Control of replication associated DNA damage responses by Mismatch Repair

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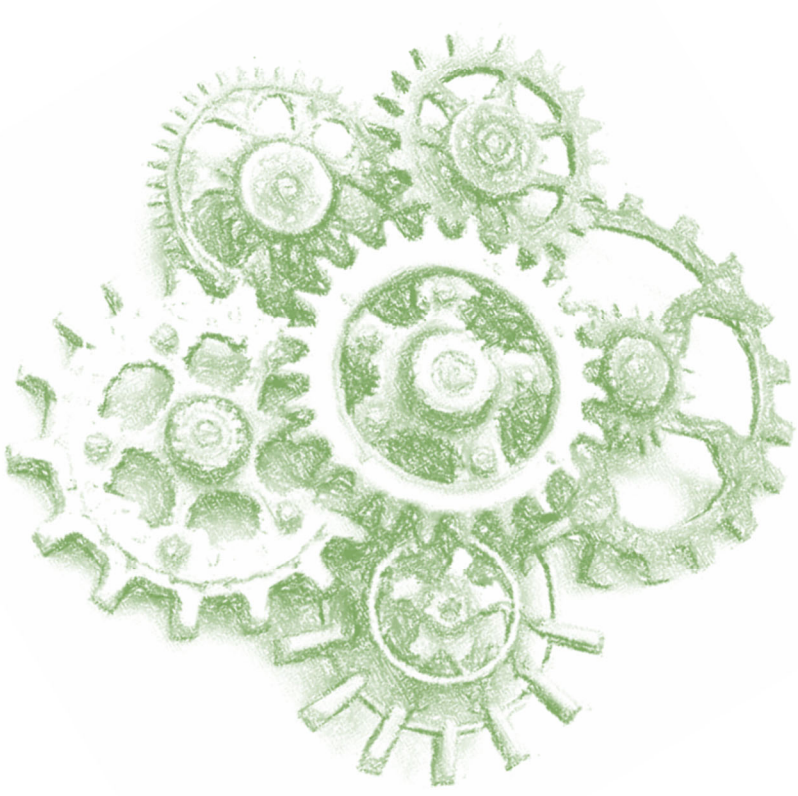

Chapter 6:
Discussion and future perspectives

DNA replication is a complex process that can lead to mutagenesis and disease when performed incorrectly. This thesis aimed to investigate how mutagenesis induced by bulky, helix-distorting DNA damage is regulated with a particular focus on DNA mismatch repair (MMR), a pathway that removes replication errors made by replicative DNA polymerases. Contrary to undamaged DNA, helix-distorting DNA lesions are replicated by translesion synthesis (TLS) polymerases with a significantly lower fidelity. This led to the hypothesis that besides its canonical function, MMR may also control TLS-related DNA damage responses.

The work presented in **chapters 3** to **5** show that the four main MMR subunits, Msh2, Msh6, Mlh1 and Pms2, all participate in reducing mutagenesis caused by ultraviolet (UV) radiation and the dietary mutagen 2-Amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (PhIP). Moreover, it was shown that loss of the Msh2/Msh6 heterodimer, also known as MutSα, resulted in loss of UV and PhIP-induced DNA damage signaling and a reduction in DNA damage-induced ssDNA formation. Interestingly, loss of the Mlh1/Pms2 heterodimer, also known as MutLα, showed no such phenotype, revealing an uncoupling of DNA damage signaling and mutagenesis induced by UV-light. The data in **Chapter 4** shows that the gene *Polh,* which encodes for the TLS polymerase eta (Polη), and *Msh6* or *Mlh1* act synergistically with respect to suppressing UV-induced mutagenesis. Moreover, **Chapter 4** indicates that in *Polh*-defective cells *Mlh1* is not required for activating UV damage responses or for the formation of ssDNA, supporting the findings of **Chapter 3**.

