DNA mismatch repair and the cellular response to UVC radiation

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"En? Heb je onder water gekeken? Heb je de bodem gezien?"

W.F. Hermans, Nooit meer slapen

voor Kees en Ineke

voor Marcel

Contents

Aim and outline of the thesis

Cellular DNA is continuously damaged by genotoxic compounds. These compounds commonly originate within the cell itself during metabolic processes such as hydrolysis, oxidation and methylation. Thus it has been estimated that per day more than 10,000 DNA lesions are induced per mammalian cell [1]. Also the spontaneous deamination, depurination or hydrolysis of nucleotides are endogenous causes of DNA alterations. Finally, the chemical and physical genotoxic agents from the extracellular environment pose a serious threat on the integrity of cellular DNA.

Various DNA repair mechanisms have evolved that can deal with this continuous induction of DNA modifications [2]. Each repair mechanism recognizes a specific subset of DNA lesions, although some overlap exists between the different repair pathways as substrate specificity is concerned. Examples of repair systems are base excision repair (BER) for the repair of (amongst others) oxidized bases, deamination products and abasic sites; nucleotide excision repair (NER), involved in the repair of photolesions and other bulky adducts that heavily distort the DNA helix structure, and double strand break (DSB) repair.

In addition to DNA damaging agents and the chemical instability of DNA, DNA replication can also threaten genomic integrity. Duplicating the cell's genome is not a completely error-free process and nucleotide misincorporations by replicative polymerases δ and ε do occur. Cells are equipped with a specialized repair machinery that repairs these replication errors: the DNA mismatch repair (MMR) system. The replicative DNA polymerases are not the only polymerases that can introduce mismatches. A separate class of polymerases comprising the so-called translesion synthesis (TLS) polymerases are the major source of misincorporations [3]. These polymerases come into play when the regular replicative polymerases are not able to replicate past a damaged nucleotide in the DNA template. Due to their low stringency TLS polymerases are able to bypass these nucleotides. TLS polymerases display either error-free or error-prone replication activity when replicating a damaged DNA template. In the latter case TLS results in mismatched adducted nucleotides, also called compound lesions [4].

No definite proof has been gathered yet in order to be able to answer the question if MMR is involved in the correction of TLS-associated compound lesions. The aim of the studies described in this thesis was to answer this question by studying the cellular response of MMR-proficient and MMR-deficient cells to ultraviolet (UV) light. The results of these studies are described in chapters 2 and 3. Our results confirm that MMR plays an important role in the cellular response to UV. Already for years, evidence is accumulating showing that MMR counteracts genotoxic agent-induced mutagenesis and apoptosis. In the following paragraphs the most important data on the cellular response of MMR-proficient and MMR-deficient cells to several different classes of genotoxic agents will be reviewed. These data are of particular importance to hereditary non-polyposis colorectal cancer (HNPCC) patients. These patients are carrier of a germ line mutation in one of the MMR genes and hence at a high risk to accumulate cells in their body that have lost MMR due to the inactivation of the wild-type allele of the particular MMR gene. In chapter 4 we show that mouse ES cells heterozygous for the MMR gene *Msh2* have indeed a high propensity to lose the wild-type allele of *Msh2* both spontaneously and upon treatment with several genotoxic agents. We hypothesize that these processes also occur in HNPCC patients contributing to their predisposition to cancer.

Chapter 1

Introduction

1.1 DNA mismatch repair (MMR)

For the proper functioning of an organism, faithful duplication of the cellular genome is of crucial importance. In order to achieve this, the cell is equipped with DNA mismatch repair (MMR) machinery (reviewed by [5]). In *Escherichia coli (E. coli*), MMR contributes almost 1000-fold to the overall fidelity of replication [6]. MMR proteins repair mismatches that arise during DNA replication when the polymerase incorporates an incorrect nucleotide opposite a nucleotide in the template strand. Other replication errors repaired by MMR are insertion/deletion loops (IDLs). These are formed when the replicative polymerase slips and subsequently misaligns during the replication of simple sequence repeats (also called microsatellites). As a consequence, small loops arise in the template or nascent DNA strand. These loops are substrates for MMR. In the absence of MMR, the loops persist resulting in the expansion/deletion of the microsatellites upon replication, a phenomenon called microsatellite instability (MSI). MSI is a hallmark of MMR deficiency (reviewed in [7]).

