Chapter 1

General Introduction

GENERAL INTRODUCTION

Epigenetics

Epigenetics (*epi-* from the Greek word *επί* meaning "over" or "above") refers to heritable meiotic and mitotic changes in gene expression that occur without a change in the DNA sequence. The best understood mechanisms that account for this form of expression regulation are DNA methylation and covalent modifications of histones.

DNA methylation

DNA methylation is a covalent modification of the fifth carbon within the cytosine DNA base; the resulting base is often referred to as the 'fifth base' in the human genome (Figure 1). In adult mammalian somatic cells, this modification occurs only on the cytosine in a CpG dinucleotide pair. The CpG notation is used to distinguish the linear sequence of a cytosine preceding a guanine bound by a phosphate from the complementary base pairing between a cytosine and guanine residue (Figure 2). The methylation of these CpGs is facilitated by the DNA methyltransferases DNMT1, DNMT3A and DNMT3B[1-4]. DNMT1 resides at the replication fork and methylates CpG dinucleotides in the newly synthesized strand, making this enzyme essential for maintaining DNA methylation patterns in proliferating cells[5-8]. DNMT3A and DNMT3B are required for *de novo* methylation during embryonic development[5-7].

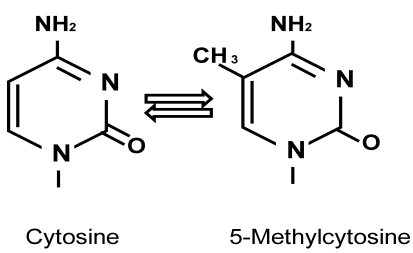

Figure 1 - Chemical structure of a cytosine nucleotide and 5-methylcytosine.

Due to spontaneous de-amination in the germ-line during evolution, CpG dinucleotides are rare within the genome[1]. However, CpG dinucleotides are enriched in DNA stretches ranging from 500 bp to several kb, and these regions are called CpG islands (GCIs)[1, 2, 4]. In contrast to the sparse CpG dinucleotides that occur throughout the genome, the majority of CGIs are hypomethylated. Approximately 60% of all genes contain a CGI within their promoter region that often expands to the first exon or intron and -regardless of the expression status of the associated geneare primarily unmethylated[4]. Although most CGIs reside in the 5' regions of genes, a large proportion of CGIs are located in inter-genic regions.

Hypermethylation of the promoter CGI is believed to down-regulate gene expression in two ways. First, DNA methylation may form a direct physical barrier against binding of the basic transcription complex or transcription enhancers (i.e., steric hindrance), thereby preventing downstream genes from being transcribed. Secondly, DNA methylation may

recruit methylation-specific proteins to the region, thus resulting in a cascade of silencing effects. Evidence for both hypotheses can be found in the literature[9]. CGI methylation is normally involved in allele-specific inactivation of imprinted genes and/or genes located on the inactive X chromosome, and aberrant CGI methylation has been found in numerous cancers[2, 10, 11].

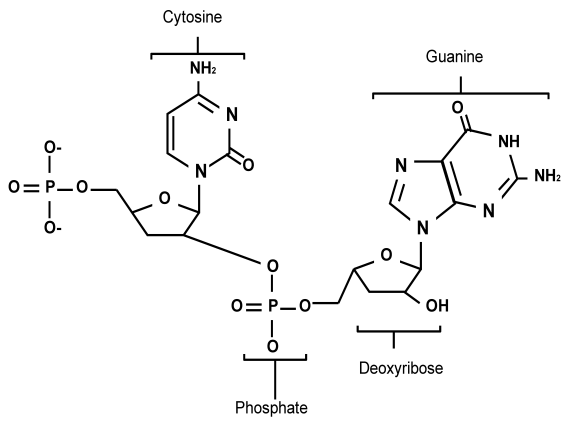

Figure 2 - Chemical structure of a CpG dinucleotide. The phosphate group (the p in CpG) indicates a deoxyribose bond between both nucleotides and thereby the 5'-3'locations of the cytosine and guanine. This annotation is used to prevent confusion with the hydrogen bonds between cytosine and guanine bases in complementary strands of DNA.

Histone modifications and chromatin state

In eukaryotes, genomic DNA is packaged with histone proteins into nucleosomes. A nucleosome consists of an octamer of histone proteins -comprised of two H2A-H2B heterodimers and two H3-H4 heterotetramers- that wrap ~146 bp of DNA around itself in 1.67 turns of a left-handed superhelix. Subsequently, these nucleosomes are themselves packed into chromatin, thus compacting DNA by approximately 10,000-fold. This 'packing' of two meters of DNA into a 1.7-µm cell nucleus is a considerable obstacle to replication, transcription and DNA repair complexes in reaching the DNA (Figure 3). To overcome this obstacle, dynamic changes in the chromatin state permit localized de-condensation from heterochromatin to euchromatin, thereby providing the nuclear machinery access to the DNA[12-16].

Condensed and de-condensed chromatin states coincide with a variety of post-translational covalent modifications of the core histone amino termini. A large number of histone modifications have been reported, among which acetylation, methylation, phosphorylation and -to a lesser extent- ubiquitination are the best characterized[12-17]. For all modifications (with the exception of arginine methylation), enzymes exist to either attach or remove the histone modification. An overview of histone modifications is presented in Table 1. The complexity of histone modifications -and our increasing understanding of their consequences- have led to the 'histone code' hypothesis. According to this hypothesis, histone modifications provide a platform for the binding of chromatin-associated regulators of gene expression[12-16].