This chapter aims to discuss these findings and to put them in a broader biological perspective. Thus, this chapter will discuss why only the four canonical MMR proteins are significantly involved in the DNA damage response, but not other MMR-related factors, such as the exonucleases and MMR homologs. Next, the findings regarding the uncoupling of UV-induced damage signaling and UV-induced mutagenesis are discussed, what role the two different MMR heterodimers, MutSα and MutLα, may play in these processes and how these findings relate to the observed cancer predisposition associated with defects in the four canonical MMR proteins. Furthermore, additional models are presented how MMR may control the DNA damage response, via (i) controlling the recruitment of TLS polymerases, (ii) reducing the mutagenicity of TLS, or (iii) controlling the activity of error-free template switching. Sporadic or inherited loss of MMR genes is associated with an increased risk of developing colorectal and endometrial cancer. As of yet, it remains unclear what causes this specific cancer tropism, since MMR should be important for suppressing mutagenesis in each replicating cell of the body. This chapter will elaborate on the possible causes of the cancer tropism associated with loss of MMR. Finally, this chapter closes with concluding remarks and advises carriers of dysfunctional MMR genes and their physicians to be mindful of the dangers of DNA damaging compounds.

Identifying genes involved in suppressing DNA damage-associated mutagenesis

Cells in the human body accumulate at least 50000 endogenous DNA lesions per day (1) and are also exposed to exogenous agents that induce DNA damage. In the past, numerous studies have been done to characterize the DNA damage response of the canonical MMR proteins Msh2, Msh6, Mlh1 and Pms2. It has been shown, using a variety of models, that MMR proteins are important in suppressing mutagenicity resulting from a broad range of DNA lesion types, including small base modifications due to methylation, ethylation and oxidation, but also from DNA lesions that distort the DNA helix, such as bulky lesions and intrastrand crosslinks (table 1). Together, these studies suggest that MMR proteins play an important role in protecting cells from the genotoxic effects of a wide variety of persistent base damages. The study described in **Chapter 3** extends these findings by measuring UV-induced mutagenesis in *Mlh1* and *Pms2*-deficient mouse embryonic stem cells (mESC). It was found that all four core MMR genes, *Msh2*, *Mlh1*, *Msh6* and *Pms2*, are important for suppressing UV-induced mutagenesis. However, canonical MMR requires not only the activity of these four core proteins, but often also requires exonuclease activity (2). Inactivating mutations in the exonucleases *Exo1* and *Fan1* did not increase UV-induced mutagenesis either. It was previously shown that deficiency for *Exo1* leads to upregulation of other exonucleases, such as Mre11, Artemis and Fan1 (3). Extensive redundancies among these exonucleases may be the reason that there is no apparent UV-induced phenotype in the Exo1 deficient cells. Knock-out of any single exonuclease may be complemented with increased activity of the others. To that end, it may also be interesting to create double and even triple knock-out cell lines to study the role of exonucleases in the UV-induced damage response.

Table 1: Summary of known literature regarding the role of MMR in dealing with DNA damage induced mutagenesis (muta) and tumorigenesis (tumor).

Agent	DNA lesions	MMR defect	Cells	Reporter gene	Muta	Tumor	Ref
MMS	methylation	Mlh1	Human cancer cell line	*HPRT*	↑		(66, 67)
		Msh6	Human cancer cell line	*HPRT*	↑		(66, 67)
Temozolomide	methylation	Msh2	Mice			↑	(68)
MNNG	methylation	Msh6	Human cancer cell line	*HPRT*	↑		(69)
ENU	ethylation	Msh2	Mice and Mouse cells	*Hprt*	↑	↑	(70)
EMS	ethylation	Pms2	Mouse kidney cell line	*Aprt*	↑		(71)
H_2O_2	oxidative	Pms2	Mouse kidney cell line	*Aprt*	↑		(71)
Cisplatin	crosslink	Msh2	Human cancer cell line	Acquired resistance assay	↑		(72)
Cisplatin	crosslink	Mlh1	Human cancer cell line	Acquired resistance assay	↑		(72)
UV	Intrastrand crosslink	Msh6	Chinese hamster cell line	Acquired resistance assay	↑		(73)
		Pms2	Chinese hamster cell line	Acquired resistance assay	↑		(73)
		Pms2	Mouse kidney cell line	*Aprt*	↑		(71)
		Msh2	Chinese hamster cell line	Acquired resistance assay	↑		(73)
		Msh2	Mice			↑	(74)
		Msh2	Mouse embryonic fibroblasts	*Hprt*	↑		(75)
		Msh6	Mouse embryonic stem cells	*Hprt*	↑		(11)
PhIP	bulky	Msh2	Human cancer cell line	*HPRT*	↑		(46)
		Mlh1	Human cancer cell line	*HPRT*	↑		(46)
		Msh6	Human cancer cell line	*HPRT*	↑		(46)
		Msh2	Mouse intestine in vivo	*lacI*	↑		(76)
		Mlh1	Mouse intestine in vivo	*cII*	↑		(77)
B[a]P	bulky	Msh2	Mice			↑	(78)