In addition to correcting replication errors, MMR is involved in the removal of 3' non-homologous tails formed during homologous recombination (HR). During meiotic HR, recombination intermediates of more than 1 kilobase (kb) can be formed, and mismatches present in these intermediates are repaired by MMR, during a process called gene conversion [2,8]. Furthermore, MMR is involved in the prevention of homeologous recombination, *i.e.* recombination between sequences that are slightly divergent. This is illustrated by the fact that recombination in MMR-deficient cells has lost dependence on complete sequence identity [9]. In conclusion, the absence of MMR has severe consequences for the cell's genomic integrity and loss of MMR results in a mutator phenotype.

MMR in prokaryotes

In *E. coli*, all proteins involved in MMR have been identified and purified and the MMR reaction has been reconstituted *in vitro* [6]. The MMR pathway is activated when a homodimer of MutS binds to a mismatch or an IDL. Solution of the crystal structures of *E. coli* and *Thermus aquaticus* (*Taq*) MutS has demonstrated

that the MutS monomers function differently from each other, *i.e.* it has been shown that only one monomer has direct contact with the mismatch [10,11]. Thus, although MutS is a structural homodimer, it is a functional heterodimer (see also paragraph 5.1). MutS binding to the mismatch is followed by binding of homodimer MutL to MutS. MutL functions as a molecular matchmaker attracting and subsequently activating the endonuclease MutH [12], which introduces a nick in the daughter strand containing the mismatch. In addition to binding of MutS, MutL binds to the surrounding DNA. Binding of MutL to DNA has been shown to be crucial for efficient MMR: Jinks-Robertson *et al.* showed that a MutL mutant, carrying a point mutation in *mutL* resulting in impaired DNA binding of the encoded protein, displayed a strong mutator phenotype [13]. The impaired DNA binding in the MutL mutant could be explained by impaired DNA-stimulated ATP hydrolysis of MutL and impaired stimulation of the downstream MMR processes such as the MutH-catalyzed nicking of the mismatch-containing DNA strand and DNA unwinding by helicase II (see below).

To distinguish between the parental strand and the daughter strand for the introduction of a nick from which mismatch excision can initiate, MutH makes use of the fact that the latter strand is, in contrast to the parental strand, transiently undermethylated after replication. After the introduction of a nick by MutH, Helicase II (=MutU/UvrD) unwinds the DNA and the mismatch containing strand is subsequently removed by any of at least four single-stranded DNA specific exonucleases: RecJ (displays 5'>3' exonuclease activity), ExoI and ExoX (display 3'>5' exonuclease activity), and ExoVII (displays both 5'>3' and 3'>5' activity). Single-stranded DNA binding protein (SSB) protects the complementary DNA strand from degradation and this strand is subsequently used by the polymerase III holoenzyme as template in order to resynthesize the excised strand. Finally, DNA ligase fills the remaining gap thereby completing the repair event ([14] and Fig.1).

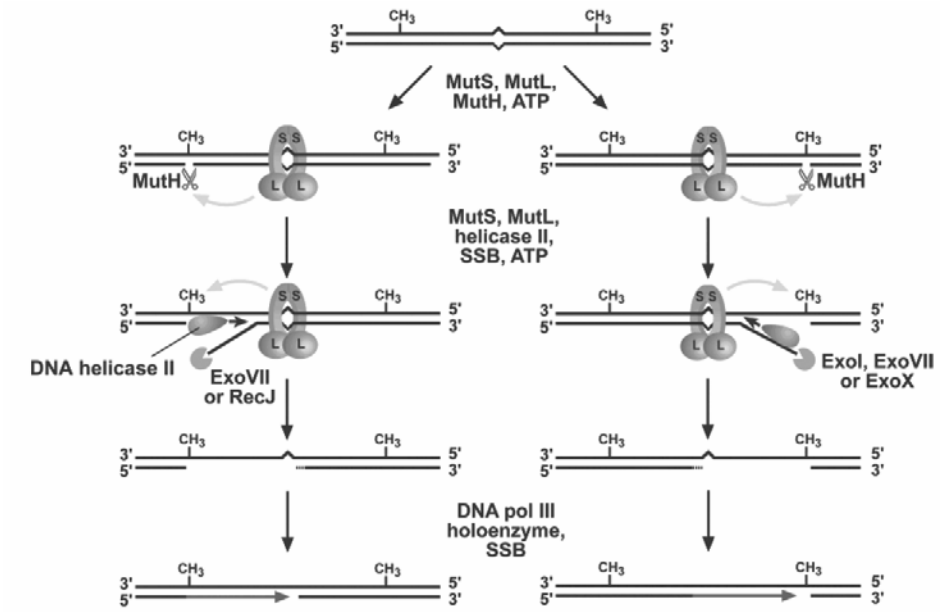


Figure 1: MMR pathway in *E.coli*. (From [14]).