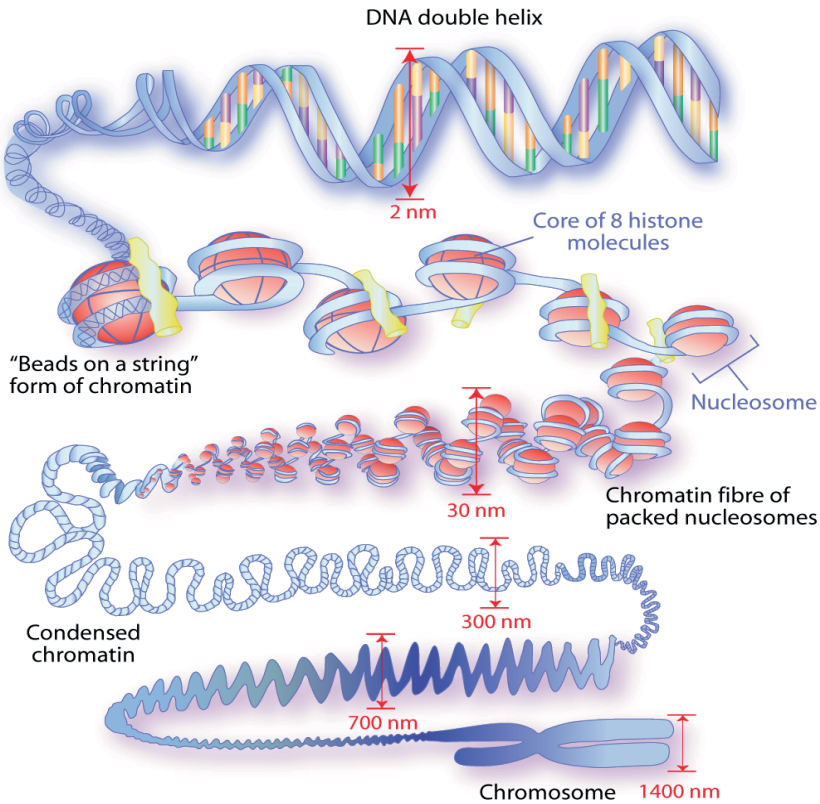

Figure 3 - Schematic representation of the sequential packaging of human DNA in the nucleus (adapted from www.epitron.eu)

Interaction between DNA methylation and histone modifications

Since epigenetic communication between DNA methylation and the chromatin state was initially described, the precise sequence of events that underlie this communication has been a subject of debate[18]. Currently, two progression models are considered to be plausible. The first model starts with initial DNA methylation that causes histone modifications via the recruitment of proteins that have methyl-DNA binding activity such as methyl-CpG-binding protein 2 (MeCP2), methyl-CpG-binding domain protein 1 (MDB1) and Kaiso (also known as the Zinc finger and BTB domain containing protein 33, or ZBTB 33). The subsequent recruitment of histone methyltransferases (HMTs) and histone deacetylases (HDACs) attach and detach histone modifications that are associated with transcriptional silencing and activation, respectively[19-25]. Finally, DNA methylation can inhibit active histone modification H3K4 methylation ($H3K4^{me}$)[26, 27].

Studies that support a model in which DNA methylation is initiated by histone modifications are increasing in number. These studies report that targets of the inactive histone modification $H3K27^{me3}$ and the enrichment of polycomb group 2 (PRC2) proteins in both embryonic (ES) and adult stem cells are pre-marked for *de novo* methylation in cancer[28-31]. Additional functional insights allowed the linking of PCR2 proteins, the presence of the

inactive histone mark H3K27^{me3} and absence of H3K4^{me3} to the recruitment of DNMTs and subsequent DNA methylation (Figure 4)[32-36]. The aforementioned studies led to a developmental model in which the balance between binding the mediators of inactivating histone mark H3K27^{me3}, PRC2 and the mediators of the activating histone mark H3K4^{me3}, the trithorax-group proteins, determine the DNA methylation and expression states of the regions to which they bind (Figure 4)[28-31, 37-40].

Although studies addressing this subject have not yielded conclusive evidence to support this model, they have revealed a high level of synergy between histone modifications and DNA methylation in regulating gene expression. Histone modifications are believed to act either sequentially or in combination with DNA methylation to generate the proposed histone code, which in turn conveys information to the nuclear machinery[15].

