H3K4me2, which confirmed the observation that Weber and colleagues described, that elevated levels of this modification are characteristics of unmethylated promoters without expression (Weber et al., 2007). In the three myeloid cancer cell lines evaluated (K562, NB4 and KG1a) there was a conformational switch at the *AWT1* promoter, from this transcriptionally permissive state to a repressed profile enriched for H3K9me3, a modification commonly associated with methylated DNA. As for the *WT1* unmethylated promoter, all 3 cell lines were enriched for the permissive chromatin marks, however, the acute myelogenous leukemia cell line KG1a, was also enriched for the repressive H3K9me3 modification, probably due to the heterochromatic spreading across the loci. We focused our analysis on the myeloid origin cell lines, but it would have been interesting to assess the histone marks of these promoters in lymphoid cell lines that are transcriptionally silent but also hypermethylated, such as RAJI, L1236 and GRANTA 519. This approach was recently performed by Vegliante and colleagues, who compare cell lines positive and negative for the *SOX11* transcription factor. This revealed enrichment of H3K4me3 in the expressing REH cell line and H3K27me3 in the *SOX11* non-expressing cell line, JVM2, indicating that lack of *SOX11* expression was associated with silencing histone marks, with or without DNA hypermethylation (Vegliante et al., 2011). The

change in histone modification enrichment at the *AWT1* CpG island in the AML cell lines is reminiscent of the chromatin profile of TSG in other forms of cancer. It is well known that there is a high degree of interdependence between DNA methylation and chromatin modifications that is exemplified by the *MLH1* and *SFRP5* promoters in colorectal cancer. These promoters are normally in an euchromatic state, being unmethylated and enriched for H3K4me2. However once hypermethylated in cancer, these promoters adopt a heterochromatic state with enrichment of H3K27me3 and H3K9me2/3. Interestingly, exposing cell lines with hypermethylated *MLH1* and *SFRP5* promoters to demethylation agents such as 5'-aza-2'-deoxycytidine results in gene expression and an intermediate chromatin profile (McGarvey et al., 2006; Jacinto et al., 2009) suggesting that DNA demethylating interventions alone are not able to restore a complete euchromatic status and a full transcriptional reactivation of the epigenetically silenced TSGs. In cancer it has been previously reported that CpG islands that undergo *de novo* promoter hypermethylation were among a subset of genes that contain bivalent promoters (H3K4me2/3 and H3K27me3). The presence of H3K27me3, catalyzed by the polycomb group protein EZH2, has been proposed preferentially recruit DNMTs, resulting in abnormal hypermethylation (Schlesinger et al., 2007; Ohm et al., 2007). Interestingly, despite bivalent domains being 14 times more likely to undergo cancer-associated hypermethylation, only one third of CIMP regions are bivalent (Roman-Gomez et al., 2006; Kaneda et al., 2011; Zhao et al., 2007; Pan et al., 2007). Our studies clearly reveal that the *AWT1* promoter lacks co-enrichment of H3K4me3 and H3K27me3 and is therefore a non-bivalent CpG region that acquires DNA hypermethylation.

Recently additional histone modifications have been shown to recruit DNMTs. The H3K36me3 modification is deposited during the transcription process and is found in coding regions of highly expressed genes (Bannister et al., 2005).

The DNMT enzymes recognize the H3K36me3 motif via the PWWP domain (Dhayalan et al., 2010; Vezzoli et al., 2010). Therefore the *AWT1* promoter regions maybe enriched for H3K36me3 in highly expressing AML cells, being located within intron 1 of the *WT1* transcript. Indeed integration ENCODE H3K36me3 ChIP-seq data in K562 cells reveals enrichment of both POLII and this histone modification across the *AWT1* CpG island. Therefore future ChIP experiments should confirm the presence of this histone mark in other AML samples. However, this histone guided hypermethylated state would only explain hypermethylation in *AWT1* expressing cells, as transcription is required for H3K36me3 deposition, and would not account for the hypermethylation in other classes of hematological malignancies.