MMR in eukaryotes

The process of MMR has been conserved during evolution and in yeast and mammals multiple homologues of MutS and MutL have been found (Table 1 and [15]). These are designated mutS homologues (MSH) and mutL homologues (MLH). Whereas MutS and MutL proteins in prokaryotes are homodimers, eukaryotic MMR proteins are heterodimers. A few years ago, the 5′-directed human MMR excision reaction was reconstituted [16,17], culminating in the recent *in vitro* reconstitution of the entire nick-directed human MMR process [18,19]. In eukaryotes, two mismatch recognition heterodimers have been identified. A dimer of MSH2 and MSH6 (this dimer is also designated as MutSα) recognizes mismatches and small (1-2 nucleotides) IDLs and a dimer of MSH2 and MSH3 (this dimer is also called MutSβ) recognizes IDLs of up to 16 nucleotides [5]. Thus, a redundancy exists between MutSα and MutSβ as the repair of small IDLs is concerned. Four MutL homologs have been identified in eukaryotic cells: PMS1, PMS2, MLH1, and MLH3 (the PMS proteins have been originally discovered after

analysis of yeast mutants with a defect in gene conversion resulting in post meiotic segregation). The central orthologue is MLH1 and this protein interacts with three other MutL orthologues forming three different heterodimers. The heterodimer of MLH1 and PMS2 (orthologue of yeast PMS1) is the major player in mammalian MMR (this dimer is also called MutLα). MutLα has been shown to play an important role in the termination of the mismatch-provoked EXOI-mediated excision of the mismatch containing strand upon removal of the mismatch [18], in addition to its role in connecting the damage recognition step with the mismatch excision step.

No role in mammalian MMR has thus far been identified for the MutLβ dimer [20], consisting of MLH1 and PMS1 (MLH1-MLH2 in yeast), although in yeast this dimer appears to function in IDL repair [21]. The MLH1-MLH3 heterodimer (also referred to as hMutLγ) functions in IDL repair in yeast as well [22]. In mice MutLγ has a substantial role in meiosis but only a marginal role in MMR [23,24]. However, it is possible that the reason for the marginal role found thus far for the mammalian MutLβ and MutLγ heterodimers in MMR is that a potentially more significant role can only be demonstrated in the absence of MutLα [25].

Strand discrimination in eukaryotic MMR

An additional difference between prokaryotic and eukaryotic MMR is that in eukaryotes, the strand discrimination signal for targeting MMR to the daughter strand containing the mismatch is not mediated by undermethylation of the nascent strand. Genome methylation in eukaryotes does occur, but this methylation is mainly observed in promoter regions, thus it can not serve as a general strand discrimination signal in eukaryotic MMR. Correspondingly, no homologue of MutH has been found in eukaryotes. Since *in vitro* MMR reactions on mismatched substrates initiate at a pre-existing DNA nick or gap, it is hypothesized that during replication, the 3′ end of the leading strand and the 5′ ends of Okazaki fragments in the lagging strand may be used as strand discrimination signals in eukaryotic MMR. In addition, since it has been observed that MSH3 and MSH6 physically interact with the polymerase processivity factor

PCNA (proliferating cell nuclear antigen) and that certain mutations in PCNA result in elevated mutation rates characteristic of MMR-deficient cells [26-28], it is believed that PCNA plays an important role in targeting MMR to the newly-replicated strand. Interestingly, during replication PCNA is loaded onto DNA in an orientation-dependent manner with its C-terminus facing the direction of strand elongation [29] such that it always has the same orientation relative to the daughter strand. Since it has been found that PCNA also interacts with the mammalian exonuclease EXOI that has been shown to remove the mismatch containing DNA strand *in vitro* [30] and to play a role in mutation avoidance in mammalian cells [31], the orientation dependent loading of PCNA could also be a determinating factor for EXOI as it comes to the direction (5'>3' or 3'>5') in which it will perform its exonuclease activity [17]. PCNA has also been shown to be important for DNA re-synthesis after mismatch excision [32].

