Epigenetic alterations in the predisposition to and progression of melanoma

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6

Summary and Discussion

Melanoma is an aggressive form of skin cancer, causing significant mortality, due to its capacity for metastatic dissemination. It develops from the pigment-producing cells, the melanocytes. In order to become malignant, these cells accumulate multiple genetic and epigenetic alterations.

During the last decade the advent of next generation sequencing methodology has allowed the identification of novel inherited pathogenic gene variants in patients with familial melanoma and patterns of somatic alterations in sporadic cases.

In the present thesis, we explored inherited and acquired epigenetic alterations in melanoma susceptibility and development. Epigenetics literally means 'on top of genetics' and refers to mechanisms that regulate gene expression not involving alterations in the DNA.[1] There are 3 main epigenetics mechanisms: DNA (hydroxy)methylation (covalent modifications of DNA bases), histone modifications (post-translational modifications on the amino-terminal tail of histones) and chromatin remodelling (repositioning of nucleosomes by protein complexes).[2] The epigenetic enzymes can be classified as writers, readers and erasers depending on their function and have different implication in cancer. The *writers* add various chemical modifications on DNA and histone tails; the *readers* are the proteins that bind the modifications and interpret them; and the *erasers* are responsible for removing these chemical tags.[3]

We aimed to address epigenetic alterations in melanoma susceptibility by studying a novel putative melanoma-susceptibility gene encoding a histone 3 lysine 79 (H3K79) methyltransferase, a *writer* according to the above-mentioned classification, was functionally assessed. In addition, epimutations as a putative cause of familial melanoma using genome-wide analyses. We also aimed to explore the epigenetic regulation of an essential gene in melanoma pathogenesis, *TERT*. Finally, we analysed the genomic distribution and functional significance of DNA hydroxymethylation in melanoma.

CAN A MUTATED EPIGENETIC ENZYME BE RESPONSIBLE FOR MELANOMA PREDISPOSITION?

To reveal new candidate susceptibility genes in melanoma we performed whole exome sequencing (WES) on DNA isolated from blood cells from two members of a family with four melanoma cases, not explained by established high penetrance melanoma-susceptibility genes. WES identified 10 rare, co-segregating, predicted deleterious missense gene variants. Subsequent co-segregation analysis revealed that only variants in the *DOT1L* (R409H) and

the *SLCO4C1* (P597A) genes were present in the other two affected members of this family. The lack of expression of *SLCO4C1* in melanocytes combined with gene function did not encourage us to explore this gene variant further. In Chapter 2, we describe a new germline missense variant in the *DOT1L* gene co-segregating with melanoma in all affected members of a Dutch melanoma family. DOT1L is the unique histone methyltransferase responsible for methylating the nucleosome core on lysine 79 of histone H3 (H3K79).[4-6] In addition, DOT1L regulates transcription elongation, establishes cell cycle checkpoints, and maintains genomic stability.[7,8] Dysregulation of DOT1L has been associated with a number of cancers either as an oncogene or tumour suppressor gene.[4] In ovarian cancer cells, when *DOT1L* expression was knocked-out by CRISPR/Cas9 technology, cell invasion and cancer stem-like cell properties were significantly promoted.[9] Studies in gastric cancer showed that *DOT1L* expression is significantly higher in gastric malignant tumours correlating to the degree of differentiation, lymph node metastasis and TNM staging.[10] Recently, in colorectal cancer DOT1L has been described as an important player in DNA double-stand break repair via homologous recombination.[11]

