Concluding remarks and future perspectives

Chapter 6

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

DNA methylation profiling: different techniques, different targets

The past decade has seen major advances in the methods for detecting DNA methylation on a genome-wide level. Enzyme-based (e.g., HELP[1], MMASS[2], DMH[3] and CHARM[4]) and chromatin immunoprecipitation methods[5] are most commonly used in combination with custom or commercial CpG island or promoter microarrays. Recently, captured methylated DNA[6] and bisulfite-converted reduced representations[7] were analyzed using high-throughput sequencing strategies. Although bisulfite-converted reduced representation still relies on restriction enzymes, the combination of antibody-mediated pull-down techniques with sequencing (ChIP-seq) does not. Combining restriction site-independent techniques with high-throughput sequencing can help to approach a true genome-wide analysis of the methylome. However, these ChIP-seq analyses are not without a bias. An over-representation of high CpG content by these techniques can lead to a false negative result of less dense CpG-rich loci. Interestingly, studies using the enzyme-based technique CHARM have shown that conserved regions up to 2 kb from the promoter (CpG island shores) undergo more cancer-related differential methylation than traditional promoter CpG islands do[8]. Additionally, ChIP-on-chip experiments have shown that CpG islands of lower density (intermediate-CpG islands) also undergo more cancer-related differential methylation[9]. Because differential methylation of these regions is tightly correlated to altered gene expression, the importance of these regions might have been underestimated in previous studies. These findings highlight another bias in microarray methodology for detecting DNA methylation. Following the general focus on promoter CpG island regions, the use of arrays that contain only such sites has led to the aforementioned interesting sites being ignored.

The DMH method was used in **Chapters 2, 4 and 5**. This technique -particularly in combination with the home-spotted 8.5 k array (**Chapter 2**)- can be regarded as a low-resolution approach in comparison with ChIP-seq or set-ups using arrays that contain larger genomic coverage. Despite the low genomic representation on the 8.5-k home-spotted microarray, the represented loci were experimentally selected based on their ability to be methylated *in vitro*[10, 11]. Moreover, pre-selection fragmentation was performed using *Mse*I. Because the *Mse*I recognition site rarely occurs in GC-rich regions, this pre-digestion leaves intact most of the low- to high-density CpG island fragments. These aspects of our experimental set-up allowed the identification of colon tumor-specific methylation of the *PTPRGint1* locus in **Chapter 2.** Additionally, in **Chapters 4 and 5**, we used the DMH method in combination with a commercial 244-k oligonucleotide microarray platform containing broader coverage of the genome. This increase in resolution allowed us to identify *BRAF* mutation-specific promoter methylation of *SMO* and *FOXD3* (**Chapter 4**) and *MLL* translocation-specific patterns of DNA methylation (**Chapter 5**).

DNA methylation and early detection of colon cancer

Chapter 2 described the colon tumor-specific methylation of a low CG-dense CpG island that is located in the first intron of the *PTPRG* gene (*PTPRGint1*). No direct biological implication of *PTPRGint1* methylation on colon tumorigenesis could be given in this thesis despite a loss of CTCF binding to the region. However, in sporadic colon lesions ranging from early adenomas to carcinomas, high levels of *PTPRGint1* methylation were observed (**Chapter 2**). In addition, Lynch syndrome-associated colon lesions contained similar high levels of *PTPRGint1* methylation (**Chapter 2**).

As mentioned in the Introduction, early detection greatly increases the survival rate for patients with colorectal cancer. Given the long asymptomatic preclinical phase, population screening of the general public would greatly increase the early detection rate of sporadic colorectal cancer. The available screening tests that are most commonly used for the detection of colorectal cancer include colonoscopy and fecal occult blood tests. Although colonoscopy is a highly sensitive method, its high costs, invasiveness and risk of complications such as bowel perforation make it less applicable for screening the general population. However, it remains a valuable method for screening high-risk patients in Europe and other views on colonoscopy use pervade in the US. Fecal occult blood testing is relatively simple, and although it has low predictive value, several randomized trials[12] have shown a decrease in the mortality rate of colorectal cancer patients by up to 25%. According to published reports, tests using epi- and genetic biomarkers for screening serum and feces promise to hold high value in the development of accurate, non-invasive screening methods[13, 14].