Exo1 is known to have both catalytic and structural domains that are suggested to play a role in the DNA damage response and MMR, respectively (4). It is important to note that the *Exo1* mutants in this thesis were generated by disrupting the catalytic domain resulting in mutants that still expressed mRNA which might encode for a truncated protein containing the structural domain. However, these *Exo1* mutants display tolerance to methylating agents such as N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) (5), indicative for defective canonical MMR. Thus, the *Exo1* mutants described in this thesis are most likely functionally deficient for *Exo1*. For unknown reasons, however, an experiment to generate cell lines with a complete knock-out of the *Exo1* gene using CRISPR did not produce any viable clones.

Additionally, Pms1 and Mlh3 are MMR homologs that can bind to Mlh1 and may also play a role in the UV-induced DNA damage response (6). Previous publications have shown that the yeast ortholog of Pms1 co-localizes with Msh2/Msh6 and is important in activation of platinum-induced DNA damage responses (7). Moreover, a dominant *PMS1* mutation was found that conferred a mutator phenotype in human cells (8). For *Mlh3* it was previously observed that mice with an *Mlh3*-deficiency develop colon cancer at an increased rate (9) and that Mlh3 also seems to relocate to sites of UV-damage (10). **Chapter 3** investigated the role of these proteins and found that deficiency of either *Mlh3* or *Pms1* caused no apparent phenotype in mESC, thus these genes are seemingly not involved in any significant UV-induced DNA damage response. Previous work has also shown that the phenotypes induced by *Mlh3*-deficiency are minor and are exacerbated by additional loss of *Pms2* (9). Therefore, it may be that Mlh3 still plays a minor backup role for Pms2 and that a double knock-out cell line may reveal this additional functionality. Additionally, it may be of interest to investigate other MMR binding partners with respect to their role in the DNA damage response. For instance, it has previously been shown that in human cancer cell lines the MCM9 helicase was important for canonical MMR activity (11) and thus may be similarly involved in the MMR-mediated DNA damage responses. Likewise, the bloom helicase BLM binds directly to both human MSH6 and MLH1. Although BLM does not seem to be important for canonical MMR (12, 13), it may be involved in MMR-mediated DNA damage responses. Finally, it may also be of interest to study the role of DNA2, a nuclease that is known to play a role in Exo1-independent MMR and may also play a role in the MMR-mediated DNA damage response (14).

The uncoupling of DNA damage signaling and mutagenesis in MMR-deficient cells: consequences for colorectal cancer development?

Chapters 3 and **4** show that both MutSα and MutLα participate in the suppression of UV-induced mutagenesis. To obtain mechanistical insights in how these MMR genes control UV-induced mutagenesis, the spectrum of UV-induced mutations at the *Hprt* coding region in cell lines deficient for *Msh6* or *Mlh1* was studied (**chapter 3**). No difference was observed between the *Msh2* and *Mlh1*-deficient cell lines in terms of the kinds and types of mutations induced by UV light. Most of these mutations were