Three new somatic mutations (M55L, P271L, and P505L) in the *DOT1L* gene that negatively affect the catalytic activity of the methyltransferase were identified in melanoma cells.[12] Loss of *DOT1L* (by silencing or mutation) impaired the DNA damage repair induced by UV-B radiation, thereby promoting melanoma development *in vivo*. Accordingly, we found that mouse ES cells express a catalytically inactive *DOT1L* mutant (G165R) were more sensitive to UV radiation, consistent with a protective role of DOT1L. However, when we functionally explored the germline R409H variant, we could neither detect histone methyltransferase activity reduction in melanoma and mESCs nor an effect on UV-induced survival in mESCs. Different explanations for this lack of effects can be pointed out: the dynamic changes in and alternative functions of H3K79me were missed in the assays used; the R409H mutant is not by itself pathogenic; the role of the R409H variant in melanocytes is not recapitulated in the cell model used in our study; the R409H variant affects a methyltransferase-independent function of DOT1L. However, in mammalian cells this activity of DOT1L has been shown to be required for several critical functions, such as reactivation of repressed genes, cell cycle progression in lung cancer cell lines, and leukemic transformation in CALM-AF10 MLL-rearranged leukemia.[13-15]

Subsequently, several variants in *DOT1L* gene have been observed in independent familial and sporadic melanoma cases from the UK (personal communication, Dr. Harland, Leeds University). The variants G1320R and Y115F, both assessed as damaging/deleterious by Polyphen and SIFT prediction tools, were described in a family with 2 cases of melanoma. The variant A591V co-segregated with melanoma in a family with 3 affected members.

Furthermore, two somatic variants in the *DOT1L* gene (S1440T and L871P) were found in independent sporadic patients with early-onset of melanoma and no family history (see Table 2, Chapter 2[16]).

Although our studies on the R409H variant do not conclusively prove pathogenicity, we believe that a variant co-segregating with melanoma in a family with four affected members reinforce the findings by Zhu *et al.* of *DOT1L* as a melanoma susceptibility gene. We consider it worthwhile to examine whether this variant has methyltransferase-independent functional consequence and to investigate *DOT1L* variants in future studies involving large familial melanoma cohorts.

MAY HERITABLE EPIGENETIC EVENTS EXPLAIN MELANOMA SUSCEPTIBILITY?

Whole exome and whole genome sequencing analyses of familial melanoma have identified a few rare pathogenic gene variants, but failed to explain the cause of melanoma predisposition in more than half of the families.[17] This might be due to the fact that most of the identified variants are extremely rare and segregate in only a few families, along with polygenic inheritance mechanisms as well as the effects of UV radiation. In addition hereditary epigenetic alterations have been proposed to explain the 'missing heritability' of melanoma susceptibility.

Over the years, the focus has been redirected to epigenetic mechanisms that may explain the melanoma predisposition in these families. Holliday defined epimutations as heritable promoter hypermethylation leading to gene silencing.[18] In hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome) not caused by mutations in DNA mismatch repair genes, epimutation of the *MLH1* gene has been identified as causative mechanism in multiple families.[19] Subsequently, epimutation of the *MSH2* mismatch repair gene was demonstrated in another family with HNPCC.[20] In several other cancer types with familial occurrence, including ovarian cancer and retinoblastoma, candidate epimutations have been found.[21,22]

Whether a heritable epimutation can be the result of transgenerational epigenetic inheritance in the absence of an underlying genetic cause is controversial.[23,24] In fact, epimutations were described as primary, those occurring in the absence of an underlying DNA sequence alteration, or secondary, where genetic variation drives the propensity for hypermethylation at a specific locus.[25] The extent to which heritable epigenetic alterations might confer predisposition to cancer and melanoma in particular remains to be established.

Therefore, besides the putative genetic cause, we have aimed to assess whether heritable epigenetic events could account for melanoma predisposition in families where no genetic cause could be found. Possible epimutation of the *CDKN2A* gene has been explored as a mechanism to explain the melanoma susceptibility in melanoma families. For this gene, our group and others concluded that in 64 Swedish, 22 Dutch and 114 American patients with hereditary melanoma, *CDKN2A* epimutation was not the causative mechanism for melanoma susceptibility.[26-28]