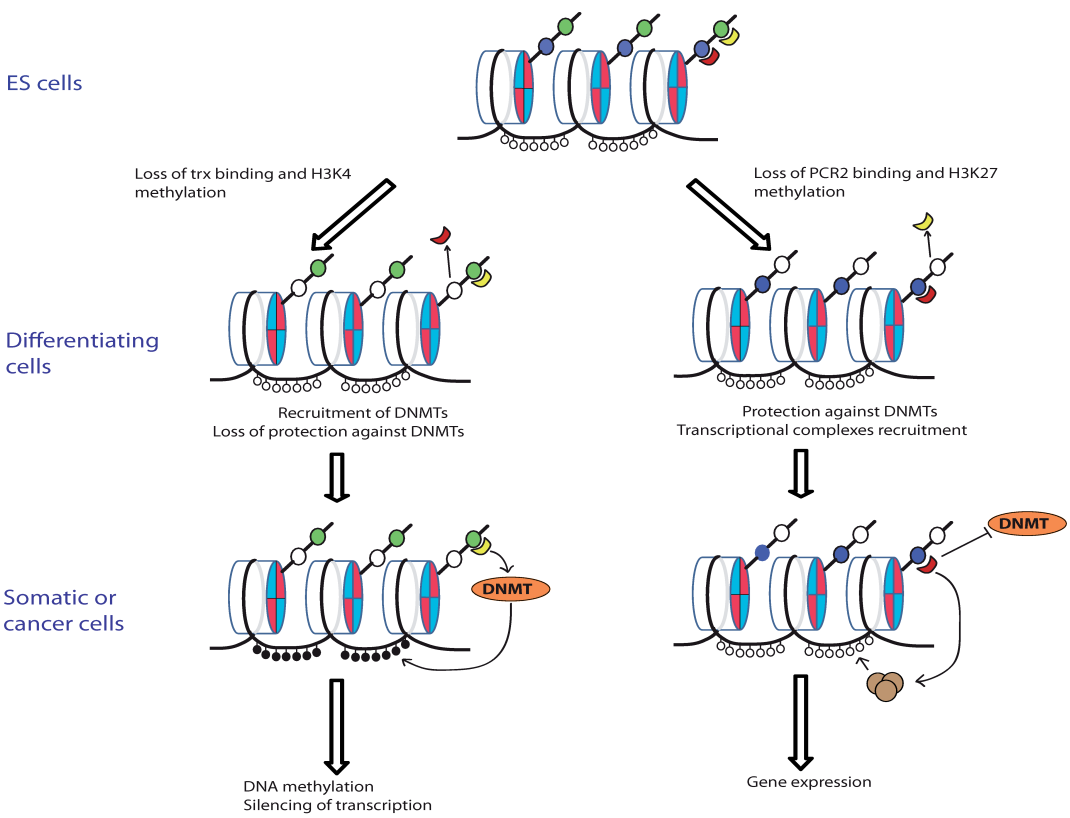

Figure 4 - Model of epigenetic regulation of gene expression in differentiation and tumorigenesis. Three nucleosomes that are composed of an H3-H4 hetero-tetramer (blue), two H2A-H2B dimers (red), the DNA (black line) with CpG dinucleotides (open circles attached to the DNA) and a histone tail with H3K4 (purple circle) and H3K27 (green circle) methylation are represented. A loss of PCR2 (yellow crescent) association during differentiation results in the loss of repressive H3K27 methylation, thereby allowing the binding of transcriptional complexes (light brown). The disassociation of trx family proteins (red) results in the loss of H3K4 methylation-mediated protection against DNMT (orange) recruitment. The remaining H3K27 methylation actively recruits the DNMT complexes, thereby resulting in methylation of the associated CpG dinucleotides (black circles attached to the DNA). The association of the trx or PCR2 complexes during differentiation can determine both the transcription of genes and downstream DNA methylation in somatic or cancer cells.

Table 1 - Histone modifications, locations and modifiers

Histone	Modification	Site	Enzyme	Proposed function
H2A	Acetylation	K5	TIP60/PLIP, HAT1, CBP/p300	Transcriptional activation
	Phosphorylation	S1	MSK1	Transcriptional repression
		T120	NHK-1	Mitosis
		S139	ATR, ATM, DNA-PK	DNA repair
	Ubiquitination	K119	HR6A	Spermatogenesis
H2B	Acetylation	K5	ATF2	Transcriptional activation
		K12	CBP/p300, ATF2	Transcriptional activation
		K15	CBP/p300, ATF2	Transcriptional activation
		K20	CBP/p300	Transcriptional activation
	Phosphorylation	S14	Mst1	Apoptosis
	Ubiquitination	K120	RNF20/hBRE1, RNF40, HR6A, HR6B,	Transcriptional activation
H3	Acetylation	K9	PCAF, GCN5	Transcriptional activation
		K14	PCAF, GCN5, TIP60/ PLIP, hTFIIIC90, TAF1, CBP/p300	Transcriptional activation
		K18	CBP/p300, PCAF, GCN5	Transcriptional activation
		K23	CBP/p300	Transcriptional activation
		K27	GCN5	Transcriptional activation
	Phosphorylation	T3	HASPIN	Mitosis
		S10	TG2, MSK1, MSK2	Transcriptional activation
		T11	DLK/ZIP	Mitosis
		S28	MSK1, MSK2	Transcription activation
	Methylation	K4	MLL($me^{1/2}$)	Transcriptional activation
			MLL2-4($me^{1/2/3}$)	Transcriptional activation
			SET1A, SET1B($me^{1/2/3}$)	Transcriptional activation
			SMYD3($me^{2/3}$)	Transcriptional activation
			SET7/9($me^{1/2}$)	Transcriptional activation
		K9	CLL8, RIZ1, SUV39h1, SYV39h2, ESET, G9A, EZH2	Transcriptional repression
		R17	CHARM1	Transcriptional activation
		K27	EZH2, G9A	Transcriptional silencing, X-inactivation (tri-methylation)
		K36	NSD1, SMYD2, SET2	Transcription activation, De-acetylation(single methylation)
		K79	DOT1L	Transcription activation, elongation / memory
H4	Acetylation	K5	HAT1, TIP60/PLIP, CBP/p300, HBO1	Transcriptional activation
		K8	TIP60/ PLIP, CBP/p300, HBO1	Transcriptional activation
		K12	HAT1, TIP60/PLIP, HBO1,	Transcriptional activation
		K16	TIP60/PLIP	
	Phosphorylation	S1	-	Mitosis
	Methylation	R3	PRMT1	Transcriptional activation
		K20	SET7/8, SUV4-20H1-2	Transcriptional repression

Chromatin state, activity and nuclear position

As mentioned above, chromatin status coincides with specific histone modifications (and thus to DNA methylation). These modifications are believed to regulate chromatin density either directly or by providing a surface substrate for interactions with other proteins[12, 16, 41]. Gene-rich and transcriptionally active regions can therefore be maintained as euchromatin, whereas gene-poor and transcriptionally inactive regions can be condensed to form heterochromatin.

Chromatin density -and thus transcriptional activity- is associated with specific interphase locations within the nucleus' volume. Heterochromatin generally clusters into condensed chromocenters that are located in the vicinity of the nucleolus, whereas active euchromatin is located in the central region and nuclear border[42]. This organization is not random, as differences have been reported based on cell type, shape, quiescence, commitment, functional status or transformation[43]. The availability of euchromatin to the interchromatin compartment -a channel network that is connected to the nuclear pores- has been postulated to facilitate transcription by the nuclear machinery that is located within this interchromatin compartment[44, 45]. Chromatin domains that contain transcriptionally active genes form euchromatic chromatin loops that migrate from the chromocenters to -or into- the interchromatin compartment[46-48].

Because histone modifications determine transcriptional activity and chromatin condensation, a reciprocal impact on nuclear architecture would be expected. Cremer et al. studied the relation between histone methylation and nuclear location in breast cancer interphase nuclei and reported clustering of histone methylation in close proximity to the nucleoli and -to a lesser extent- in the nuclear periphery[49]. Studies that investigated the relation between nuclear location and specific histone modifications for active (i.e., H3K4^{me3}, H4K20^{me1} and H4K20^{me3}) and inactive (i.e., H3K9^{me1}, H3K9^{me3} and H3K27^{me3}) chromatin revealed that methylation patterns are arranged in distinct nuclear layers, with a certain degree of overlap that depends on the type of epigenetic modification[50, 51].