To date, a number of DNA methylation markers have been tested in both stool and serum samples (for an overview, see Table 2). The addition of even more specific and sensitive markers -and a combination of these markers- will provide a screening method that is more accurate, more cost effective and more comfortable than current screening methods. The possible addition of the *PTPRGint1* locus to the current list of stool and/or serum markers will depend on the successful adaption of the locus to an applicable method for such testing. Currently, the use of (quantitative) methylation-specific PCR protocols is predominantly reported in the literature.

Table 2 – DNA methylation markers in colorectal cancer patient serum/plasma and stool

Gene	Sample material	Patients (percentage methylated)	Healty donors (percentage methylated)
ALX4[81]	Stool	25/30 (83%)	16/52 (30%)
CDH4[82]	Peripheral blood	32/46 (70%)	0/17(0%)
CDKN2A/p16[83]	Stool	Adenomas 9/29 (31%)	3/19 (16%)
CDKN2A/p16[84]	Serum	12/17 (71%)	0/10(0%)
GATA4[85]	Stool	20/28 (71%) Cohort 2: 24/47 (51%)	7/45 (16%) 2/30 (7%)
HIC1[86]	Stool	11/26 (42%) Adenomas 4/13 (31%) Hyperplastic polyps 0/9 (0%)	1/32 (3%)
HLTF[87]	Serum	16/49 (32.7%)	3/41(7%)
HLTF[88]	Serum	22/103(21%)	0/20(0%)
ITGA4[89]	Stool	9/13(69%)	6/28(21%)
MGMT[83]	Stool	Adenomas 14/29 (48%)	5/18 (27%)
MLH1[90]	Peripheral blood	35/262 (13.4%)	
MLH1[87]	Serum	19/49 (39%)	1/41 (2%)
NDRG4[91]	Stool	17/28 (61%) Cohort 2: 25/47 (53%)	7/45 (16%) 0/30 (0%)
NGFR[92]	Plasma	68/133 (51%)	29/179 (16%)
OSMR[93]	Stool	26/69 (38%)	4/81 (5%)
PGR[94]	Stool	18/23 (78%)	8/26 (31%)
RUNX3[84]	Serum	11/17 (65%)	0/10 (0%)
SEPT9[92]	Plasma	92/133 (69%)	25/179 (14%)
SFRP2[94]	Stool	19/23 (83%)	6/26 (26%)
SFRP5[94]	Stool	18/23 (78%)	9/26 (35%)
TPEF/HPP1[92]	Plasma	87/133 (65%)	56/179 (31%)
TPEF/HPP1[88]	Serum	13/103 (13%)	0/20 (0%)
Vimentin[95]	Stool	43/94 (46%)	20/198 (10%)

CTCF binding regulation by *PTPRGint1* methylation

The methylation status of *PTPRGint1* was not found to have a direct effect on the transcription level of the *PTPRG* gene (**Chapter 2**). However, the annotation of *PTPRGint1* as a CTCF-binding locus suggests possible downstream effects on transcription and chromatin modifications through the varied functions of CTCF (**Chapter 2**). Recently, an electrophoretic mobility shift assay was performed on the *PTPRGint1* locus and validated this region as a methylation-dependent CTCF binding region (Barry Pepers, Leiden University Medical Center, personal communication).

The binding of CTCF to a region confers protection against DNA methylation; CTCF remains associated with specific chromosomal regions during mitosis, thereby suggesting a possible role in the maintenance of epigenetic marks throughout cell division[15-18]. Aberrant methylation of CTCF binding sites such as the ones that are associated with *ARF*[19, 20], *Rb*[21], *BRCA1*[22, 23], *p16*,[24] *RASSF1*[24] and *CDH1*[24] result in the spreading of DNA methylation to the promoters of these genes, as implicated previously in human cancers. CTCF can directly protect the binding sequence against DNA methylation; inhibition of CTCF binding -and consequently, its insulator function- putatively results in a boundary loss between euchromatin and heterochromatin with sequential spreading of inactivating epigenetic marks (Figure 8)[24-26].