In Chapter 3, we evaluated 5 families with a history of melanoma in multiple generations where no pathogenic gene variants in any of the currently established or candidate high penetrance melanoma susceptibility genes had been found. DNA from peripheral blood of 2 affected members of each family was assessed by 450K Illumina arrays.[29] In this study we made use of DNA methylation data from a valuable reference group of 1000 healthy individuals from the Biobank-based Integrative Omics Studies (BIOS) consortium. The occurrence of epimutation of the recently established melanoma susceptibility genes (*CDKN2A*, *CDK4*, *BAP1*, *TERT*, *POT1*, *TERF2IP*, *ACD, MBD4, POLH, POLE, EBF3, GOLM1, MC1R* and *MITF)* was examined in these familial melanoma patients. Secondly, loss-of-imprinting, a distinct epigenetic mechanism of inheritance by which certain genes are silenced based on its parental origin epigenetic mark, was examined.[30] Genomic imprinting is involved in fetal growth and plays a role in carcinogenesis[31], being the association between the *IGF2–H19* locus and Wilms tumour the best-described in cancer.[32] Thirdly, a genome-wide analysis of approximately 450,000 CpGs located in all human gene promoters was performed. This unbiased genomic analysis of single CpG sites was complemented by investigation of differentially methylated regions (dmrs).

In summary, there was no significant difference from the reference group in methylation level at any of the melanoma susceptibility genes. Nor did we find any indication of loss-of-imprinting at the CpG sites located at the imprinted loci. The exploratory analysis found 6 hypermethylated CpGs in both affected members of a melanoma family compared to a healthy cohort as well as 35 hypomethylated CpGs. Although we did find the 6 hypermethylated CpGs located in the promoter regions of *RABGGTB, SND1, SCAF11, ZNF638, THAP1* and *SFSWAP* genes, none of them have been reported as a cancer predisposition gene or functions related to melanoma.[33] Besides these also the dmrs in the promoters of *CCNI, CD47* and *USP46* genes showed minor differences compared to healthy controls. Two of 35 hypomethylated CpGs were located in cancer-related genes, but were not found to be plausible candidates either. This was the first time that heritable epigenetic events in melanoma have been analysed in a genome-wide fashion interrogating 99% of human genes. We conclude that none of the hyper/hypomethylated CpG sites and dmrs in these

families provide a relevant pathogenic explanation for melanoma inheritance. However, it is still possible that pathogenic heritable DNA methylation alterations might occur in a minor proportion of melanoma families or that epimutations may affect other potentially regulatory regions. Recently, constitutional mosaic epimutations in *MGMT, MLH1* and *BRCA1* genes have been reported as the epigenetic cause of a significant number of glioblastoma, colorectal and ovarian/breast cancers, respectively.[34] Therefore, constitutional methylation of key tumour suppressor genes may represent an initiating event of carcinogenesis that might be present in a mosaic fashion due to a post-zygotic epigenetic event in melanocyte precursor cells and not be retrievable in DNA from blood.

Uncovering the causes of predisposition to melanoma in families is of major clinical relevance since it could improve the risk estimation, genetic counselling and testing and enable targeted clinical surveillance of patients at high risk of melanoma. Accordingly, further studies for such constitutional epigenetic events could aid cancer prevention programs.

CAN AN ALTERED DNA HYDROXYMETHYLATION PATTERN BE A BIOMARKER OF MELANOMA?

The role of DNA methylation in cancer has been widely explored. Cutaneous melanoma demonstrates altered patterns of DNA methylation that are associated with genetic instability and transcriptional repression of tumour suppressor genes. A more recently discovered epigenetic modification, that has since received increasing attention of the cancer research field, is DNA hydroxymethylation. Active DNA demethylation entails a hydroxymethylation step mediated by TET enzymes, which catalyze conversion of 5-methylcytosine (mC) to 5-hydroxymethylcytosine (hmC). Hydroxymethylcytosine can thus be considered as an intermediate step in the active demethylation of DNA.