It is possible that in eukaryotic cells, like in *E. coli*, several exonucleases play a role in MMR in addition to EXOI. An indication for this comes from Wei *et al.* [31] who show that the *Hprt* mutation rates in *ExoI*$^{-/-}$ mice are lower than those observed in *Mlh*$^{-/-}$ mice. Also, it was shown that in *Saccharomyces cerevisiae* the 3'> 5' exonuclease activities of Pol δ and ε play a role in MMR [33]. In addition, it has been found that 3'-directed mismatch-provoked excision is partially inhibited by the polymerase inhibitor aphidicolin in HeLa cell extracts [34]. However, experiments in which the nick-directed human MMR process was reconstituted in a purified system showed that the contribution of polymerase δ to MMR-provoked excision was limited in comparison to the role of EXOI in this reaction [19].

During excision of the mismatch-containing strand in eukaryotic cells, the complementary DNA strand is protected by replication protein A (RPA), similar to the protection of the parental strand by SSB in prokaryotes. In addition, RPA and the high mobility group protein I (HMGBI) have been found to stimulate the EXOI-catalyzed excision in the presence of a mismatch [18]. The RPA-coated strand serves as a template for resynthesis of the excised strand, a reaction that is mediated by polymerase δ [35]. Repair DNA synthesis by polymerase δ depends on the presence of RPA, PCNA and PCNA clamp loader RFC (replication factor C;

[18,19]). As a final step in the eukaryotic MMR process, DNA ligase I seals the gap and the MMR event is completed [18].

Table 1: MMR-associated proteins in *E. coli* and their mammalian homologues

E. coli	Mammalian cells
MutS	MSH2/MSH6 (=MutLα)
	MSH2/MSH3 (=MutLβ)
MutL	MLH1/PMS2 (=MutLα)
	MLH1/PMS1 (=MutLβ)
	MLH1/MLH3 (=MutLγ)
MutH	No known homologue
β-clamp	PCNA (clamp loader: RFC)
Helicase II (= MutU/UvrD)	No known homologue
ExoVII, RecJ, ExoI, ExoX	EXOI
SSB	RPA
Pol III	Polδ
DNA Ligase	DNA LigaseI

(Adapted from [36])

Signal relay between recognition and excision steps in MMR

A thus far unresolved issue in the study on MMR concerns the way in which the mismatch recognition proteins signal to the excision machinery. Three models have been proposed that differ with respect to the dynamics of the MutS-MutL complex (model 1&2 versus model 3) and the role of ATP hydrolysis in the dynamics of this complex (model 1 versus model 2):

Hydrolysis-dependent translocation model [6,37,38].
This model is based on electron-microscopic analysis of the interaction of *E. coli* MutS and MutL with DNA containing a mismatch (heteroduplex DNA) and on the observed dynamics of MutS interacting with these heteroduplexes. It was shown that complexes between MutS and heteroduplex DNA were converted to α-shaped loop structures with the mismatch located within the

DNA loop (Fig. 2A). Loop formation was enhanced by MutL and shown to be dependent on ATP hydrolysis. These observations suggested that loading of the MutS-MutL complex onto the mismatch results in ATP-hydrolysis-dependent translocation of MutS and MutL away from the mismatch. It was subsequently hypothesized that the ATP-hydrolysis dependent DNA tracking serves to detect a strand discrimination signal, in order to determine the orientation of the mismatch. The mismatch containing strand would subsequently be nicked by MutH, creating an initiation site for the exonuclease.

Molecular switch model [39-41].