_WT1_ AND _AWT1_ PROMOTERS SHOW DISTINCT METHYLATION SIGNATURES IN PRIMARY LEUKEMIA SAMPLES.

In the panel of leukemia and lymphoma cell lines we observed frequent hypermethylation at the *WT1* locus. Consequently, we wanted to confirm our observations in cell lines were reflecting the epigenetic state in primary samples. The DNA methylation of both promoters was quantified by pyrosequencing in a large cohort of well-characterized hematological malignancies of both myeloid and lymphoid origin. It is very clear that similar profiles exists in the primary samples, however in most instances the cell lines exhibit higher levels of CpG methylation. This maybe because immortalize cells exhibit increased DNMT1 activity and an increased methylation, which in turn may allow for clonal selection over time (Vertino et al., 1996; Smiraglia et al., 2001) or because the primary samples are a mix of leukemic blasts and normal cells. Unfortunately we do not have blast count information for the majority of our primary samples. Therefore we considered an average methylation of above 40% for all the CpGs within the pyrosequencing amplicon to represent a hypermethylated state.

Prior to determining the methylation profile in the primary samples, we performed the pyrosequencing assay in a panel of normal leukocytes to determine the base-line methylation. We compared DNA extracted from peripheral leukocytes from 3 different age groups. This is because the onset of certain hematological malignancies varies with age, and our primary samples collected at diagnosis reflect this. The 3 groups analyzed represent extremes of age; newborns, adults (25-40 years) and centenarians. The methylation values of both *WT1* and *AWT1* promoters were extremely stable, being uniformly unmethylated, even though it is known that methylation at specific loci can increases with age and that DNA methylomes of newborns and centenarians are distinct (Heyn et al., 2012).

In general the myeloid origin samples, which is composed of myeloproliferative (CML, ET and PV) and AML neoplasms had a very distinct methylation profile, which consisted of an unmethylated *WT1* CpG island and a severely hypermethylated *AWT1* promoter. Despite being significantly methylated compared to controls, this hypermethylation was less prominent in the myeloproliferative neoplasms. Interestingly, the hypermethylation of *AWT1* in AMLs was similar for all the known fusion genes, suggesting that *AWT1* hypermethylation is a universal marker for AML. In the lymphoid neoplasms, the promoters did not present such distinct pattern as in the myeloid origin samples, and in most of the cases the promoters were only mildly methylated. The exceptions are the B-cell leukemias caused by MLL translocations that presented with a *WT1* and *AWT1* profile indistinguishable from the AMLs. The only sample of T-lymphoblastic leukemia was hypermethylated at both regions. Recent large-scale epigenetic studies of leukemia patient cohorts have demonstrated that aberrant DNA methylation is a hallmark of specific sub-types (Figueroa et al., 2010). The methylation profiles can be used to classify leukemias in distinct clusters defined by specific patterns of methylation, often associated with known translocations or genetic mutations, such as inv(16),

t(8;21), t(15;17), *NPM1* mutations among many others. These methylation profiles can also predict clinical outcome. For example, hypermethylation of *DBC1* is a prognosis marker for AML with normal karyotype (Alvarez et al., 2010). Recently, mutations found in *IDH1/IDH2*, that disrupt the function of TET2, cause a specific hypermethylation signature in AML contributing to the pathogenesis (Figueroa et al., 2010).

IS *AWT1* HYPERMETHYLATION A USEFUL BIOMARKER FOR LEUKEMIA?.