In Chapter 4, we aimed to identify CpG sites and regions with differential hydroxymethylation and methylation levels when comparing melanoma (metastatic and non-metastatic) and melanocytic nevus samples that might be used as diagnostic and prognostic markers. In addition we intended to gain insights into the functional significance of this DNA modifications by studying its distribution. A study from Lian *et al.* had previously reported the loss of hydroxymethylation as an epigenetic hallmark of melanoma.[35] Oxidative bisulfite chemistry combined with arrays that simultaneously interrogate hmC and mC at 850,000 CpG sites throughout the genome were used in our study. This methodology followed by bioinformatic analysis revealed significant differences in the global hmC patterns of melanoma and nevus samples, being more pronounced than in mC patterns. Although the

levels of hmC were uniformly lower in metastatic than in non-metastatic melanoma, the patterns of distribution were not significantly different. DNA hmC loss may be explained by passive dilution of the hmC mark due to DNA replication in proliferating melanoma cells and by insufficient active demethylation.[36,37] Within the pattern of global hmC depletion, specific CpG sites and regions could be identified with significantly lower hydroxymethylation in melanoma than in nevus, pointing to epigenetic deregulation at specific loci. We identified 22,164 single differentially hydroxymethylated CpG sites and 68 regions that are strong candidate biomarkers for diagnosis.[38-40] Among the 68 DhMRs, 5 localized to cancer-related genes: *PTEN, DAXX*, *GAS7*, *GNAS* and *TPM4*. *PTEN* is an essential tumour suppressor gene in melanoma. Many previous studies report downregulation of PTEN in melanoma compared to nevus, *PTEN* inactivation through promoter hypermethylation, and enhanced melanoma formation upon PTEN downregulation.[35,41-45] These findings strengthen our hypothesis that hmC depletion at the *PTEN* regulatory region in melanoma has functional significance by affecting expression of this tumour suppressor gene. Briefly, in normal skin, the development of melanocytic nevi is caused by activating mutations of the *BRAF* or *NRAS* oncogenes, which provides a proliferative stimulus to the cells at first but eventuates in a state of stable cell cycle arrest (oncogene-induced senescence). We found that at this stage the *PTEN* promoter region shows high hmC and low mC levels. The accumulation of mC due to reduced active demethylation (loss of hmC) at this region regulating *PTEN* expression may lead to transcriptional silencing of this essential tumour suppressor gene facilitating escape from oncogene-induced senescence and contribute to malignant transformation.

Recently, Bonvin *et al.* made use of a well-established genetically engineered *Nras* mutation–driven mouse model of spontaneous melanoma to disclose the roles of specific epigenetic alterations in melanomagenesis. They showed that in mice, genetic ablation of *Tet2* in combination with *Nras*^{Q61K}-driven melanoma mouse model promotes melanoma initiation by an overall decrease in hmC and specific hmC gains in selected gene bodies, accelerates melanoma progression and decreases melanoma-free survival. They also suggest that the presence/absence of hmC modulates the binding of epigenetic factors resulting in changes in chromatin structure rather than directly affecting gene transcription.[46] Similar to our results a striking hmC depletion in melanoma compared to nevus samples was observed exceeding those of mC patterns. Altogether these studies suggest that active demethylation may protect promoter and enhancer regions from methylation-associated silencing and therefore loss of hmC might contribute to malignant progression. Our findings call for further functional studies of the role of DNA hydroxymethylation at the *PTEN* locus and other tumour suppressor gene loci.

The reasons behind hmC reduction have been widely explored and some studies report that mutational inactivation and/or downregulation of *TET2* might explain the loss of hmC as well as mutant IDH proteins.[35,47] While wild-type IDH protein produce αKG, the co-substrate of TET enzymes, the mutant IDH transform it into 2-HG, an oncometabolite that is a competitive inhibitor of TET. Resetting the differential mC and hmC levels towards a functional demethylation pathway might be an interesting target to cancer therapy.[48] Recently, a new combinatorial treatment between vitamin C and ML309, *IDH1*-mutant inhibitor, showed a synergistic effect in colon cancer cells, significantly decreasing the 2-HG levels to levels comparable with WT cells, leading to increased levels of global hydroxymethylation and even increased expression of tumour suppressors.[49]

HOW IS *TERT* GENE EPIGENETICALLY REGULATED?