In humans, the *CTCF* gene maps to the cancer-associated chromosomal locus 16q22.1 (Ref. 27), which is a region that is often lost in primary breast carcinomas[28-30], prostate adenocarcinomas[31], ovarian cancer[32] and Wilms' tumors[33]. However, the biallelic loss of CTCF expression is not observed in human cancers. CTCF null mice exhibit early embryonic lethality; conditional knock-down of CTCF in cultured fibroblasts rapidly leads to apoptosis[34, 35], thereby suggesting that a loss of CTCF is incompatible with cell survival. However, tumor-specific mutations in the third and seventh zinc fingers of the *CTCF* 11-zinc finger coding domain have been found in breast, prostate and Wilms' tumors[36]. All of the identified mutations were accompanied by the loss of the second *CTCF* allele and resulted in a missense codon at a position that is predicted to be critical for either zinc finger formation or DNA sequence recognition[36]. CTCF can use various combinations of zinc fingers to bind to a wide range of DNA sequences and proteins. Theoretically, the mutations that were identified in *CTCF* could confer a differential loss of binding to specific DNA recognition sites and/or proteins.

Such a model wherein specific CTCF functions are affected without interfering with other CTCF functions that are essential for cell viability is potentially interesting. However, mutations in *CTCF* are extremely rare and cannot account for the wide-scale DNA methylation differences that are observed in cancers[33, 35-37]. An alteration in the protein partners that CTCF requires to perform one or more of its many functions is an alternative explanation.

Although the spreading of heterochromatin marks can be a consequence of a loss of CTCF binding, evidence for the sequential progression between CTCF binding and DNA methylation of its own binding site remains elusive. Given that CTCF binding to a CpG-containing binding site has been shown to be sensitive to methylation, whether methylation of the CTCF binding site is caused by a loss of CTCF binding or whether aberrant methylation precedes CTCF binding loss remains an open question.

Before tackling these important fundamental questions, the impact of losing CTCF

binding after methylation of the *PTPRGint1* region must first be investigated. Mapping of CTCF binding sites in normal colon epithelium would serve as an essential validation of the region as a colon-specific CTCF binding site, as the *PTPRGint1* region has only been described as such in a cell line model system (**Chapter 2**). Applying the chromosome conformation capture technique to both normal colon epithelium and colon cancers with *PTPRGint1* methylation might reveal putative differences in chromatin loop formation. An analysis of DNA/histone modifications, Lamina-associated domains and transcription changes with respect to these putative chromatin loop formation alterations might provide a clear view of the consequences of *PTPRGint1* methylation. However, apart from assessing DNA methylation and mRNA levels, the aforementioned analyses are difficult to perform on tissues other than cultured cells due to the high numbers of cells that are required.

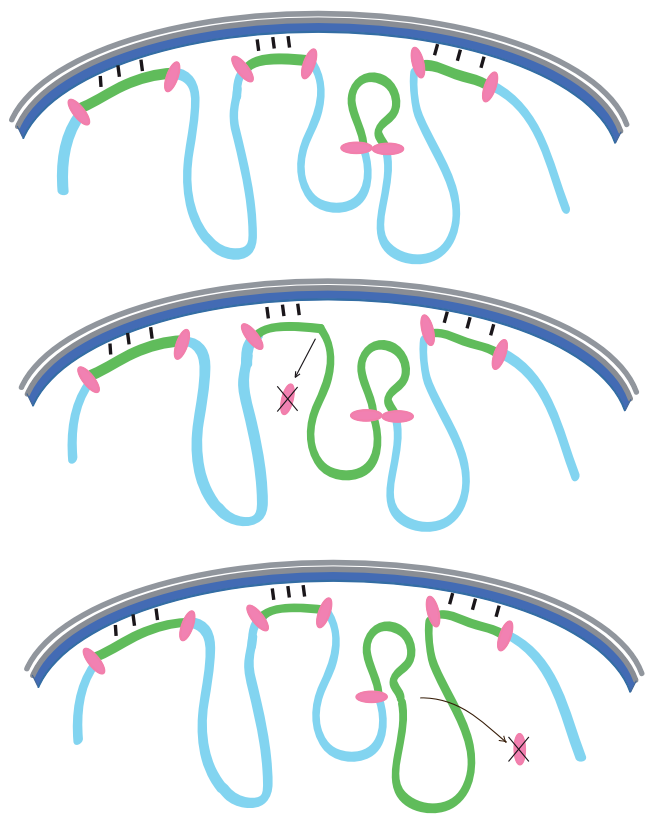

Figure 8 – Heterochromatin spreading caused by disassociation of CTCF binding. Upper panel: normal CTCF protein (pink) binding demarcates the boundaries between heterochromatin (green line) and euchromatin (light blue line). Middle panel: disassociation of CTCF protein (pink) binding causes spreading of lamina-associated heterochromatic marks. Lower panel: spreading of non-lamina-associated heterochromatic marks by the regional loss of CTCF binding. Figure adapted from de Wit et al.[25].