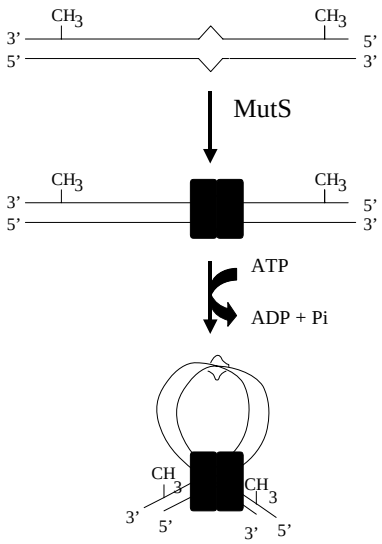
Studies of the human MSH proteins led to an alternative model. It was shown that ATP binding by MutSα bound to hetereroduplex DNA resulted in a conformational change of MutSα and the formation of a sliding clamp capable of diffusion over several thousands of nucleotides ([42] and Fig. 2B). Iterative loading and sliding away from the mismatch was proposed to mark the mismatch region and to instruct EXO1 such that subsequent degradation of the mismatch-containing strand by EXO1 would not proceed beyond the mismatch. In support of this, Zhang *et al.* found that for efficient excision of the mismatch-containing strand and termination of excision upon mismatch removal a molar excess of the MutSα and MutLα protein complexes was required, suggesting that more than one ternary complex of MutSα-MutLα is needed for efficient MMR [18]. The main difference between the first and the second model is that in the translocation model the DNA tracking induced upon MutS binding is driven by ATP hydrolysis and can occur only in one direction, whereas the DNA-bound MutS-ATP complex in the molecular switch model can freely diffuse in both directions along the DNA helix, with ATP hydrolysis occurring only after dissociation of the MutS complex from DNA.

Static transactivation model [43].

A study of the computer-predicted protein-protein interfaces of MutS, MutL, and MutH [10,11,44] led to still another model for mismatch signalling in MMR. In this model, interaction of MutS with heteroduplex DNA leads to an

enhancement of the lifetime of ATP-bound MutS on the DNA in comparison to the lifetime of ATP-bound MutS on homoduplex DNA. This gives MutL and MutH the opportunity to bind MutS, resulting in the formation of a static MutS-MutL-MutH complex on or near the mismatch. This ternary complex subsequently interacts *in trans* with a GATC-site, inducing strand incision by MutH ([43,45] and Fig. 2C). This model was supported by the observation that a MutS mutant defective in the hydrolysis of ATP and therefore immobile after binding to a mismatch, supported MutL-dependent activation of the MutH endonuclease in a DNA transactivation assay [43]. More support for the static transactivation model has come from Wang *et al.* [46], who show that the presence of streptavidin barriers between a mismatch and a pre-existing nick still results in mismatch-provoked 3'> 5' excision. However, Baitinger *et al.* re-evaluated the MutS mutant used by Junop *et al.* [43] and found that the mutant protein is defective in cisactivation of MutH endonuclease, displaying only 1-2% of the activity of wild type MutS, thus questioning the observation of transactivation by this protein [47].

A)



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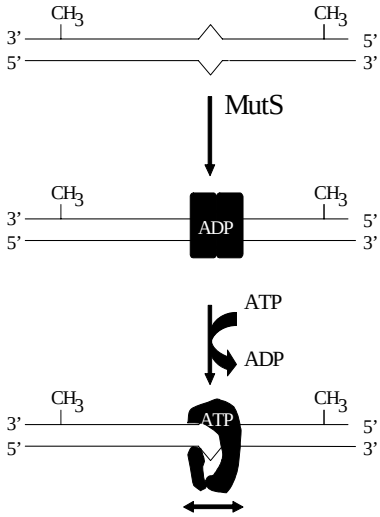

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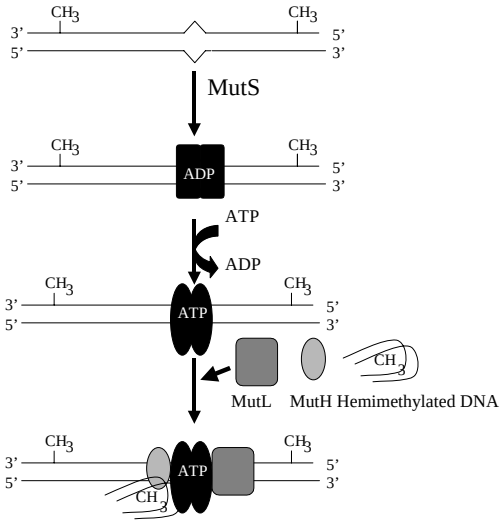

Figuur 2: Three models for the initial steps of MMR
A) Translocation model; B) Molecular switch model; C) Static transactivation Model
(Adapted from [43])