Located on chromosome locus 5p15.33, telomerase reverse transcriptase (*TERT*) encodes the catalytic subunit of the ribonucleoprotein telomerase and is capable of extending the repetitive, non-coding DNA sequence of telomeres following mitosis.[50] Telomeres become shorter at each cell division, until reaching the Hayflick limit, which induces replicative senescence and growth arrest.[51] Through *TERT* reactivation, the cells keep the ability of extending their telomeres or prevent their shortening allowing immortalization of malignant cells and tumour progression as occurs in 90% of human cancers. This limitless replicative potential by active telomere elongation is one of the hallmarks of cancer.[52-54]

Expression of TERT is a key determinant of telomerase activity in human cells. Activating *TERT* promoter (*TERT*p) mutations are reported in 30–80% of familial and sporadic melanomas.[55,56] The well-known influence of epigenetic mechanisms in the regulation of gene expression in cancer led us to explore how the *TERT* gene can be epigenetically reactivated. As mentioned above in this thesis the canonical effect of hypermethylation in the promoter regions of cancer cells is to repress genetic transcription.[57,58] Contrary to common concepts, CpG methylation in the *TERT*p was previously correlated with higher *TERT* mRNA expression.

In Chapter 5, we aimed to characterize the genetic-epigenetic interaction in the regulation of the *TERT* gene in healthy skin samples and melanoma cell lines. The two hotspot mutations in *TERT*p, dubbed C228T and C250T, together with *TERT*p methylation, chromatin accessibility and *TERT* expression were assessed. The methylation fraction was evaluated by NGS in a set of 31 CpGs in the *TERT* promoter region and validated by custom ddPCR-based assays, namely for the CpG cg11625005 that deserved special attention. Previously

in brain tumours and melanoma this CpG showed absence of methylation in normal cells.[52,59] However, in our study, the methylation fraction was quite high in healthy skin samples, naevi and cultured keratinocytes. Furthermore, our results suggest that context-related methylation of a genomic region might be biologically more relevant as opposed to methylation of one specific CpG. The two point mutations in the *TERT*p described in melanoma create new transcription factor binding motifs and correlate with upregulation of the *TERT* gene. In our cohort, we checked the presence of these mutations through ddPCR-based assays. Altogether, *TERT* expression was only found in tumour cell lines, with or without *TERT*p mutation and a broad range of promoter methylation levels (5%-100% methylation fraction). Thus, we hypothesise that another level of regulation was responsible for mRNA expression. Since distinct histone modifications influences chromatin remodelling and ultimately gene accessibility to transcription factors, such as ETS/TCF, we investigated to which extent the chromatin was accessible and whether this had occurred in a mono- or biallelic fashion. We observed a positive correlation between chromatin accessibility and methylation levels as well as mRNA expression leading us to conclude that mutated alleles were more accessible, possibly favouring the binding of transcription factors and consequently *TERT* mono-allelic expression of the mutant allele. Our results are in line with the study from Stern *et al.* and Huang *et al.*, where the authors found that the mutant allele was hypomethylated and allowed monoallelic *TERT* expression.[60,61] Thus, these observations are consistent with the canonical influence of methylation on transcriptional regulation. On the contrary, the WT *TERT*-expressing uveal melanoma cell lines show a methylation fraction close to 100% and significantly higher chromatin accessibility. These characteristics of WT *TERT*p cell lines may lead to biallelic *TERT* activation. In fact, in this case *TERT* seems to be an exception, since possible biallelic hypermethylation leads to upregulation.

Taking everything into account we might conclude that *TERT* activation is under distinct epigenetic regulation in mutated and WT *TERT*p cell lines. While in mutant *TERT*p, methylation executes its canonical function, repressing transcriptional regulation, in WT *TERT*p, methylation carries out a non-canonical role, leading to transcriptional activation in these cell lines. The dynamics of epigenetic mechanisms in *TERT* genetic regulation is complex, however we believe that our results contribute to the full understanding of all (epi) genetic mechanisms that collectively reactivate *TERT*.