located at dipyrimidine sites, suggestive of *Msh6* and *Mlh1* working on misincorporations opposite UV lesions. However, also in unexposed cells most mutations occurred at dipyrimidine sites. Thus, from these data it cannot be concluded that UV-induced mutations were actually targeted at UV lesions. These mutational spectra analyses were restricted to the exons of the *Hprt* reporter gene encompassing a coding sequence of only 654 nucleotides. Therefore, studying the UV-induced mutational profile of MMR-deficient cells by using whole genome sequencing may be more informative. Nevertheless, previous work with *Msh6*-deficient cells showed that UV-induced mutations are predominantly targeted to a site-specific 6-4PP in a replicating plasmid (15). In addition, it has been shown that MutSα more strongly binds to incorrectly paired 6-4PPs rather than 6-4PPs that are paired with the correct bases (16, 17). Moreover, using antibodies that recognize CPDs (**Chapter 4**) or 6-4PPs (15) in ssDNA configuration, it was shown that loss of Msh6 resulted in less ssDNA gaps opposite UV-lesions, suggesting Msh6-mediated excision of ssDNA opposite UV-damaged DNA. Taken together, these data argue for a model in which misincorporations opposite UV lesions are removed by MMR, thereby suppressing UV-induced mutagenesis. To further evaluate the hypothesis that MMR excises replication errors opposite damaged DNA, additional experiments are of interest. Previous studies have shown that MutSα is able to bind various damaged DNA substrates, including those containing 'mismatches' opposite UV photolesions (16, 17) and O^6-methylguanines (18) and substrates that contain cisplatin intrastrand crosslinks (18), deoxyguanosine adducts of aminofluorene and acetylaminofluorene (19), benzo[*a*]pyrene-7,8-hydrodiol-9,10-epoxide adducts at guanines (20) and benzo[c]phenanthrene adenines (21). It would be interesting to analyze whether MutSα can also recognize replication errors opposite PhIP-damaged guanines to support the findings summarized in **chapter 5**. Moreover, it would be of interest to study the structure of such a binding using crystallography to better understand how and why MutSα binds to (mis-)paired PhIP adducts. It would also be valuable to study MMR activity in the panel of MMR-deficient cell lines described in this thesis by using a replicating plasmid containing a site-specific UV-lesion or PhIP adduct, similar to what was previously published in *Msh6*-deficient cells (15). Finally, it may be of interest to perform *in vitro* experiments to measure MMR activity in nuclear extracts using a nicked DNA substrate that contains 'matched' or ''mismatched' nucleotides opposite a photolesion or PhIP adduct, reminiscent of the cell-free *in vitro* MMR activity (CIMRA) assay that measures G-T mismatch repair in a test tube (22).

The generation of UV-induced mutations is the outcome of a cellular response to unrepaired photolesions that includes, amongst others, the activation of the Atr/Chk1 and the Atm/Chk2 signaling axes, induced by the formation of ssDNA and double strand DNA breaks (DSB), respectively (23). While studying the phosphorylation of Chk1 and Kap-1 as markers for ssDNA and DSB-induced activation of DNA damage signaling (24, 25), an interesting observation was made: while loss of MutSα resulted in reduced UV-induced phosphorylation of Chk1 and Kap-1, loss of MutLα showed no such phenotype. This result suggests that MutSα mediates both formation of ssDNA

and DSB upon UV-irradiation, independent of MutLα. This suggestion was supported by studies on the UV-induced formation of chromatin-bound Rpa, indicative for the generation of ssDNA (**chapters 3** and **4**)**.** Combined with an investigation of the level of UV lesions in ssDNA configuration, as a more direct measure of ssDNA gap formation opposite UV lesions, the results indicated that knock-out of *Mlh1* did not reduce the formation of UV-induced ssDNA. Taken together these data suggest an uncoupling of UV-induced mutagenesis and ssDNA formation in the case of *Mlh1* and *Pms2*-deficient cells. This is a puzzling finding, since in the canonical MMR model mutagenesis is controlled by removing the misincorporation, thus by generating ssDNA, in which MutLα is an important factor. The possible mechanisms that may explain the UV-induced activation of the Atr/Chk1 and the Atm/Chk2 signaling axes in the absence of MutLα are extensively discussed in **chapters 3** and **4**. These mechanisms include Msh2-dependent stimulation of Mre11-mediated degradation of nascent DNA at stalled replication forks, Mlh1-independent removal of misincorporations in the lagging strand and Exo1 hyper-excision of DNA in the absence of Mlh1. The latter was tested experimentally by inactivating *Exo1* in *Mlh1*-deficient cells. However, these double knock-out cells displayed only slightly reduced UV-induced damage signaling as compared to *Mlh1*-deficient cells (**Chapter 3**), indicating that Exo1 hyper-excision only slightly contributes to the activation of checkpoint signaling after UV exposure. Thus, additional experiments and considerations are required to better understand how MutLα controls UV-induced mutagenesis independent of activation of UV damage signaling.