Aberrant histone modification machinery: initiation of cancer specific DNA methylation?

In support of a direct link between histone modifications and DNA methylation, H3K4 methylation was found to protect against DNA methylation[38]. In addition, binding of the PRC2 member EZH2 to a region recruits DNMTs[39]. Evidence for a developmental model (see Figure 4 in the General Introduction) resulted from the discovery that polycomb-mediated H3K27 methylation in ES cells pre-marks genes for *de novo* DNA methylation in colon cancer[40-45]. In this model, the balance between mediator binding of inactivating (Polycomb Repressive Complex 2 ; PRC2) and activating histone (Trithorax-group ; TrxG) marks dictate the downstream DNA methylation and expression states of chromosomal regions (see Figure 4 in the General Introduction). This balance between the antagonistic histone modifications is most pronounced in ES cells in which almost all H3K27^{me3} bound promoters are bivalent[46, 47]. The loss of this bivalent binding in both adult differentiated colon tissue and colon cancer supports the conventional view that such histone modifications act as 'plastic' epigenetic switches for transcription and *de novo* methylation during development[40-45]. In this hypothesis, DNA methylation serves as a definitive epigenetic lock that is preceded by histone modifications and their associated machinery.

Recent studies have reported a possible pre-marking of cancer-specific hypermethylated genes by H3K27^{me3} and binding of the PRC2 member SUZ12 in both ES cells and differentiated normal colon mucosa[42-44]. The presence of repressive histone modifications at promoters in normal colon epithelium suggests that the associated genes are in a transcriptionally silent state prior to tumor formation, thereby reducing the relevance of their promoter's methylation on expression and thus tumorigenesis. To focus on genes that are silenced by tumor-specific promoter methylation (rather than lineage-specific methylation), we excluded from our analysis in Chapter 4 the loci with H3K27^{me3} in embryonic stem (ES) cells.

In model systems, abnormal TrxG or PRC2 function often results in aberrant gene expression that leads to tumor development, which suggests that dysregulation of these epigenetic programs can initiate tumorigenesis[39, 48-50]. Although there is no evidence of alterations in the balance between TrxG proteins and PRC2 in colon cancer, such an alteration exists in infant B-ALL. In Chapter 5, we described the translocation-based grouping of DNA methylation patterns from *MLL*-rearranged B-ALL patients. Although patients who contain the translocation t(4;11) or t(11;19) have comparable high levels of DNA methylation, patients with a t(9;11) translocation have DNA methylation patterns that are comparable to normal controls and B-ALL patients without *MLL* mutations. As the histone methyltransferase moiety of one copy of *MLL* is lost in all three translocation subtypes, their differing DNA methylation levels suggest that this loss has minimal impact on DNA methylation. In addition, 90% of all genes that are expressed in t(4;11) rearranged leukemia cells contain the H3K4^{me3} modification[51]. Therefore, perturbation of MLL H3K4 methyltransferase activity by the alteration of one copy of *MLL* also has a low impact on H3K4 methylation levels[52]. Finally, recent studies have shown that a loss of the normal H3K4 histone methyltransferase activity in one copy of *MLL* is not sufficient to initiate leukemia[53, 54]. It is therefore likely that the combined functions of the reciprocal fusion partners facilitate both aberrant DNA methylation and tumorigenesis in infant B-ALL[54, 55].

The fusion protein that results from the aforementioned translocations is a combination of a C-terminal transcriptional activation domain provided by the fusion partner (AF4, AF9 or ENL) fused with the DNA binding N terminus of MLL. Recruitment of DOT1L and sequential H3K79 methylation to regions that are bound by N-terminal MLL is contributed to these fusion proteins[51, 52, 56-59]. This addition of H3K79^{me} -a modification that is associated with transcription elongation- could explain the high expression of MLL target genes that are associated with leukemogenesis such as *MEIS1* and *HOXA9*[51, 52, 58]. Co-occupancy of both the germline MLL protein and the MLL fusion protein could provide both the activating H3K4^{me3} histone mark and the transcription elongation mark H3K79^{me}. The combination of both histone modifications might be sufficient to enable such aberrant high expression (Figure 9). However, the effect of the fusion proteins on the DNA hypermethylation that has been observed in infant B-ALL patients with t(4;11) and t(11;19) translocations (Chapter 5) remains unknown.