TERT was first recognized for the telomere maintenance and over the years many other non-canonical roles have been reported, such as involvement in regulation of non-telomeric DNA damage responses, promotion of cell growth and proliferation and control of mitochondrial integrity following oxidative stress. Moreover, TERT behaves as a regulator of

genetic transcription through chromatin modulation.[62,63] Beyond the telomere maintenance, *TERT* is crucial to other aspects of the tumour microenvironment, such as angiogenesis, inflammation and cancer cell stemness.[64] Although TERT repression may sensitize cells to conventional chemotherapy in a telomere independent manner[65-67], due to the above mentioned telomere-independent functions this approach can lead to severe side effects.[63] Therefore, further study is required to elucidate the conventional and alternative molecular mechanisms of telomerase beyond telomere maintenance, in order to develop new anticancer strategies targeting telomerase or telomeres.[63,68]

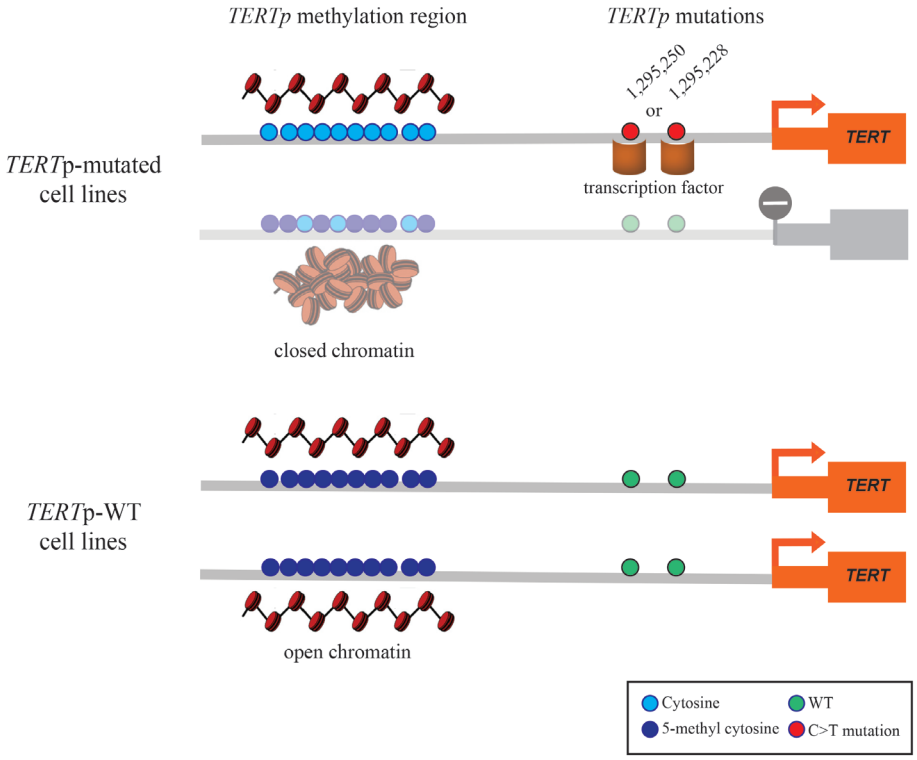

FIGURE 1. Proposed model of *TERT* transcriptional regulation. In *TERT*p-mutated cell lines, active mutant allele is hypomethylated, highly accessible and allows monoallelic *TERT* expression. In *TERT*p-WT cell lines, the MF is close to 100% with a significantly higher chromatin accessibility leading to high expression levels due to biallelic *TERT* activation.

HOW DO OUR FINDINGS CONTRIBUTE TO THE UNDERSTANDING OF EPIGENETIC ALTERATIONS IN THE SUSCEPTIBILITY TO AND DEVELOPMENT OF MELANOMA?