To be a useful biomarker, the assay has to differentiate between affected patients from people without the disease. To date only a handful of molecular tests that aid clinical decision-making are commercially available. Biomarkers can be used for multiple clinical settings: estimating risk of disease, screening occult primary cancers, distinguishing between benign and malignant forms, determining prognosis and recurrence, or even following response to therapy. In order for a biomarker to be applied into clinic, there are a number of obstacles to surpass such as analytic validity, clinical validity, and clinical utility (Teutsch et al., 2009). The expression of *WT1* is currently used as a biomarker for MRD following chemotherapy and/or allogenic BM transplantation in AML. However, determining the expression levels of *WT1* is more laborious that detecting DNA methylation changes, and has the benefit that the bisulfite analysis can be performed on stored samples. Moreover, hypermethylation markers are much more indicative that the use of a hypomethylated markers since it is much easier to detect a gain of methylation rather than a reduction (Heyn et al., 2012). For example, hypermethylated promoters such as *MGMT* in glioblastomas are used for treatment choices, and in prostate cancer, *GSTP1* for diagnosis.

In order to use the *AWT1* hypermethylation as a biomarker for the diagnosis, positive predictive value (PPV), negative predictive value (NPV), sensitivity and specificity values need to be calculated. Briefly, the sensitivity is the ability of a

test to correctly classify an individual as diseased. The specificity is the ability to correctly classify an individual as disease free. The PPV is the percentage of patients with a positive test who actually have the disease and, finally, the NPV is the percentage of patients with a negative test who do not have the disease. The values of these parameters for the *AWT1* and *WT1* promoter in the various hematological malignancies are listed in the following Table D.1.

Table D.1: Values obtained for sensitivity, specificity, PPV and NPV for the *AWT1* and *WT1* promoters in various hematological malignancies.

	AWT1				***WT1***			
DISEASE	SENSITIVITY	SPECIFICITY	PPV	NPV	SENSITIVITY	SPECIFICITY	PPV	NPV
COMPLETE COHORT	45,88%	100%	100%	38,05%	27,57%	100%	100%	31,39%
CML	33,33%	100%	100%	93,39%	7,69%	100%	100%	90,40%
AML	85,84%	100%	100%	87,60%	1,77%	100%	100%	50,45%
B-ALL	73,08%	100%	100%	94,17%	3,85%	100%	100%	81,88%
CLL	13,92%	100%	100%	62,43%	79,75%	100%	100%	87,60%
BL	27,50%	100%	100%	79,58%	25%	100%	100%	79,02%
FL	20%	100%	100%	66,86%	21,29%	100%	100%	68,07%

In normal controls, peripheral blood leukocytes, both promoters were hypomethylated never being more than 15% methylated, therefore using a cut-off of 40% methylation the specificity and PPV values are 100%. This indicates that the analysis of these promoters can correctly detect and classifies a healthy individual. The sensitivity and NPV values change according to the disease and promoter analyzed. In general, the methylation at the *WT1* promoter has poor sensitivity and NPV except for CLL, which were 79,75% and 87,6% respectively. The hypermethylation of the *AWT1* promoter is much better, with high sensitivity and NPV values for AML and B-ALL. The *AWT1* hypermethylation was clearly more severe in AML than B-ALL (range 10-93% and 5-82% respectively), making this an ideal marker to detect and correctly classify AML.

When comparing these results with other well-characterized methylation markers in other forms of cancer, the outcomes are extremely encouraging. For example, one study revealed that methylation of *APC*, *GSTP1*, *RASSF1A*, *CDKN2A*, *RUNX3* and *SFRP1* are all significantly elevated in hepatocellular carcinoma (HCC) compared to both adjacent tissue and normal liver samples and that the presence of at least one hypermethylated maker is found in almost all HCC cases. However, when combined and using 30% methylation cut-off, the *APC*, *GSTP1*, *RASF1A* and *SFRP1* hypermethylation test only reach 76.6% of sensitivity, 91,5% of specificity, 92,3% of PPV and 80% of NPV (Hua et al., 2011). In glioblastoma, hypermethylation of the *MGMT* promoter could be detected with 95% of sensitivity and 60% of specificity, as well as being able to detect cancer progression, overall survival and predicting response to chemotherapeutic treatment (Balaña et al., 2011). In prostate cancer, *GSTP1* promoter methylation is used as a diagnosis marker, with 82% of sensitivity and 95% of specificity (Van Neste et al., 2012). Also, it has been demonstrated that combining additional hypermethylated genes, such as *APC*, *RASSF1, PTGS2* and *MDR1* increased sensitivity up to a 100% (Yegnasubramanian et al., 2004). In a small cohort of NSCLC, the use of the combination of the *MGMT*, *CDKN2A*, and *GSTP1* hypermethylation promoters detected a sensitivity of a73% (Esteller et al., 1999).