Recent studies have shown that unlike the MLL-AF4 fusion protein, the t(4;11) reciprocal fusion protein AF4-MLL, which consists of N-terminal AF4 and C-terminal MLL, is sufficient to induce leukemogenesis in mouse models[53]. The N-terminal domain of AF4 has been suggested to retain its ability to bind RNA polymerase II while losing the ability to recruit DOT1L. However, the downstream effects of this reciprocal translocation protein and its importance in leukemogenesis warrant further investigation.

Our future understanding of the full impact of *MLL* rearrangements on histone modifications and their role in aberrant DNA methylation will require multi-dimensional studies that combine histone modification, DNA methylation, insulator binding, transcription and MLL fusion protein binding data. The involvement of the TrxG member MLL supports a direct link to the disrupted balance between the antagonistic histone modifiers PRC2 and TrxG in infant B-ALL. However, down-regulation of MLL histone-methyltransferase activity has little effect on H3K4 methylation levels[52]. Complete mapping of DNA and histone modifications -combined with transcription data from the various stages in hematopoiesis and *MLL*-rearranged leukemogenesis- will provide a clearer understanding of the true identity of the oncogenic drivers. The identification of translocation-specific MLL binding sites might provide additional insight into the differences in DNA methylation that have been observed between infant B-ALL patients who harbor different types of translocations (Chapter 5).

A sequential model for colon differentiation and tumorigenesis would provide similar insights and could reveal possible links between aberrant DNA methylation, histone modification and their modifier complexes. Linking this data to transcription and insulator binding data should help expand our understanding of the role of DNA methylation in the etiology of colon cancer.