Although the relations between gene activity, chromatic condensation and spatial location in the nucleus are less pronounced in quiescent cells than in proliferating cells, genomic loci that are found in the same chromosome territories during S phase are likely to be replicated at the same time and come into contact with the same chromatin factors following replication[52]. This provides a means to re-establish a given transcriptional and/or spatial pattern of organization in the daughter cells, as the factors that mediate the chromatin state are proposed to act coordinately on newly replicated loci[52]. As such, subnuclear compartments may not be critical for immediate biological events but may provide a mechanism for an accurate heritable transmission of the chromatin state and transcription patterns[52].

Lamina binding

A mechanism for anchoring chromatin to subnuclear compartments -and more specifically, to the nuclear envelope- occurs via binding of chromatin to the nuclear lamina (NL). The core of the NL consists of nucleus-specific, type V intermediate filament lamin proteins. These lamin proteins can be divided into A-type lamins, which are found predominately in differentiated cells, and B-type lamins, which are essential for cell viability[52, 53]. Stable

interactions between lamins and lamin-associated polypeptides (LAPs) are integral for both maintaining mechanical integrity of the nuclear envelope and providing anchor points for the aforementioned chromatin binding to the NL[53, 54]. The interaction between the NL and chromatin-associated proteins is mediated through LAPs, which bind to both the NL and to chromatin-associated proteins such as BAF, HP1 and Rb (see references 52, 54, and 55 for an overview).

Both genomic and proteomic experimental approaches have identified an association between the NL and heterochromatin. Although a putative role for the NL in the formation and/or maintenance of heterochromatin remains unclear, the NL is believed to anchor heterochromatin to the nuclear periphery, thereby providing structure and associated replication timing (Figure 5). A genetic approach to the study of B1-type lamin-associated DNA in human fibroblasts has identified 1,344 sharply defined DNA domains of 0.1-10 Mb each[56]. These lamina-associated domains (LADs) are characterized by hallmarks of heterochromatin such as a low level of gene expression, low gene density, high levels of H3K27^{me3} and low levels of H3K4^{me2}. Interestingly, these LADs are demarcated (Figure 5) by CpG islands, promoter regions driving transcription away from LADs and binding regions of the insulator protein CCCTC-binding factor (CTCF)[56].

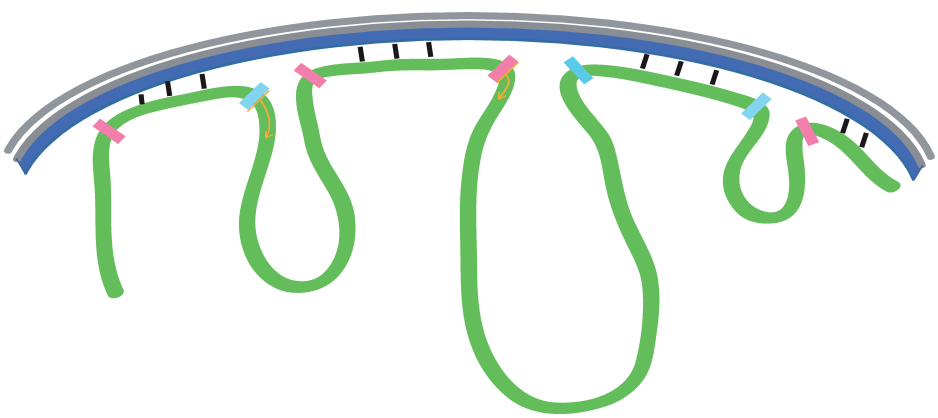

Figure 5 – Model of chromatin binding to the nuclear lamina. Large chromatin domains (green line) are dynamically associated (depicted as black lines) with the nuclear lamina (dark blue) adjacent to the nuclear envelope (gray). The LAD regions are demarcated by putative insulator elements, including CTCF binding sites (light blue), CpG islands (pink) and promoters that are orientated away from the lamina (orange arrows)[56]. Adapted from de Wit et al.[192].

The insulator protein CTCF

In vertebrates, CTCF is a ubiquitously expressed, 11-zinc finger protein that has been shown to bind to a larger number of binding sites in the genome; the number of binding sites ranges from 13,804 to 26,814 sites, depending on the cell type, technique and method of analysis[57-61]. This 'Jack-of-all–trades' protein has been implicated in diverse roles in gene regulation, including promoter activation/repression, enhancer blocking and/or barrier insulation, hormone-responsive silencing, genomic imprinting and -most recently- long-range chromatin interactions[62]. In addition to the aforementioned correlation between LAD boundaries and CTCF, a recent genome-wide mapping study uncovered a significant proportion of CTCF binding sites that are localized to the boundaries between euchromatic

and heterochromatic domains that are marked by H2AK5^{Ac} and H3K27^{me3}, respectively[61].

The discovery of CTCF-mediated intra- and inter-chromosome loop formation at the *IGF2/H19*[63, 64] and β-*globin* loci[65, 66] gives insight into how CTCF might form loops of condensed chromatin. Although the variability of CTCF loop formation by either homo- or hetero-dimerization with one of the many suggested protein partners makes it difficult to portray CTCF in a universal model, the high number and high variation of CTCF binding sites throughout the genome suggest a key role for CTCF in nuclear architecture. It has been reported recently that CTCF binding sites are generally located in chromatin linker regions that are flanked by at least 20 symmetrically distributed nucleosomes, thus revealing both a genome-wide role for CTCF in nucleosome positioning and a link to the regulation of chromatin structure[67]. Among CTCF's many protein partners, the recruitment of the Polycomb Repressor Complex 2 member Suz12 by DNA-bound CTCF is associated with the subsequent acquisition of H3K27^{me3}, indicating that CTCF binding might initiate local heterochromatin formation[68].