Counterintuitively, the MutLα-independent activation of protective DNA damage signaling pathways contrasts strongly with the observation that germ line mutations in MLH1 or MSH2 are associated with a higher penetrance and earlier mean age of cancer onset than MSH6 or PMS2 mutations (26). In addition, **Chapter 5** shows that there is no significant difference among the four core MMR proteins in the level of protection against diet-derived PhIP-induced mutagenesis, which is thought to play a role in the development of CRC (see below). However, **chapter 5** also reveals that loss of Msh2 results in reduced PhIP-induced DNA damage signaling which may give Msh2-deficient cells a growth advantage. Thus, one interesting experiment for the future could be to grow a mixed population of wild-type and *Msh2*-deficient cells in the presence of a chronic low-dose of PhIP and monitor if *Msh2*-deficient cells will dominate the culture of cells over time. Recently, others have shown that a defect in MSH2 in colonic organoids or human embryonic stem cells results in reduced replication stress as well as a growth advantage compared to MMR-proficient cells (27). Reduced DNA damage signaling, hypermutability and a growth advantage of MSH2 defective cells may possibly explain the strong contribution of MSH2 carriers in the population of LS patients. In contrast to loss of MSH2, which renders cells completely defective in MMR, loss of MSH6 leads to a somewhat milder phenotype as these individuals still express the MSH2/MSH3 heterodimer involved in the recognition and removal of insertion-deletion loops. Possibly, for this reason carriers with a MSH6 mutation are less likely to contract CRC as compared to carriers with a MSH2 mutation.

But what about MLH1? As mentioned before, contrary to *Msh2* deficiency, *Mlh1*-deficient cells do activate DNA damage signaling (**Chapters 3** and **4**) that can result in decreased cell cycle progression (see also **chapter 4**), increased DNA repair and increased senescence or apoptosis in order to prevent mutagenesis and, consequently, tumorigenesis (28). Yet, *Mlh1*-deficient cells display hypermutability after exposure to UV light and other DNA damaging agents (**Chapters 3-5**, Table 1) and *Mlh1*-deficient mice show enhanced mutagenesis following PhIP exposure (Table 1). Together these data suggest that tumorigenesis in the absence of Mlh1 may occur more frequently, despite the activation of DNA damage signaling. This paradoxical phenotype is somewhat reminiscent of that found in UV-exposed mice containing a defect in TLS polymerase Rev1. Although a *Rev1* defect results in enhanced replication stress, increased DNA damage signaling and reduced mutagenesis (29), UV-exposed *Rev1*-mutated mice develop skin tumors much more rapidly than *Rev1*-proficient mice. This is accompanied with hyperproliferation in the skin and increased expression of IL-6, a cytokine that can stimulate cell proliferation in a paracrine fashion (29). Apparently, enhanced cell proliferation compensates for increased DNA damage signaling and reduced mutagenesis in the *Rev1*-mutant mouse skin. Interestingly, IL-6 signaling is triggered by the presence of cytosolic DNA (30) resulting from processing of ssDNA and DSB. Since *Mlh1*-deficient cells generate increased levels of ssDNA, and likely also DSB, following the formation of bulky and helix-distorting DNA damage (**chapters 3** and **4**), processing of ssDNA and DSB may result in the formation of cytosolic DNA (31). Consequently, it might be possible that Mlh1-deficient cells compensate for the anti-proliferative effects of checkpoint signaling by enhancing cell proliferation via the expression of cytokines such as IL-6. Thus, the higher risk for developing CRC in carriers with an *MLH1* mutation might be explained by (i) DNA damage-induced hypermutagenesis in *Mlh1*-deficient cells and (ii) enhanced cell proliferation that is promoted following the formation of cytosolic DNA derived from processed ssDNA and collapsed replication forks.