In our study, we discovered R409H variant in the *DOT1L* gene using WES in a family. Although we were not able to definitely prove the functional effect of the R409H variant in the *DOT1L* gene, encoding a H3K79 methyltransferase, our findings reinforce the ones by previous studies, which pointed to *DOT1L* as a melanoma susceptibility gene with a role in DNA damage repair.[11,12] *DOT1L* is not involved in transcriptional regulation of the DNA repair genes, but rather promotes the assembly of the nucleotide excision repair complex on chromatin by interacting with XPC and stimulating its recruitment to DNA lesions.[12] Loss of *DOT1L* impaired DNA damage repair induced by UV-B radiation, thereby promoting melanoma development *in vivo*. To fully disclose the functional consequences in regard to melanoma risk of the variant identified in our study, an engineered mouse model to harbour the *DOT1L* R409H variant might be valuable tool.

As previously observed in patients with hereditary nonpolyposis colorectal cancer and familial chronic lymphocytic leukemia, heritable epigenetic events might be a plausible explanation for melanoma predisposition.[19,20,69-72] For the first time we performed genome-wide analysis assessing all CpGs located in gene promoters of candidate and established susceptibility genes, imprinted genes and gene promoters throughout the genome. We conclude that none of the hypermethylated CpGs and differentially methylated regions identified do constitute a pathogenic epimutation in predisposition to melanoma in these families. Nevertheless, it is still possible that pathogenic heritable DNA methylation alterations might either exist in a mosaic pattern through a post-zygotic epigenetic event, potentially only affecting melanocytes or occur in other potentially regulatory regions rather than in promoters or in a small number of melanoma families.

Beyond the epigenetic silencing of tumour suppressor genes or inherited epigenetic alterations involved in the predisposition to cancer, we also explored how epigenetic alterations may contribute to progression towards malignancy. Specific CpG sites and regions identified with differential distribution and significantly lower hydroxymethylation levels in melanoma than in nevus can be used to aid in distinguishing malignant from benign melanocytic lesions.[38-40] Moreover, the presence of hmC at the *PTEN* regulatory region in nevus, and its loss in melanoma, may signify that active demethylation can protect promoter and enhancer regions from methylation-associated silencing of tumour suppressor genes. Loss of hmC might therefore contribute to malignant progression, in accordance with hmC

depletion observed at the *PTEN* promoter region in melanoma.

Genetic regulation comprises different processes and pathways. We have shown that a holistic model encompassing *TERT*p mutation, methylation and other epigenetic mechanisms as chromatin accessibility are crucial to dictate *TERT* gene expression. Either a widely open chromatin state in *TERT*p-WT samples due to hypermethylation throughout the promoter or mono-allelic expression of the accessible mutated (C228T or C250T) allele in combination with moderate (probably allele-specific) methylation fraction are required.
In general, the studies presented in this thesis show associations between epigenetic states and cellular gene expression patterns or phenotypes. However demonstration of causality would require methods to specifically introduce such epigenetic alterations and investigate their consequences. Whereas CRISPR/Cas9 technology has become a valuable tool to engineer genetic alterations, similar methods to introduce epigenetic changes still need further development.

SUMMARY AND FUTURE PERSPECTIVES

Over recent years, our understanding of the genetic variation underlying cutaneous melanoma susceptibility has increased, with the discovery of pathogenic gene variants that explain half of melanoma susceptibility in families. The focus has been redirected to the epigenetic mechanisms that may either explain the melanoma predisposition in the remaining families and to reveal how certain genes are repressed or activated in melanoma. Nowadays, the integration of genomic/epigenomic/transcriptomic/metabolomic data seems to be of major importance. We generated a comprehensive view of interaction between genome and epigenome of cancer cells by addressing hydroxymethylation as a possible protection against hypermethylation-associated silencing at promoter regions of relevant genes as well as by revealing how mutations, methylation and chromatin organization may interact to regulate the expression of a gene involved in the immortalization of cancer cells. Many questions still remain for instance on hydroxymethylation distribution at the promoter region that may interfere with allele-specific chromatin accessibility and expression. We were also able to identify potential epigenetic diagnostic markers for melanoma and (epi) genetic alterations that must be taken into consideration in future studies of melanoma predisposition.

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