Studies of CTCF binding to the imprinting control region of *IGF2/H19* have shown that CTCF binding is DNA methylation sensitive[69, 70]. Additionally, methylation of a single CpG dinucleotide within the CTCF consensus sequence of the chicken *β-globin* gene is sufficient to block CTCF binding. This finding has led to the classification of CTCF binding sites into the following three groups: sites without CpG dinucleotides, sites that contain DNA methylation and unmethylated sites. A small-scale comparison between pre-B and thymocyte cell lines found that sites with unchanged CTCF occupancy are generally unmethylated, whereas sites that display differential binding between lineages may acquire CpG methylation[69, 71]. Not only does the binding of CTCF appear to be DNA methylation sensitive, but the recruitment and activation of the DNMT1 inhibitor PARP-1 by DNA-bound CTCF seem to indicate a protective function against methylation of CTCF binding sites that contain CpG dinucleotides[72, 73]. Interestingly, a specific subset of CTCF remains associated with chromosomes during mitosis, suggesting a possible role in the maintenance of epigenetic marks throughout cell division[74, 75]. Together with its insulator function, the protection of CTCF's own binding sites throughout cell division could link epigenetic transcriptional regulation and nuclear architecture and could explain epigenetic heritability through cell division in differentiated cells. Naturally occurring DNA sequence variations can also influence CTCF binding. For example, a polymorphism in a CTCF binding site downstream of *MMP-7* that leads to differential CTCF binding is a possible genetic factor in breast cancer[76].

DNA methylation in cancer

Aberrant methylation of CpG dinucleotides is commonly seen in cancer and -shown by studies of this phenomenon- is recognized as an important step in tumorigenesis[4, 77]. In carcinomas, hypomethylation of the genome is accompanied by regional hypermethylation of CGIs compared to the normal epithelium cells from which they arise[2, 4, 77]. Global hypomethylation has been linked to both genomic instability and increasing mutation rates, whereas hypermethylation of promoter CGIs can lead to transcriptional inactivation of the associated gene[78, 79]. This aberrant CGI hypermethylation is accompanied by the recruitment of methyl-CpG binding domain (MBD) proteins and histone deacetylases (HDACs) and is associated with histone modifications that are associated with expressional

down-regulation[80]. In various types of cancers, promoter hypermethylation of tumor suppressor genes (TSGs) such as *p16INK4a*[81-83], *MLH1*[84-87], *BRCA1*[88, 89] and *Rb*[90] have been described.

Hypermethylation of CGIs in tumors is part of a cascade that can lead to the down-regulation of expression through changes in the histone code and possibly even via the nuclear location of the associated DNA. Due to the robust nature of DNA methylation, changes in the DNA methylome can be detected using various techniques, and there exists a huge potential for the use of DNA methylation as a diagnostic and/or prognostic marker[91]. Additionally, the identification of aberrancies in epigenetic regulation might provide new insights into tumorigenesis and perhaps pave the way for the development and application of new cancer treatments that reverse DNA methylation.

The initiation of cancer-related DNA methylation has been a focus for researchers since it was first discovered. The aforementioned complex interplay between DNA methylation with histone modifications and their mediators yields a large group of epigenetic machinery proteins that can play a role in epigenetic tumorigenesis. A complete understanding of the initiation and impact of DNA methylation in tumorigenesis is needed to distinguish between randomly accumulated DNA methylation and the methylation of targets that are important in the development of cancer.

Colorectal cancer: clinical context

Colorectal cancer (CRC) is the third and second most common type of cancer in males and females, respectively, and one of the leading causes of cancer-related deaths in both Europe and the US[92, 93]. In the Netherlands, the lifetime risk for developing CRC is 6% (an incidence of approximately one in 17) among both genders. In recent years, the number of new CRC cases and associated deaths has seemingly decreased in developed countries, and this is possibly due to improved screening methods and early diagnosis[92, 93]. However, in Japan and other developing countries, the incidence of CRC is increasing, and this is believed to reflect a combination of factors that are related to a Western lifestyle, including changes in dietary patterns, obesity and an increased prevalence of smoking[92-96]. Worldwide, it is estimated that approximately one million new cases are diagnosed annually[92, 93, 96]. Over 95% of colorectal cancers are adenocarcinomas, and approximately half of these patients develop a local recurrence or a distant metastasis during the course of the disease. Survival depends greatly on early detection, particularly before the tumor has metastasized[97]. The five-year survival rate ranges from 93.2 to 82.5% for the early stages in which no lymph node metastasis has occurred yet[98]. In cases of lymph node metastasis (stage III; see www.UICC.org) or distant metastasis (stage IV), the survival rates are 59.5 and 8.1%, respectively. Stage III and stage IV tumors are typically treated with chemotherapy consisting of 5-fluorouracil compounds either with or without oxaliplatin or irinotecan[97, 99]. In recent years, insights into the molecular pathogenesis of colorectal cancer have led to the use of targeted therapeutics that are specific for the epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF)[97, 99]. Although the success of these therapies in CRC is limited, these examples illustrate how molecular biological research contributes to the development of promising new therapies.

Tumorigenesis of CRC

The accumulation of genetic and epigenetic changes results in the progressive transformation of normal colon epithelium to hyperplasia, dysplasia and eventually adenocarcinoma. This stepwise progression of tumorigenesis in colorectal cancer has served as an example of other types of tumors. The recently updated yet classic Vogelgram[100] shows that colorectal neoplasias can be characterized based on molecular features. The predilection for specific molecular alterations at different sites in the colon is remarkable. Right-sided (proximal) and left-sided (distal) CRC[100-103] can be seen grossly as the following two classic and distinct genetic pathways (Figure 6): tumors with high levels of chromosomal instability (CIN) or microsatellite instability (MSI or MSI high/MSI-H). The CIN pathway (which comprises 50-70% of sporadic colon cancers) is characterized by a change in chromosomal copy number such as a chromosomal gain, loss or a copy-neutral loss of heterozygosity (cnLOH)[104]. Tumors that arise via this pathway are often located in the left-sided colon (i.e., distal to the splenic flexure) and are often aneuploid. Although these CIN colon tumors progress through the adenoma-carcinoma progression pathway, the facilitating mechanism is not completely understood. Specific mutations in genes that are involved in mitotic spindle checkpoints and DNA replication checkpoints (e.g., *hBUB1* and *hBUBR1*) have been proposed to underlie CIN, and self-propagating genomic instability can occur in the absence of genetic mutations[104-108]. To date, no data have been provided compelling evidence that mutations in any of these genes provide more than a permissive role for CIN, despite the tight association between CIN and mutant APC and p53[106].