Post-replicative control of DNA damage-induced mutagenesis

Persistent DNA damage induces mutations in the genome via different routes. Unreplicated ssDNA formations may become DSB resulting in genomic alterations when repaired in an error-prone fashion (32), DNA repair pathways can be error-prone (33) and DNA damage tolerance pathways may bypass the damaged DNA incorrectly (34). **Chapter 4** investigated how MMR may control the mutagenicity of TLS and two hypotheses were formulated: MMR-dependent recruitment of the “correct” TLS polymerases or MMR-dependent excision of TLS errors. **Chapter 4** concluded that neither model is an exact fit and that the two models may be interwoven; MMR-mediated excision of TLS-induced misincorporations will activate DNA damage signaling, cell cycle arrest and DNA repair to prevent excessive mutagenesis in surviving cells, whilst post-excision recruitment of “correct” TLS polymerases by MutSα may quench DNA damage signaling and, in addition, prevent mutagenesis.

As an alternative to TLS, cells can employ replication fork reversal and template switching to bypass replication-blocking DNA lesions. Template switching prevents DNA damage-induced mutagenesis and involves recombination of a nascent strand with the complementary, newly synthesized strand of the sister chromatid. Although template switching in mammalian cells is still poorly understood, template switching in yeast depends on the Rad5 protein (35). Rad5 is an E3 ubiquitin ligase as well as a DNA helicase that catalyzes fork reversal and strand invasion. Recently, it was not only shown that both yeast Msh2 and Mlh1 interact with Rad5 but also that the human homologs of Rad5, HLTF and SHPRH, are binding partners of MSH2 and MLH1, respectively (36). Although the biological relevance of these interactions is still unclear, it should be noted that HLTF and SHPRH prevent mutagenesis in a DNA damage-specific manner: HLTF suppresses UV-induced mutagenesis, whereas SHPRH contributes in suppressing methylation-induced mutagenesis (37, 38). Experimental evidence suggests that (i) HLTF might stimulate the recruitment of Polη to allow relatively error-free bypass of UV damage, (ii) HLTF might prevent the interaction of SHPRH with error-prone Pol κ at UV damage, and (iii) SHPRH can interact with Pol κ, which acts relatively error-free at methylation damage (38). In addition, HLTF, like Rad5, can reverse stalled replication forks resulting in chicken foot structures that allow bypass of DNA damage in an error-free fashion. Furthermore, HLTF promotes strand invasion and D-loop formation that are intermediates during template switching (39). The interactions of MMR proteins with HLTF and SHRPH could play two important roles in suppressing DNA damage-induced mutagenesis. First, following the recognition of ‘compound lesions’ caused by error-prone TLS across nucleotide lesions, MMR proteins may stimulate error-free TLS by recruiting Pol η or Pol κ via HLTF or SHPRH. Second, recognition of ‘compound lesions’ by MutSα may activate HLTF-mediated fork reversal or template switching. It may be of interest to perform follow-up studies by knocking-out HLTF and SHRPH in MMR-proficient mESC as well as in cells defective for MutSα or MutLα and analyze mutagenic and signaling responses following UV irradiation.

Aberrant responses to food-derived DNA damage and the Lynch syndrome tropism

The narrow colorectal/endometrial cancer tropism of LS is an interesting phenomenon. Lynch syndrome is caused by loss of MMR in individuals who inherited one dysfunctional copy of an MMR gene (26). In principle, tissue types with many proliferating cells should all be affected as these tissues will garner many replication errors that are substrate for MMR. Yet, cancer in the hematopoietic system, where cells are constantly dividing to create mature blood and immune cells (40), is infrequently found in LS patients. To make matters even more interesting, inheriting not one but two faulty copies of a MMR gene (also known as constitutional MMR deficiency) does predispose carriers to a large range of cancers in highly dividing tissues, including the hematopoietic system and the (still developing, young) brain (41).