It must be noted that when assessing all samples together, hypermethylation of the *AWT1* promoter it does not perform well as a diagnostic factor for unspecified leukemias/lymphomas. This test is therefore ideally suited to monitor MRD in which the diagnosis of AML has already been performed, and the recurrence of the disease during remission needs to be accurately monitored. To ensure that this is correct, future work should focus on quantifying *AWT1* hypermethylation in samples following treatments to determine if it correctly predicts treatment outcomes, similar to the expression of *WT1*. Indeed our small-scale analysis of cell lines treated with Decitabine, a

FDA approved treatment for AML, CML and MDS, revealed a decrease in *AWT1* methylation upon exposure to this epigenetic agent, suggesting that this marker may also be useful for monitoring specific drug treatments.

DEREGULATION OF MIR15A/16-1 AND MIR-222 DO NOT CORRELATE WITH *WT1/AWT1* EXPRESSION.

After an in depth analysis of the epigenetic profiling of the *WT1/AWT1* promoters in various cell lines we failed to identify a signature that correlates with the high expression levels of *WT1/AWT1* in AML. In the last years, it has become apparent that mRNA levels are also regulated by miRNAs. These 19-24nt ncRNAs regulate gene expression by imperfect base-pairing with complementary sequences located mainly, but not exclusively, at the 3'UTRs of target mRNAs (Calin et al., 2008). Since *WT1* and *AWT1* transcripts seem to be co-expressed and share the same 3'UTR, we hypothesized that they could be deregulated by microRNAs. Altered miRNA expression is a key signature of leukemias (Fabbri et al., 2008), with abnormal expression profiles often clustering with disease subtypes and correlating with cytogenetic and molecular subtypes (Li et al., 2008; Dixon-Mclver et al., 2008). In prior studies, Calin and colleagues demonstrated that by transfecting the miRNA15a/16-1 into the megakaryocytic cell line, MEG-01, WT1 protein levels were reduced (Calin et al., 2008). From this study we hypothesized that deregulation of the miRNA15a/16-1 tumor suppressing microRNAs, by deletion or promoter silencing, could result in an increase expression of *WT1/AWT1.* While performing our experiments it was demonstrated that miR-15a/16-1 inhibits growth of leukemia cell lines by down-regulating WT1 protein levels through an indirect, unspecified, mechanism (Gao et al., 2011). The inhibition of translation rather than *WT1/AWT1* mRNA stability by miR-15a/16-I is consistent with the absence of seed sequences in the 3'UTR of WT1 when assessed by microRNA prediction programs, such as TargetScan, EMBL, and

MiRanda. We did not identify deletions of 13q14.3 in the myeloid cancer cell lines or hypermethylation of the *DLEU2* CpG island, the promoter of the pri-miR-15a/16-1. We subsequently quantified the expression levels of these miRNAs, which failed to reveal a correlation between indigenous miRNA levels and *WT1/AWT1* mRNA abundance. In addition to the miR15a/16-1, we also quantified the expression of miR-222, a miRNA with a predicted seed sequence in the 3'UTR of *WT1/AWT1.* This microRNA was also expressed in all cell lines and controls. Due to the lack of a negative correlation, the direct involvement of these 3 miRNA is unlikely, however to discount a role of these miRNAs in leukemia initiation would require additional experiments such as GFP-WT1 3'UTR reporter analysis in cells transfected with the mature miRNAs and the depletion of the miRNAs in immunosorted CD34+ cells.

GATA-2 CO-EXPRESSION AND REGULATION OF _WT1/AWT1_ EXPRESSION.