Tumors that arise via the MSI pathway (comprising ~15% of sporadic colon cancers) are typically diploid, right-sided (i.e., before the splenic flexure) and carry small deletions and/or insertions in short repetitive sequences (A_n or CA_n, where n is the number of repeats) as a result of a loss of function of any of the DNA mismatch repair (MMR) genes[106]. In colon cancer, MSI is found in the context of Lynch syndrome (previously known as hereditary non-polyposis colorectal cancer, or HNPCC) with germline mutations in one of four MMR genes, primarily in *MLH1* or *MSH2*[109] and -to a lesser extent- in *MSH6*[110] or *PMS2*[111]. Deletions in *EPCAM/TACSTD1*, which is upstream of *MSH2*, cause sequential *MSH2* methylation[112, 113]. Although rare, several studies have described inherited and *de novo* germline methylation of *MLH1* in patients with Lynch-like colon cancer[114-120]. Approximately 15% of all sporadic colon cancers are due to somatic biallelic or hemiallelic methylation of the *MLH1* promoter[121].

A growing understanding of the impact and level of promoter, inter- and intra-gene CGI methylation that is described as aberrantly methylated in MSI colon cancer has led to the classification of colon cancers into the following CpG island methylator phenotypes (CIMP), regardless of MSI status: CIMP1 (CIMP-high), CIMP2 (CIMP-low) and CIMP0 (CIMP-negative)[4, 122-124]. Although the definition of CIMP has been debated in the literature, an integrated genetic and epigenetic analysis provided definitions for each of these three phenotypes[124]. The phenotype with the highest frequency of aberrant methylation, CIMP1, is associated with sporadic MSI, somatic *BRAF* mutations and the methylation of a debated set of methylation markers. The methylation status of the second phenotype, CIMP2, has also been the subject of debate. Methylation has been found among cancers in this group, albeit to a lesser extent than among CIMP1 tumors. Although methylation

markers have been suggested for both groups, indecisiveness regarding a defined marker set has led to *MLH1* methylation (and thereby sporadic MSI) and *BRAF* mutations as being the best indicators for CIMP1, whereas *KRAS* and *TP53* mutations are often found in CIMP2 and CIMP0 tumors, respectively[122-126].

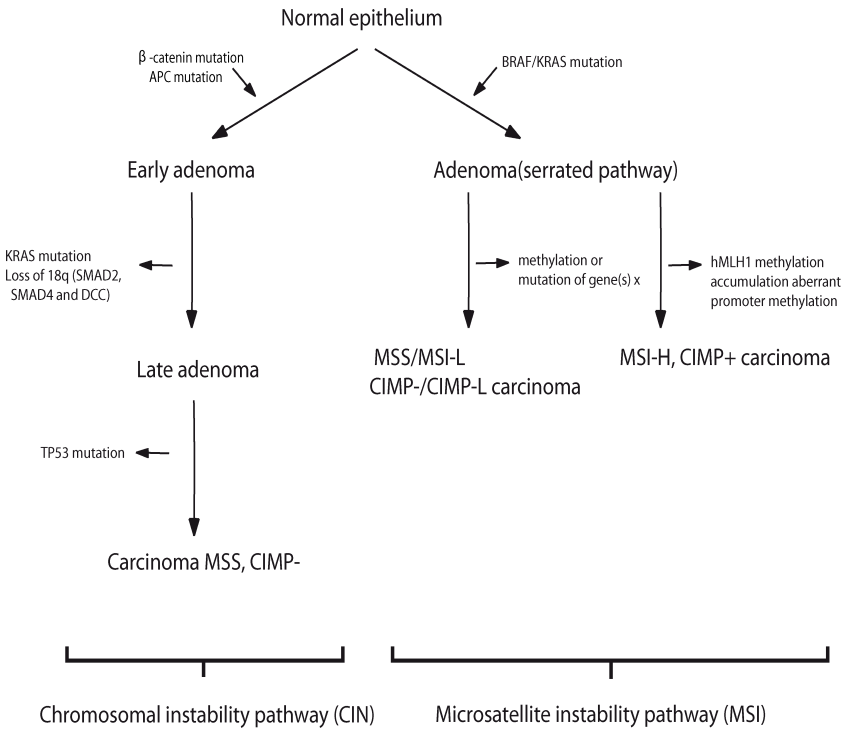

Figure 6 – A model of the CIN and MSI tumorigenesis pathways

The cause of aberrant DNA methylation in CRC

The underlying causes of aberrant methylation and subsequent sporadic MSI colon cancer remain largely unknown. Both *BRAF* and *KRAS* mutations have been observed in the earliest identified colonic neoplasms, and recent studies have provided evidence that induction of the ras oncogenic pathway results in DNA hypermethylation[127-132]. Although activating *KRAS* and *BRAF* mutations are present in early colonic neoplasia, they give rise to different types of polyps. *KRAS* mutations are primarily found in adenomatous polyps, whereas *BRAF* mutations occur primarily in polyps that have a serrated architecture and have been suggested as precursor lesions for MSI carcinomas[129, 130, 132-135]. In early neoplasia, *BRAF* mutation was are associated with CIMP, which has been suggested to precede MSI by *MLH1* promoter methylation[128-130, 132, 136]. This association of *BRAF* mutations with sporadic MSI colon cancer, their precursor lesions and CIMP (in contrast to *KRAS* mutations) suggests that the two mutations (*BRAF* and *KRAS*) follow distinct tumorigenesis pathways despite being members of the same signaling pathway[128, 130, 132, 136].