1.2 Hereditary non-polyposis colorectal cancer (HNPCC)

At the beginning of the 20th century several families were described who appeared to have a predisposition to gastric, colon and endometrium cancer [48,49]. Lynch *et al.* demonstrated an autosomal dominant pattern of inheritance of the cancer predisposition in one of the families, with the majority of the occurring cancers being adenocarcinomas of the colon, endometrium, and stomach [50,51]. In the 1980's, the observed cancer family syndrome was subdivided into Lynch syndrome I (families with mainly colorectal cancers) and Lynch syndrome II (families with colonic cancers and extracolonic cancers) [52]. Both syndromes have been unified and named hereditary non-polyposis colorectal cancer (HNPCC) to emphasize the lack of multiple colonic polyps in patients and to separate it from the polyposis syndromes in which patients present with numerous polyps, such as familial adenomatous polyposis coli (FAP). In 1993, the observation of extensive MSI in hereditary non-polyposis colorectal cancer (HNPCC) tumors led to the identification of the human orthologue of the MMR gene *mutS*, *hMSH2*, as the gene being mutated in many HNPCC patients ([53], reviewed in [15]). This was confirmed by large-scale genetic analysis of HNPCC patients [54]. Soon thereafter, mutations in the *E.coli mutL* homolog *hMLH1* were found to be able to cause HNPCC as well [55,56]. In order to come to a valid diagnosis of HNPCC, diagnostic criteria have been formulated [57,58]:

- At least three close relatives should be affected with colorectal cancer or cancer outside the colon such as cancer of the endometrium, stomach, small intestine, hepatobiliary system, kidney, urethra, ovary or brain in two successive generations;
- The age at diagnosis should be <50 years in at least one family member;
- FAP should be excluded.

HNPCC and MMR

HNPCC is an autosomal dominantly inherited disorder of cancer susceptibility with high penetrance [59,60]. HNPCC is caused by germ line

mutations in one allele of four mismatch repair genes- *hMSH2*, *hMSH6*, *hMLH1* and *hPMS2*. The majority of germ line mutations in HNPCC patients are found in *hMSH2* and *hMLH1* (±90% of all known mutations in HNPCC [61]). *hMSH6* harbours ±10% of all known HNPCC mutations and is frequently mutated in patients with endometrial cancer [62]. Mutations in *hPMS2* are relatively rare [63]. In a fraction of HNPCC patients no mutations can be found in any of the MMR genes mentioned above. This indicates that mutations in a yet unidentified (MMR) gene might cause HNPCC as well.

Since HNPCC patients are carrier of a germ line mutation in a MMR gene, they are heterozygous for the particular MMR gene in all of their cells. Thus, mutational inactivation, loss of heterozygozity (LOH) or silencing of the wild type allele rendering a cell in the body MMR-deficient, is likely to occur [64]. This results in a mutator phenotype of the particular cell. Upon subsequent acquisition of additional mutations particularly in growth-controlling genes this cell can develop into cancer. Tumors linked to germ line mutations in MMR genes account for around 5% of all colon cancers [65,66]. Microsatellite instability (MSI, see paragraph 1.2) is seen in nearly all HNPCC patients and also in 10-15% of sporadic colon and other cancers [67-70]. The majority of these sporadic tumors showing MSI (also designated as MSI-high, MSI-H) are caused by inactivation of *MLH1*, which mostly results from promoter hypermethylation rather than from somatic mutations or LOH [70,71]. Studies on MSI-displaying cancer cell lines have shown that in many cases, both alleles of *MLH1* are inactivated by promoter hypermethylation [72].

Tumorigenesis in HNPCC patients

Both HNPCC and sporadic cancers with MSI are diploid or near diploid [73]. This is in direct contrast with the majority of colorectal cancers (and most carcinomas in general) that have an aneuploid karyotype. It has been proposed that HNPCC and sporadic MSI-H tumors develop in a different way compared to other colorectal cancers. Thus, a distinction has been made between tumors that show chromosomal instability (CIN) and tumors that show microsatellite

instability (MIN), like the tumors associated with HNPCC [74]. MIN tumors have a 'mutator phenotype' due to the underlying MMR defect. Mutation rates in tumor cells with MMR deficiency are 100 to 1000 fold increased as compared to normal cells [75]. Among the genetic targets in the transformation process of MMR-deficient cells are genes that harbour mononucleotide repeats that can become unstable in the absence of MMR. Some of these genes are found to be mutated in tumors of diverse origin [76], whereas others show tissue specificity. For example, frameshift mutations in the tumor suppressor gene *TGFβRII* [77,78] and the transcription factor gene *TCF4* [79] are often found in gastrointestinal malignancies but not in endometrial cancer. This can reflect the importance of the encoded proteins for the growth control in these particular tissues and could be one explanation for the specific tumor spectrum of HNPCC patients [80].