So far, our data suggests that the mechanisms leading to expression of these transcripts in AML remains elusive. Recently Furuhata and colleagues demonstrated that the transcription factor GATA-2 binds to an enhancer located 1kb past the *WT1* 3'UTR activating the transcription (Furuhata et al., 2009). *GATA-2* gene encodes a transcription factor, which plays a critical role at various stages of hematopoiesis, and determines the fate of distinct myeloid lineages, therefore being tightly regulated (Vicente et al., 2011). Alterations in the expression of this transcription factor have been reported in patients with AML, CML and MDS (Fadilah et al., 2002; Tenen, 2003; Luesink et al., 2012). In one recent study, Vicente and colleagues reported *GATA-2* overexpression in a large series of patients with poorer outcomes and that individuals with abundant *GATA-2* and *WT1* expression had an even worse prognosis (Vicente et al., 2012). Our analysis in normal cells and AML cell lines revealed a correlation between the presence of *GATA-2* and *WT1/AWT1* with high transcription levels being observed solely in myeloid cell lines. This observation

is consistent with GATA-2 associated activation of *WT1/AWT1* expression by binding to the 3' enhancers, however definitive proof of this mechanism would require ChIP experiments and abolition of G*ATA-2* expression using siRNAs in myeloid cell lines.

A POSSIBLE MECHANISM OF *AWT1* HYPERMETHYALTION IN AML, AUTOREGULATION AND RECRUITMENT OF DNMT1.

Up to this point, the expression of the *WT1* and *AWT1* transcripts is not due to promoter epigenetic deregulation, but could be due to GATA-2 specific enhancer activation. Nevertheless, what is cause of hypermethylation at the *AWT1* promoter? Based on our results so far, in combination with the wealth of knowledge already in literature, we propose that AML-specific hypermethylation could be due to GATA-2 induced *WT1* expression, with subsequent *WT1* promoter autoregulation. WT1 is a zinc finger transcription factor that binds to CG rich sequences, for which there numerous functionally redundant, degenerative WT1-binding consensus sequences positioned both upstream and downstream of the *WT1* and *AWT1* transcription start sites. It was reported nearly twenty years ago that the WT1-KTS (lacking KTS) protein can bind to its own promoter by out-competing EGR-1, consequently self-regulating expression during kidney development (Malik et al., 1994; Rupprecht et al., 1994). Furthermore, in mouse embryonic fibroblast cells, the WT1 protein can recruit the EZH2 and the DNA methyltransferase DNMT1 (Xu et al., 2011). Therefore we speculate that WT1-DNMT1 protein complex may bind to degenerative 5′-GCGTGGGAGT-3'sequences within the *AWT1* promoter resulting in hypermethylation. Preliminary results suggest that this is plausible, since co-immunoprecipitation experiments revealed interaction between WT1 and DNMT1 in various co-expressing cell lines. Subsequently, we verified that in myeloid cell lines with *AWT1* hypermethylation, both WT1 and DNMT1 were present at this region. To fully confirm this mechanism, overexpression of WT1

in a normal myeloid progenitor cells without endogenous expression would be needed to determine if this is sufficient to initiate the cascade of DNMT recruitment and hypermethylation. However transfection/infection of myeloid precursors is notoriously difficult and which of the 36 WT1/AWT1 isoforms to express is unknown. It would also be interesting to determine whether WT1 can recruit the *de novo* methyltransferases, rather than just the maintenance factor, DNMT1. It was recently shown that WT1 directly regulates transcription levels of DNMT3A (Szemes et al., 2012). Unfortunately commercial antibodies directed against DNMT3A and B are notoriously bad, and we were unable to optimize them for ChIP, co-IPs or western blotting.

Taken together, accumulating evidence suggests that WT1 may be a key developmental regulator of DNA methylation, either by direct recruitment of the enzymes or by managing the DNMTs transcription. However, similar to our hypothesis for H3K36me3, this theory only applies to cell lines expressing *WT1* and not those with *AWT1* hypermethylation lacking expression.