Although *KRAS* and *BRAF* mutations are observed in early colonic neoplasia, the sequence of events regarding DNA methylation remains unclear. Promoter methylation

of O6-methylguanine DNA methyltransferase (*MGMT*) often occurs in many tumor types, including colon cancer[137-139]. Additionally, epigenetic down-regulation of *MGMT* expression is often seen in tumor-adjacent normal colon mucosa[140]. MGMT is a DNA base excision repair protein that removes mutagenic and cytotoxic adducts from the O6 position of guanine. O6-methylguanide often mispairs with thymine during replication, resulting in the conversion from a GC pair to an AT pair if the adduct is not removed. Inactivation of the *MGMT* gene via promoter hypermethylation can result in G-to-A transitions in the mutational hotspots within codons 12 and 13 of the *KRAS* oncogene, as well as in *TP53*[137, 139, 140]. Therefore, methylation of the *MGMT* promoter might initiate tumor progression through secondary *KRAS* and/or *TP53* mutations, a theory that might argue against the initiation of aberrant DNA methylation via the occurrence of activating *KRAS* mutations.
Although *BRAF* mutations cannot be explained by *MGMT* inactivation, methylation of the *IGFBP7* promoter has been shown to facilitate the oncogenic potency of activated BRAF. Active IGFBP7 is required for oncogene-induced cellular senescence (OIS), an important tumor suppressor mechanism[141-143]. Escaping the OIS pathway could favor selection for activating *BRAF* mutations. The accumulation of aberrant promoter hypermethylation might provide a favorable environment for the oncogenicity of mutated *BRAF*, which could explain the association between *BRAF* mutations and CIMP. However, the association between *BRAF* mutations and MSI remains a molecular puzzle. More research is needed to determine the initiating factor and the role of *MLH1* methylation in this model.

MLL-rearranged B-lineage leukemia

Acute lymphoblastic leukemia (ALL) is the most common malignancy in children under the age of 15 and accounts for 26.8% of all childhood cancers[144, 145]. This lymphoid leukemia can be divided into B and T cell leukemia depending on the cancer cell lineage. Over past few decades, treatment with a combination of chemotherapies has led to a considerable decrease in childhood cancer-related deaths and a 5-year survival rate that is currently between 78 and 83% in developed countries[144, 145].
However, upon age stratification of childhood ALL, a subgroup of infants who are younger than one year of age at diagnosis only attains a 5-year survival rate of approximately 50%[146, 147]. Although complete remission is achieved in most of these patients, a high relapse rate is the principal cause of this decrease in survival odds[146, 147]. Approximately 80% of infants with ALL carry chromosomal translocations that involve the mixed lineage leukemia (*MLL*) gene and typically exhibit an immature CD10-negative precursor B-lineage immunophenotype[146-148]. Within this infant ALL subgroup, the presence of *MLL* rearrangements and an age of younger than six months are described as the most important factors for predicting poor outcome[146, 147].
The most prevalent chromosomal translocations in infant ALL patients are t(4;11), t(11;19) and t(9;11), which fuse the N terminus of MLL to the C-terminal regions of AF4, ENL and AF9[146, 149]. Interestingly, these different translocations are characterized by distinct mRNA levels[150, 151] and DNA methylation patterns[152]. Genome-wide studies of DNA methylation levels as well as studies into the functions of MLL and fusion partner proteins have indicated that epigenetic changes play a major role in *MLL*-rearranged ALL and might be the driving force behind the expression differences between the translocation-stratified groups and control samples.

The normal function of MLL

The human *MLL* gene was discovered in the early 1990s by isolating the chromosomal breakpoints at chromosome 11q, cytoband 23[153-156]. A sequence comparison revealed three regions of sequence similarity with the *Drosophila melanogaster* gene *trithorax* (*trx*); thus, both are members of the trithorax group, an evolutionarily conserved family of proteins[157]. Similar to the function of trx in *Drosophila*, in mammals MLL acts as a transcriptional regulator of the class I homeodomain (*Hox*) genes and counters the repressive effects of the Polycomb group (PcG) proteins (Figure 4)[158-161]. The *Hox* genes, in turn, are transcription factors that direct cell fate during development. *MLL* is ubiquitously expressed both during development and in most adult tissues, including myeloid and lymphoid cells, and is required for definitive hematopoiesis[162-164]. In both *Mll*$^{-/-}$ mice and *trx*$^{-/-}$ flies, *Hox* gene expression is initiated correctly but deteriorates during embryogenesis, suggesting an essential role in maintaining expression patterns following initiation by other factors[157].

Identification of the different active domains of the large (3,968 amino acids) MLL protein has provided much insight into how MLL-mediated transcriptional regulation is facilitated (Figure 7). The MLL protein is cleaved by the protease taspase I into 320-kDa N-terminal and 180-kDa C-terminal fragments, both of which are core components of the MLL complex[165-168]. Two N-terminal domains -a region of three AT-hook domains and a region containing a CXXC zinc-finger domain- are believed to be involved in DNA binding[169-172]. The AT hook domain is a minor groove DNA binding motif that preferentially recognizes DNA that is distorted with bends or kinks, whereas the CXXC domain is the major determinant of subnuclear localization and target gene selection and recognizes and binds specifically to unmethylated CpG dinucleotides[173-175]. Although MLL can bind directly to DNA, MLL recruitment to chromatin can be mediated by DNA-binding protein partners such as menin (encoded by the *MEN1* gene)[176]. In addition to the CXXC, another domain targets MLL to sites that are associated with active chromatin. A central region between the third and fourth fingers contains three cysteine-rich plant homeodomain (PHD) zinc fingers and a fourth divergent PHD finger. This bromodomain has been shown to bind lysine-acetylated histone-derived peptides, thus suggesting preferential binding to acetylated histones by MLL[170, 172, 177-179].

Although the MLL protein has been associated with proteins that suppress gene expression, the recruitment of MLL to chromatin is most often associated with transcriptional activation. Both of the activating domains -namely, the transcription activation (TA) domain and the SET [Su(var)3-9, enhancer of zeste, and trithorax] domain- are located on the protein's C terminus[169-171]. The activating functions of both of these domains are mediated through epigenetics; the SET domain is directly responsible for methylating H3K4, and the TA domain recruits the histone acetyltransferases CREB-binding protein (CBP) and p300[180-183].