Another way to explain the particular tumor spectrum of HNPCC patients is based on the fact that MMR-deficient cells display a reduced sensitivity to several genotoxic agents (this will be extensively discussed in the following paragraphs). Since the concentration of genotoxic agents is relatively high in the colon due to either a direct intake of genotoxic substances or due to generation of these compounds as by-products of metabolism/digestion, MMR-deficient cells may have a growth advantage in this environment, resulting in the accumulation of MMR-deficient cells displaying a mutator phenotype. In addition, as will be discussed in the next paragraphs and following chapters of this thesis, MMR-deficient cells are hypermutable by a wide range of genotoxic compounds, some of which circulate in the gut and this can increase the mutator phenotype of the MMR-deficient cells culminating in the development of malignancy [81].

Alternatively, the genotoxic compounds circulating in the gut could cause tumor formation in HNPCC patients by increasing the inactivation rate of the wild type allele (this will be discussed in more detail in chapter 4). Taken into account that the newly-generated MMR-deficient cells could subsequently have a growth advantage over wild type cells in the presence of the particular mutagen or will display a hypermutable phenotype to the compound as described above, the selection of MMR-deficient cells in the gut can be easily envisioned. An interesting fact in this respect is the observation that carriers of heterozygous germ-line

mutations in MMR genes do not develop hematological malignancies, whereas carriers of homozygous germ line mutations in MMR genes do [82,83], indicating that the induction of LOH and the subsequent selection of the MMR-deficient cells could indeed be tissue specific and depend on the presence of mutagenic compounds. Alternatively, since mutations arise during DNA replication, the development of hematological malignancies in carriers of homozygous germ line mutations in MMR genes could be due to the high turnover of T cells in the thymus during the development of the T cells early in life. The tissue-specific proliferation rate could similarly explain the high incidence of gastro-intestinal (GI) tumors in adult HNPCC patients, since the epithelium of the GI-tract has the highest turnover rate of all cell types (every 3-5 days, [84]).

A related, but different explanation for the observed HNPCC tumor spectrum could be a tissue-specific difference in surveillance by the immune system. HNPCC tumors are characterized by the presence of infiltrates of lymphocytes [85], indicating that the immune system does recognize these tumor cells. This recognition is probably caused by the presence of many new antigens on the surface of HNPCC tumor cells representing mutated proteins that arose due to the mutator phenotype of HNPCC tumor cells. In order to investigate whether the HNPCC tumor spectrum is controlled by the immune system, de Wind *et al.* studied tumor formation in *Msh2*$^{-/-}$ mice that also carried a homozygous disruption in *Tap1* [86]. Tap1 encodes a subunit of the complex of transporters associated with antigen processing (TAP complex). In *Tap1*-deficient mice, the cytotoxic T lymphocyte (CTL) response is impaired [87]. *Msh2*$^{-/-}$ mice generally develop lymphomas and also, albeit to a lesser extent, tumors of the intestine. Absence of the CTL-mediated response did not significantly affect the time of onset, tissue distribution (apart from the fact that these mice did not develop lymphomas due to the absence of the thymus), and number of tumors in *Msh2*$^{-/-}$ mice, indicating that in mice, the immune surveillance is not an important factor in the etiology of HNPCC-related tumors. However, Duval *et al.* screened a large series of Non-Hodgkin lymphomas for MSI and found that (the few) patients which scored positively for MSI were immuno-compromised either due to HIV infection or due to a posttransplant-lymphoproliferative disorder, indicative of a possible role of

the immune-system in the suppression of MMR deficiency-related malignancies [88].