CONCLUSIONS

1. Expression of the *WT1*, *AWT1* and the antisense *WT1-AS* transcripts are present in fetal kidney and not in any other normal tissue evaluated. This expression is not regulated by DNA methylation as all normal tissues are associated with unmethylated CpG islands.

2. The hemimethylation reported at *WT1-ARR* regulatory element is not enriched on the maternal allele, and therefore cannot be an imprinting control region. The methylation profile of this region is associated with a mosaic profile that gains methylation in a temporal fashion in kidney.

3. Both, the RNA and protein levels of *WT1* and *AWT1,* are present in all of the myeloid leukemia cell lines evaluated, but not in peripheral blood or immunosorted cells (CD19+, CD3+).

4. The promoter CpG island associated with the *AWT1* transcript is frequently hypermethylated in leukemia and lymphoma cell lines, despite aberrant overexpression. The hypermethylation of this region is associated with a concomitant change in histone modifications, with the replacement of H3K4me2 for H3K9me3.

5. The hypermethylation at *AWT1* promoter is not 5hmC.

6. In a large cohort of well-characterized primary hematological malignancies, the *WT1* locus is frequently hypermethylated, with the profiles similar to the cell lines. In 149 AML samples, the *AWT1* promoter is hypermethylated in 89%, suggesting that methylation profile of this region maybe an excellent molecular marker.

7. Decitabine treatment in AML cell lines induces demethylation of the hypermethylated *AWT1* promoter.

8. The aberrant overexpression of *WT1/AWT1* in AML is not associated with loss of regulatory miRNAs. The miR15a/16-1 and miR-222 have been suggested to regulate WT1, but we observe no inverse correlation between *WT1/AWT1* mRNA and miRNA levels.

9. The overexpression of *WT1/AWT1* in AML is associated with the aberrant expression of the GATA-2 transcription factor, which binds to an enhancer located 1Kb past the *WT1/AWT1* 3'UTR inducing expression.

10. The WT1 protein co-precipitates with DNMT1, and both are enriched at the hypermethylated *AWT1* promoter region suggesting an epigenetic autoregulation.

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APPENDIX

APPENDIX

PRIMER SEQUENCES

Mutation analysis

PRIMER	SEQUENCE	PRODUCT SIZE
WT1 exon 1 F	5’-GCTTGGGCTGCTGAGTGAATG	1034bp
WT1 exon 1 R	5’-GGTCAAAAGGGGTAGGAGAGG	
AWT1 exon 1 F	5’-CAGGGACAGCGCGCTCTAATC	384bp
AWT1 exon 1 R	5’-CGAAGAAGTTTTCTCTAGACG	
WT1 exon 2-3 F	5’-GGTCCGAATGCGGGGTTCAG	891bp
WT1 exon 2-3 R	5’-GACCCTGCATGCCCGCAGTCAG	
WT1 exon 4 F	5’-CATTCTGGAAAATGTGGAGGC	282 bp
WT1 exon 4 R	5’-CTTCATAAGTTCTAAGCACC	
WT1 exon 5 F	5’-CTGGGGACTTAGTTCAGCACTC	289bp
WT1 exon 5 R	5’-TGCTACCCTGATTACCCAC	
WT1 exon 6 F	5’-GCTTTAAAAACCATCATTCCC	298bp
WT1 exon 6 R	5’-GAGGCTGCCAGGGCCAAAGAG	
WT1 exon 7 F	5’-CACCTGCCACCCCTTCTTTGG	372bp
WT1 exon 7 R	5’-GCAGTGCTTACTTTCCATCCTGG	
WT1 exon 8 F	5’-CCTAACAAGCTCCAGCGAAG	514bp
WT1 exon 8 R	5’-CAAATTGGATTCCGCTCTCCATC	
WT1 exon 9 F	5’-GGCAGATGCAGACATTGCAGG	240bp
WT1 exon 9 R	5’-TGTGAAGAAAAGTTTACGCA	
WT1 exon 10 1F	5’-GATGACTTGCACTCGGGCCTTG	793bp
WT1 exon 10 1R	5’-GGTAAAATTCTTTTAGATTTCTATAG	
WT1 exon 10 2F	5’-GTCAGCCAGGCTGCTAACCTGG	850bp
WT1 exon 10 2R	5’-CGGTGGCGTCTTCTCTTACCTG	