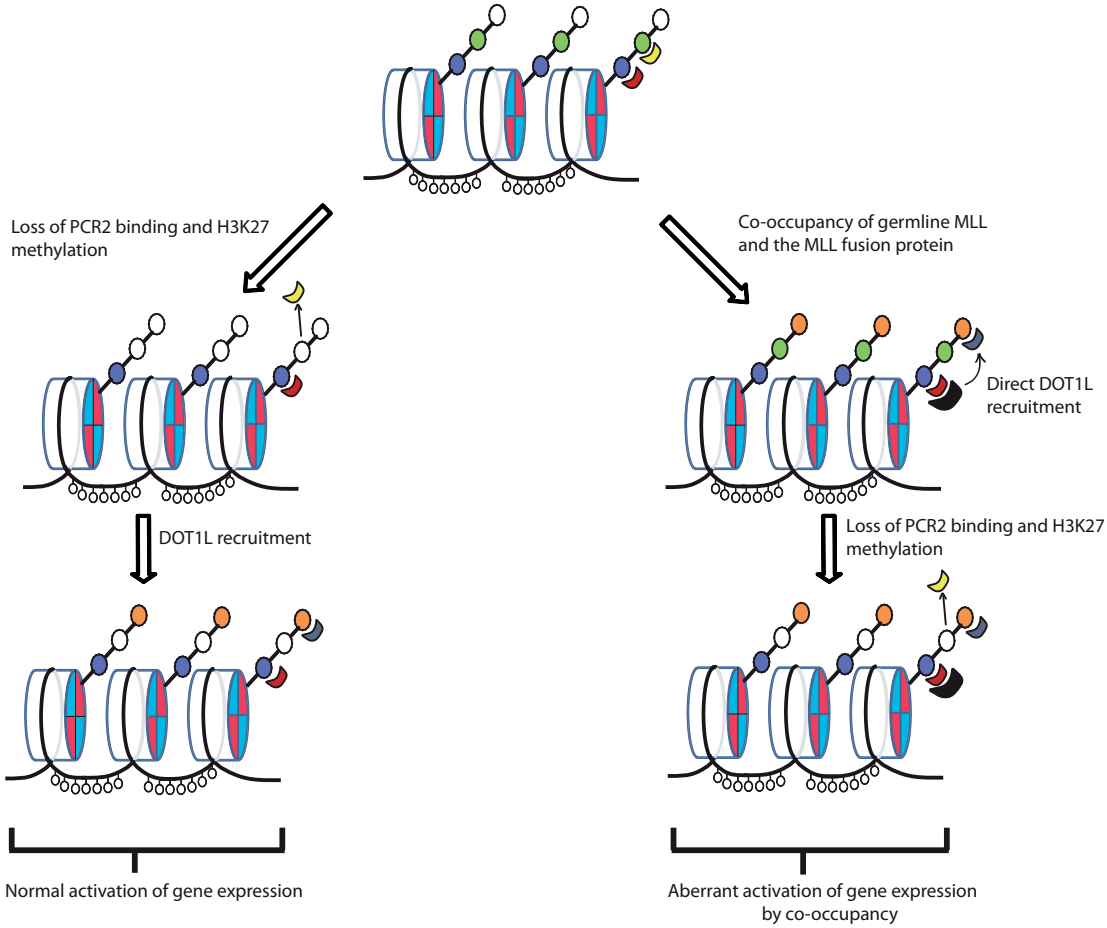

Figure 9 – Progression model showing normal and aberrant recruitment of the H3K79 histone methyltransferase DOT1L. Co-occupancy of both the unaffected MLL (red crescent) as well as the MLL fusion protein (large red crescent with gray stripes) recruits DOT1L (grey crescent). This direct recruitment of DOT1L and its histone (H3K79) mark (orange) for transcription elongation can aberrantly activate gene expression by circumventing the sequential progression and balance between the TrxG/MLL, PRC2 (yellow crescent) proteins and associated histone marks (H3K4 methylation in purple and H3K27 methylation in green).

The TrxG family and BRAF, is there a connection?

The aforementioned enrichment of PRC2 components and H3K27^{me3} in targets for colon cancer-specific DNA methylation suggests a possible dysregulation of the balance between TrxG and PRC2 during colon tissue differentiation (**Chapter 4**). Although this balance is perturbed by deregulation of the TrxG protein MLL in *MLL*-rearranged ALL, such an association has not been described in colon cancer. One study described a direct link between colon cancer and dysregulation of TrxG in both colon cancer cell lines and tumors. The TrxG member *MLL2* was found to be overexpressed in colon tumors compared to corresponding adjacent tissue[60]. In addition, colon cancer cell lines that were derived from highly invasive, poorly differentiated tumors exhibited altered sub-cellular distribution and proteolytic processing of MLL2 compared to non-tumor cell lines

and less invasive tumor cell lines[60]. In contrast to increased *MLL2* expression, the altered sub-cellular distribution and proteolytic processing indicate a decrease in normal MLL2 activity. Although the relationship between CIMP and MLL2 has not been investigated, CIMP is known to be associated with poor differentiation, thus indicating a possible association between CIMP and the proteolytic dysregulation or sub-cellular distribution of MLL2. Unfortunately, no distinction of CIMP, MSI or *BRAF* mutational status was made in the study in relation to the overexpression of *MLL2* in colon tumors and cell lines. Grouping these factors would provide a clearer insight into the role of MLL2 in the etiology of tumors with aberrant DNA methylation. Interestingly, in **Chapter 4** we described an association of colon cancer DNA methylation with H3K27^{me3} targets in ES cells and showed that this enrichment is less pronounced in CIMP- and *BRAF*-associated DNA methylation.