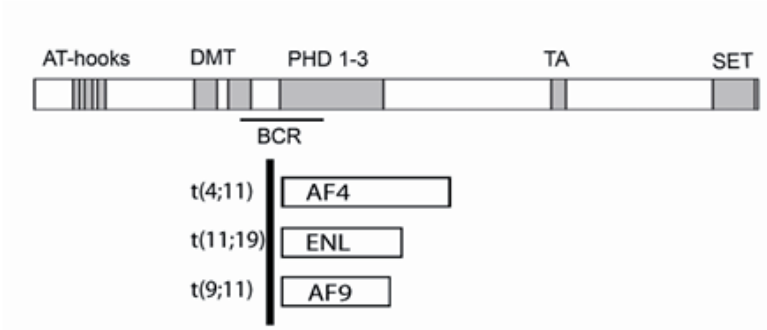

Figure 7 – Schematic representation of the MLL protein. The 89-kb *MLL* gene consists of 37 exons and encodes a 3,969-amino acid nuclear protein. MLL is cleaved at two cleavage sites (CS1 at amino acid 2666 and CS2 at amino acid 2718), resulting in two non-covalently associated subunits (N-terminal MLL (300 kDa) and C-terminal MLL (180 kDa)). The DNA-interacting domains (AT-hooks and the DNA methyltransferase homology domain (DMT) containing the zinc finger) are located in the N-terminal cleavage fragment. The PHD zinc-finger motifs facilitate the binding of proteins that are suggested to regulate MLL protein activity. This domain can be either present in an MLL fusion protein or completely absent, depending on the precise site of translocation in the breakpoint cluster region (BCR) spanning exons 8-13. Located on the C-terminal MLL domains are the transcriptional activation site (TA) and the SET domain (SET), both of which are involved in transferring marks of transcriptional activation to histone tails. The C-terminal parts of the fusion partners are shown beneath the MLL protein.

MLL fusion proteins: what do they add?

Given that MLL functions as an epigenetic transcriptional activator, a disruption in normal MLL function can be linked directly to the differences found in expression and DNA methylation between *MLL*-rearranged ALL groups and controls. All of the *MLL* rearrangements that have been found in ALL are believed to arise from a failure of DNA double-strand break repair during hematopoiesis. Most of the *MLL* rearrangements target the breakpoint cluster region that is located between exons 8 and 13, resulting in a fusion protein that contains N-terminal MLL and the C terminus of a fusion partner[184, 185]. Mouse studies have revealed that a truncation of MLL after exon 8 is not sufficient to induce leukemia but requires a functional C-terminal portion of a fusion protein[186]. The perturbed H3K4 methylation of one *MLL* copy is therefore not sufficient to initiate leukemogenesis. The aforementioned translocations with fusion partners *AF4*, *AF9* or *ENL* account for more than 80% of all *MLL*-rearranged leukemias, and all three resulting fusion partners contain a C-terminal transcriptional activation domain. These activation domains are associated with the H3K79 histone methyltransferase DOT1L[187-191]. H3K79 methylation levels are increased in targets that are crucial for *MLL*-rearranged leukemogenesis[187-191]. Given that the various methylation marks regulate transcription in unique ways[12, 14-16, 41], the addition of H3K79 methylation to normal H3K4 methylation at MLL-associated promoters could account for the aberrant over-expression and DNA methylation differences in *MLL*-rearranged leukemia. However, the high levels of DNA hypermethylation observed in patients with *MLL-AF4* and *MLL-ENL* translocations cannot currently be explained with the current understanding of *MLL* rearrangements in infant B-ALL.

SCOPE AND OUTLINE OF THIS THESIS

Tumor formation is the result of either DNA mutations in the genetic code, of chromosomal alterations or of epigenetic changes, the latter with DNA hyper- and hypomethylation. DNA mutations comprise base substitutions (point mutations) as well as relatively small insertions and deletions. Chromosomal alterations can occur as copy number variations, translocations and inversion of chromosomes. Often these alterations occur in parallel to ploidy changes of the whole genome of the cells.

Epigenetics, comprising DNA and histone modifications, is a relatively new field of study and recent technical possibilities have fuelled a growing interest in the role of epigenetics in tumorigenesis. Although the causes and effects of DNA hyper- and hypo-methylation in cancer are still being investigated, cancer-specific methylation profiles can be potentially used for clinical purposes such as pre-symptomatic screening for colorectal cancer in serum and faeces. In this thesis DNA methylation was studied in colon cancer and infant acute lymphoblastic leukemia.

In **Chapter 1** an introduction is given on this topic. In **Chapter 2**, using the differential methylation hybridization (DMH) technique, home-spotted CpG island microarrays were employed on right-sided colon cancer samples and compared with normal colon mucosa. High frequent methylation of the *PTPRGint1* sequence different types of colon cancer was seen. The *PTPRGint1* sequence turned out to be a binding site for CTCF, a protein that is involved in regulation of chromatin modifications. In **Chapter 3** a relatively young cohort of colon cancer patients with *MLH1* promoter hypermethylation was studied. Interestingly, this epigenetic down-regulation of *MLH1* is mostly seen in elderly colon cancer patients above 70 years of age. In **Chapter 4** we used the DMH technique in combination with a high density oligonucleotide CpG island microarray to obtain methylation profiles of colon cancer samples and matching normal colonic mucosa. As DNA methylation is suggested to be a consequence of pre-existing histone modifications we filtered *BRAF* mutation-specific methylation profiles for such pre-marking and identified promoter methylation of *FOX* genes involved in oncogene induced senescence.

Finally, the CpG Island microarrays were employed to study cancer-specific DNA methylation in *MLL*-rearranged B-ALL **(Chapter 5).** Infant ALL-specific as well as *MLL*-translocation-specific promoter methylation patterns were identified. These promoter methylation patterns correlated strongly with expression and outcome.

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