Methylation analysis

PRIMER	SEQUENCE	PRODUCT SIZE
WT1 bis OUT F	5’-GGAGGTGGGGGGAGGGTTGTG	270bp
WT1 bis OUT R	5’-CCTTACCCCAACTACCTAAC	
WT1 bis IN F	5’-GTGTTGGGTTGAAGAGGAGGG	199bp
WT1 bis IN R	5’-CCCTCAACTTCCCAAAACTC	
WT1-ARR bis OUT F	5’-GGTTATATGATTTGAATTTAGGG	506bp
WT1-ARR bis OUT R	5’-CTACCCACCACCCTCTTAAAAC	
WT1-ARR bis IN F	5’-GGGTGGAGAAGAAGGATATATTTAT	394bp
WT1-ARR bis IN R	5’-CAACCATCTAAACATCTATAAC	
AWT1 bis OUT F	5’-GGATAGTAGGTTTAGTTAGG	263bp
AWT1 bis OUT R	5’-CCTACTAATCCACTAATCCC	
AWT1 bis IN F	5’-GGAGGTGTTTAGAGGTTG	187bp
AWT1 bis IN R	5’-CCCTAAAACATAATTAAACC	
miR15/16 bis OUT F	5’-GGGTTTGGGAAATTTTTTTTGGGG	261bp
miR15/16 bis OUT R	5’-AATCTATACAACAAAACCCCC	
miR15/16 bis IN F	5’-GATAATATAGATAGATGGTG	215bp
miR15/16 bis IN R	5´-CTCCTTAACAAAAAAACTAA	
GATA-2 bis OUT F	5’-AGGGTAATTTTTATTATGTTAATTT	350bp
GATA-2 bis OUT R	5’- GTTGGTTTGTGAGGGTTTTTGTAGG	
GATA-2 bis IN F	5’-AGGGTAATTTTTATTATGTTAATTT	253bp
GATA-2 bis IN R	5’-GTTTGGGATTTATAGGGTTTTTGT	

Expression analysis

PRIMER	SEQUENCE	PRODUCT SIZE
WT1 qRT-PCR F	5'-CGCTATTCGCAATCAGGGTTAC	125bp
WT1 qRT-PCR R	5'-ATGGGATCCTCATGCTTGAATG	
AWT1 qRT-PCR F	5'-GAGAAGGTTACAGCACGGTCAC	109bp
AWT1 qRT-PCR R	5'-ATGGGATCCTCATGCTTGAATG	
WT1-AS qRT-PCR F	5'-AGAGTCCGTTCAGAATCCTTG	123bp
WT1-AS qRT-PCR R	5'-AGGCTGCAGGGAACTCCTCCCA	
DNMT1 qRT-PCR F	5'-AGATGGAGACGAGAAAGATGA	250bp
DNMT1 qRT-PCR R	5'-ACTGAATGCACTTGGGAGGGTGG	
GATA-2 qRT-PCR F	5'-GGCTCGTTCCTGTTCAGAAGGC	164bp
GATA-2 qRT-PCR R	5'-TGGTCGGTTCTGCCCATCATCTGT	
β-ACTIN qRT-PCR F	5'-CCTGACGGCCAGGTCATCAC	155bp
β-ACTIN qRT-PCR R	5'-GGAGCAATGATCTTGATCTTC	
GAPDH qRT-PCR F	5'-CGTATTGGGCGCCTGGTCACC	180bp
GAPDH qRT-PCR R	5'-CACCACCTTCTTGATGTCATC	