The answer to what causes the LS tropism may be found in what sets the gastro-intestinal tissue apart from the others: cells of the gastro-intestinal tract are continuously exposed to DNA reactive metabolites derived from food substances. In LS patients these DNA damaging agents may inactivate the remaining, functional, copy of the MMR gene and thus kickstart hyper-mutagenesis in intestinal cells. This hypothesis was tested in **chapter 5** in which a mESC model that mimics the LS cell situation was used to investigate whether the diet-derived mutagen PhIP may inactivate MMR. Indeed, exposing cells, hemizygous for *Msh2* or *Mlh1*, to PhIP resulted in a significantly higher frequency of MMR-deficient cells than PhIP-exposed wild-type cells. This finding may already partly explain the tropism found in LS individuals. However, the gastro-intestinal tract is not the only rapidly dividing tissue exposed to DNA damaging agents. The skin is also a rapidly dividing tissue and often exposed to UV light from solar radiation. So why do individuals with LS not develop skin malignancies? In fact, approximately 10% of individuals with LS do develop sebaceous skin tumors, a syndrome called Muir-Torre (42). Moreover, constitutional MMR deficiency is associated with sunlight-induced skin cancer in mice (43). The difference in incidence between skin and colorectal tumors in LS may be caused by the rate of cell division, which will invariably influence the rate of replication-associated mutagenesis. In normal epidermis the cell proliferation rate is approximately one cell division every 13 days (44), whereas colonic stem cells divide roughly every 2 days (45). As such MMR may play a much more important role in protecting against DNA damage-induced mutagenesis in the colorectal tract than in the skin.

Apart from inactivating MMR, PhIP may also play a role in increasing the mutational burden in MMR-deficient cells, thus further accelerating carcinogenesis. Previously, it was found by Glaab *et al.* that MMR-deficient cancer cell lines had increased levels of PhIP-induced mutagenesis compared to MMR-proficient cancer cell lines (46). **Chapter 5** further corroborates and expands upon this finding by exposing a complete panel of MMR-deficient mESC lines to PhIP. It was shown that under the experimental conditions used, 50 to 90% of all PhIP-induced mutations are suppressed by the four core MMR proteins, indicating a major role for the pathway in protecting against diet-induced mutagenesis. In contrast, Msh3 played no significant role in suppressing PhIP-induced mutagenesis. The mutational profile described in **chapter 5** shows that the PhIP-induced mutations were primarily G>T and G>A mutations, in line with previously published literature (46). Interestingly, a recent publication showed that LS tumors contain mutations mainly encompassing the COSMIC mutational fingerprints SBS1, SBS20 and SBS44 (47), which are largely characterized by G>T and G>A mutations. As such, these mutational fingerprints may be in part attributed to PhIP-exposure. Yet, other signatures are sometimes found as well, such as SBS5 that shows a mutational profile which is not at all dominated by one or two types of SBS, but is instead more varied (47). It is important to realize that the gastro-intestinal tract is not solely exposed to PhIP, but also to other mutagens from the diet (48), the microbiome (49) and bile (50, 51). This complicates the effort to correlate a spectrum of mutation in colorectal tumor DNA with exposure to specific mutagenic agents, especially considering the

status of DNA repair pathways, differential cell proliferation rates and pre-existing mutations. Therefore, a one-to-one mapping of a single genotoxin to a mutational profile should be avoided.

If exposure of colonic cells to DNA damaging agents is at the root of the Lynch tropism, what then underlies the formation of endometrial cancers (EC) associated with LS? Endometrial cells are often exposed to high levels of hormones, such as estrogen, that are known to indirectly damage the DNA (52-54). Upon oxidation, estrogens can be converted in reactive quinones that bind to guanine and adenine bases (52, 55). These base modifications are chemically unstable resulting in non-instructive abasic sites (55), which can lead to mutations upon bypass by TLS (56). In addition, estrogen quinones can induce oxidative DNA damage which may provoke mutagenesis (52). Possibly, estrogen-induced misincorporations might be recognized and excised by MMR, similar to the excision of misincorporations opposite other lesions such as those induced by PhIP and UV light. As such MMR may play an important role in suppressing estrogen-induced mutagenesis. For this reason, loss of MMR in endometrial cells may explain the high incidence of endometrial cancers in LS carriers. Moreover, previous studies have also shown that exposure to estrogen upregulates MLH1 expression, suggesting a role for MMR in regulating the outcomes of estrogen exposure (57).