An association between *BRAF* mutations and CIMP colon cancer has been documented[61] and was also described in **Chapter 3** for colon cancer patients up to 50 years of age. CIMP colon cancer with *BRAF* mutations and MSI may originate from sessile serrated polyps through a unique pathway. While *BRAF* mutations are present in sessile serrated polyps and serrated aberrant crypt foci, *KRAS* mutations are more closely associated with non-serrated polyps and non-serrated aberrant crypt foci[62-67]. Interestingly, 90% of all aberrant crypt foci with *BRAF* mutations were found to be microsatellite-stable , whereas ~70% of sporadic MSI colon cancers exhibit *BRAF* mutations. These observations led to the suggestion that *BRAF* mutations precede *MLH1* methylation and that mutationally active BRAF might play an initiating role in the manifestation of aberrant DNA methylation in colon cancer[62-64, 68-70]. Although no direct interactions between members of the RAS-RAF pathway and either the TrxG, PRC2 or DNMT family have been described, the downstream transcription factors that are activated by the RAS-RAF pathway protein MAPK (for example, c-Fos, CREB and c-MYC) interact with DNMT3A and DNMT3B[71]. Additionally, it was shown that inhibiting the ERK/MAPK signaling pathway decreases the genomic DNA methylation content in cancer cells[72]. KRAS and BRAF are directly linked in the same pathway but associate differently with CIMP in colon cancer. Therefore, interactions between these shared transcription factors and the DNA methylation machinery should be investigated further. An alternate hypothesis regarding the association between *BRAF* mutations and DNA methylation suggests that promoter methylation and silencing of specific target genes could favor the selection of activating *BRAF* mutations. This hypothesis has been suggested for *IGFBP7*, a mediator of BRAF-induced cellular senescence[73-75]. Promoter methylation and the down-regulation of *IGFBP7* could provide a favorable context in which to obtain an activating *BRAF* mutation[73-75]. In **Chapter 4,** we showed *BRAF* mutation-specific promoter methylation of *FOXD3* after stringent filtering for pre-marking and copy number changes. FOXD3 is a mediator of p21^{Cip1}- and p53-dependent cell cycle arrest, which is down-regulated by constitutively active BRAF in melanoma cells[76]. These findings suggest that FOXD3 might play a role in eluding BRAF-induced senescence in colon cancer through epigenetic inactivation. The augmented proliferation and high levels of senescence that are induced by constitutively active BRAF could favor the selection of cells that can escape senescence by the epigenetic inactivation of mediators, which might explain the link between *BRAF* mutations and aberrant DNA methylation in colon cancer.

In **Chapter 3**, we described site-specific methylation of the *MLH1* promoter in sporadic colon cancer patients with an age of diagnosis below 50 years, despite low levels of global methylation. In contrast, patients with a *BRAF* mutation who were either below

or above 50 years of age at diagnosis have significantly higher levels of CIMP marker methylation. The aforementioned *BRAF* mutation-driven selection for cells that can escape senescence suggests an accumulation of promoter methylation that ultimately results in MMR deficiency and/or escape from BRAF-induced senescence. Future studies of the interactions of activated RAS-RAF pathway proteins with histone and DNA modification machineries should yield a clearer understanding of the roles of KRAS and BRAF in the initiation of aberrant DNA methylation. The suggested interaction between RAS-RAF activated transcription factors and DNMT3A and DNMT3B in targeting specific promoter loci[71] is particularly interesting. Identifying differential RAS-RAF-associated transcription factor binding sites in *BRAF* and *KRAS* mutated cancer models might point towards specific mediators of *BRAF*-initiated DNA methylation. However, the mechanisms that underlie the observed site-specific *MLH1* promoter methylation in *BRAF* wild-type patients have been elusive. Polymorphisms such as the *MLH1* -93G>A polymorphism that was discussed in **Chapter 3**, when present in such a transcription factor binding site and thereby putatively altering binding, could provide an additional factor in site-specific DNA methylation.

The observation that promoter methylation of the Methylguanine-DNA methyltransferase (*MGMT*) gene can give rise to mutations in *KRAS* and the tumor suppressor gene *TP53*[77] is somewhat contradictory. A loss of *MGMT* expression can result in a G-to-A transition in *TP53* and codons 12 and 13 of the *KRAS* gene[78-81]. These findings argue against a role for the RAS-RAF pathway in initiating aberrant DNA methylation; elucidating the precise cascade of events is required to determine the function of RAS-RAF pathway in this process.

The role of epigenetics in the initiation of colon cancer and its association with *KRAS* and *BRAF* mutations is complex and requires additional research to unravel the molecular mechanisms that are involved. Although links between mutations in the RAS-RAF pathway and the DNA methylation machinery have been reported[68, 71], these links are indirect. In addition, cellular model systems with constitutively active BRAF fail to induce CIMP[73]. Selective pressure on neoplastic cells that carry *KRAS* or *BRAF* mutations for the epigenetic inactivation of certain genes might be a more plausible explanation for the associations between the mutations and the increased levels of promoter DNA methylation in colon cancer. The absence of *BRAF* mutations and the relatively low levels of methylation that are observed in Lynch syndrome patients[82], young sporadic colon cancer patients with site-specific *MLH1* methylation and colon cancer patients with germline *MLH1* methylation (**Chapter 3**) also hint towards such an association.

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