Allelic expression

PRIMER	SEQUENCE	PRODUCT SIZE
WT1 F	5'-CGCTATTCGCAATCAGGGTTAC	1560bp
WT1 R	5'- GTATAGAAATCTAAAAGAATTTTACC	
AWT1 F	5'-GAGAAGGTTACAGCACGGTCAC	1515bp
AWT1 R	5'-GTATAGAAATCTAAAAGAATTTTACC	
WT1-AS F	5'-CAGAGCTAATGGGATGCAGAG	433bp
WT1-AS R	5'-GCTTTCGACTAGCGCCTCTC	

Pyrosequencing primers

PRIMER	SEQUENCE	PRODUCT SIZE
WT1 F	5'-Bio-GGTTGAAGAGGAGGGT	197bp
WT1 R	5'-GAGTTTTGGGAAGTTGAGGG	
WT1 Pyrosequencing	5'-GGTTTGGGTTGTTGAGTGAATGG	
AWT1 F	5'-Bio-TAGGAAGGTGTTTAGAAGGTT	187bp
AWT1 R	5'-GGTTTAATTATGTTTTAGGG	
AWT1 Pyrosequencing	5'-TATTTGATTTTAGGGTGG	
LINE-1 F	5'-TTTTGAGTTAGGTGTGGGATATA	73bp
LINE-1 R	5'-Bio-AAAATCAAAAAAATTCCCTTTC	
LINE-1 Pyrosequencing	5'-AGTTAGGTGTGGGATATAGT	
ALU YB8 F	5'-Bio-AGATTATTTTGGTTAATAAG	85bp
ALU YB8 R	5'-AACTACYAACTACAATAAC	
ALUYB8 Pyrosequencing	5'-AATAACTAAAATTACAAAC	
Sat-α F	5'-AGTTTAATTTATAGAGTAGAGTAG	87bp
Sat-α R	5'-Bio-AAATCTTCACTTACAAATACCA	
Sat-α Pyrosequencing	5'-TGGGATTTTTTTGAGAATTT	
miR 15a/16-1 F	5'-Bio-GATGTAGAAATGTTTTATTATT	233bp
miR 15a/16-1 R	5'-ATCATACTAAAAATAACAAGATTAT	
miR15a/16-Pyrosequencing	5'-CTCCTTAACAAAAAAACTAAA	
GATA-2 F	5'-Bio-AGGGTAATTTTTTATTATGTTAA	286bp
GATA-2 R	5'-CCCTACAAAAAACCCTCACAAACCA	
GATA-2 Pyrosequencing	5'-GTGGTGATGAGAATTTAG	

ChIP analysis

PRIMER	SEQUENCE	PRODUCT SIZE
WT1 CpG HPA II F	5'-CCTCCGGCCCTGGAGACGTTCAGC	174bp
WT1 CpG HPA II R	5'-TTGCTGCAGGACCCGGCTTCC	
AWT1 CpG HPA II F	5'-CCTTCCTGGCTGAGCCTGCTGCTGTC	188bp
AWT1 CpG HPA II R	5'-GGTAGCGCCTTTCCCACGGTTAGTC	
GAPDH ChIP F	5'-TAGTGCGCAGCGGGTGCATC	181bp
GAPDH ChIP R	5'-GCTAGCTCGCTCCACCTGACTT	
HS SAT ABCAM F	SEQ NOT PROVIDED	--
HS SAT ABCAM R	SEQ NOT PROVIDED	
PAX 2 ChIP F	5'-AGCAGCCGGGCGTTCACTCA	184bp
PAX 2 ChIP R	5'-GCCGCCGGTACTCACGGTGCAT	

Sequencing primers

PRIMER	SEQUENCE	PRODUCT SIZE
pGEM vector F	5'-CGCCAGGGTTTTCCCAGTCAC	303bp (+insert)
pGEM vector R	5'-GAGCGGATAACAATTTCACAC	

“Doing a PhD shows you that you never gave up”

Hans