Taken together, as stated in **chapter 5,** food-derived DNA damage may influence the LS cancer tropism by a three-step process: (i) PhIP-induced mutational inactivation of the remaining wild-type MMR gene, (ii) reduced DNA damage signaling as a consequence of MutSα deficiency and (iii) loss of protective MMR resulting in PhIP-induced hypermutagenesis. However, further studies should be done to corroborate these findings and place them in a broader biological perspective. The work of this thesis was performed using mESCs, but it is important to validate the findings in different models to assess their biological relevance. For instance, now that human inducible pluripotent stem cells have been more widely adopted, this model may also be used to create Lynch-like cell lines. Indeed, it would be valuable to investigate whether PhIP can cause MMR-deficiency and accelerate mutagenesis in cells originating from an actual LS patient. Cell models are important tools in biological research, yet they will always lack the complexity of tissues, *i.e.* the 3D structures, different cell types and immune micro-environments. Thus, it would be important to reproduce the experiments with PhIP using *in vivo* mouse models to increase the biological relevance of the findings described in **chapter 5** even further. These findings can also be further expanded upon by performing epidemiological studies. Previously it was already shown that intake of red and processed meat increases the risk of developing CRC (58, 59). Furthermore, some studies have shown that in LS carriers dietary patterns, such as diets with high amounts of fat or meat, may slightly influence CRC incidence (60) or cancer incidence in general (61), whereas others find no significant differences (62). As such, it would be valuable to further investigate which dietary patterns are influencing cancer incidence in LS carriers by doing more extensive population studies with LS carriers and tracking their lifestyles in-depth.

Concluding remarks

The work presented in this thesis showed that the core MMR genes are invaluable for suppressing DNA damage-induced mutagenesis and as such safeguard the genome against the harmful consequences of TLS errors. Moreover, this thesis investigated what other genes are important in the suppression of DNA damage-induced mutagenesis and showed how MMR might contribute to activate DNA damage signaling. Here, an interesting new model is presented that may answer long standing questions about how MMR regulates DNA damage responses, namely, that suppression of DNA damage-induced mutagenesis is the result of MMR-mediated excision of TLS errors in combination with MMR-dependent recruitment of “correct” TLS polymerases or with MMR-dependent promotion of error-free template switching.

The work presented here also studied the DNA damage responses induced by MMR in a more clinically relevant setting by investigating the effect of PhIP, an intestinal mutagen, in a cell-based model of LS. Diet-derived mutagens accelerated the loss of the remaining wild-type copy of the affected MMR gene in Lynch-like cells. Subsequently, complete loss of MMR further increased mutagenesis coming from the same diet-derived mutagen and quenched the protective DNA damage signaling. This fundamental work may also have important implications for carriers of LS, which are at greater risk of losing the MMR pathway and are predisposed to develop colorectal cancer. To minimize the risk of developing colorectal cancer, these individuals may be advised to modulate their lifestyles in order to reduce the exposure to DNA damaging agents, for instance found in the diet. It may be of interest for individuals affected by LS to adopt a vegetarian lifestyle, but to also avoid other products known to contain DNA damaging agents, such as aflatoxins found in long storage food products, e.g. nuts and dried fruits (63). Moreover, if meat is consumed, preparation is key. Using the right herbs and cooking the meat at low temperatures reduces the amount of DNA damaging agents (64). Interestingly, a recent study has shown that consumption of resistant starch, found in for instance bananas, potatoes and grains, reduced extracolonic tumor formation in LS patients (61). Resistant starch is hypothesized to reduce total bile acid levels, a known mutagen that induces oxidative DNA damage (51), and as such the negative effect of post-TLS repair deficiency may be reduced (65). Collecting such studies about diet and lifestyle factors into one large compendium may be an interesting venture for further research to better advise LS carriers.

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