HNPCC: mice versus men

Interestingly, *Msh2*$^{+/-}$ mice do not show increased tumorigenesis in comparison to wild type mice as would be expected on the basis of the high penetrance of tumorigenesis in HNPCC patients who are hemizygous for a MMR gene. Mice homozygous for an *Msh2* mutation do not mimick HNPCC patients either, since the majority of the mice succumb to T-cell lymphomas at the age of ≤ six months. However, mice that survive longer than six months do develop adenomas and carcinomas of the GI-tract (in addition to skin tumors). The *Msh2*$^{-/-}$ *Tap*$^{-/-}$ mouse (see above) has been found to be a better model for HNPCC [89]. *Msh2*$^{-/-}$ *Tap*$^{-/-}$ mice do not develop lymphomas, but do develop GI-tract tumors and skin tumors. *Msh2* deficient mice that are heterozygous for a mutation in the adenomatous polyposis coli (*Apc*) tumor suppressor gene also have been found to be a good model for HNPCC [89]. *Msh2*$^{-/-}$ *Apc*$^{+/Min}$ mice carrying a germ line mutation in the tumor suppressor protein Apc (a mutation that results in multiple intestinal neoplasms, hence designated as 'Min') showed accelerated tumorigenesis and a higher number of adenomas as compared to mice with an *Msh2* deficiency only. In humans, germline mutations in *APC* have been found to cause familial adenomatous polyposis (FAP), the second most frequent hereditary colorectal cancer syndrome (after HNPCC), characterized by the development of hundreds to thousands of adenomas throughout the colon, of which some will develop into colorectal cancer [90]. APC has also been found to be mutated in many cases of sporadic colorectal cancer [91] as well as in tumors of HNPCC patients, although the significance of the latter observation is still a matter of debate [92].

Of interest, the rate of intestinal tumorigenesis of *Msh2*$^{+/-}$ *Apc*$^{+/Min}$ is not significantly different from *Msh2*$^{+/+}$ *Apc*$^{+/Min}$ mice [86,93]. In addition, tumors arising in *Msh2*$^{+/-}$*Apc*$^{+/Min}$ mice mostly stained positive for Msh2, indicating that tumorigenesis in *Msh2*$^{+/-}$ *Apc*$^{+/Min}$ mice is less likely to be the result of the induction of a mutator phenotype via inactivation of the wild type allele of *Msh2* followed by the inactivation of the wild type *Apc* allele. A likely explanation for this is that mice

may not live long enough to accumulate enough mutations in growth-controlling genes once the wild type *Msh2* allele is inactivated, *i.e.* after loss of the wild type allele of the *Msh2* gene in heterozygous mice, the life-span of the mouse may be too short to encompass the time needed for selection of a cell that carries mutations in all genes necessary to give the cell carcinogenic properties. HNPCC patients usually present with tumors in their third or fourth decade of life which demonstrates that considerable time is needed to lose the wild type MMR allele resulting in a mutator phenotype and tumorigenesis in the long run, time that may not be available in the mouse.

1.3 The role of MMR in the cellular response to genotoxic agents: methylating agents

The role of MMR in the toxicity of methylating agents has been studied extensively and it has been found that the absence of MMR confers tolerance to S_N1-methylating agents (see below). Methylating agents can be divided into two classes, depending on the mechanism via which they react with DNA. S_N1- methylating agents react with relatively weak nucleophiles such as O-atoms, but they also react with stronger nucleophiles such as the N-atoms. S_N2- methylating agents react preferentially with stronger nucleophiles. Treatment of cells with the S_N1-N-nitroso compounds N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and N-methyl-N-nitrosourea (MNU) gives rise to N^7- methylguanine, N^3-methyladenine, O^4-methylthymine, O^6-methylguanine and methylphosphotriesters in DNA. The cytotoxicity of S_N1-methylating agents is ascribed to the O^6-methylguanine (O^6-meG) adduct that is induced. Treatment of cells with MNNG results in the formation of an O^6-meG product in 8% of the total alkylation events. In comparison, the S_N2-alkylating agent methylmethanesulfonate (MMS) gives an O^6-meG product in only 0.3% of the total alkylation products [94,95]. The cytotoxicity of O^6-meG is illustrated by the fact that the sensitivity to S_N1-methylating agents can be abolished by expression of the alkylguaninealkyltransferase (AGT) protein that specifically reverts the damage induced by methylating agents at the O^6 -position of guanine [96-99]. It has been found that *E. coli* strains lacking alkyltransferase activity show increased sensitivity to MNNG [100] and mice that have lost AGT activity are extra sensitive to chemotherapeutic alkylating agents [101]. The O^6-meG adduct is an effective mutagenic lesion, since it efficiently mispairs with thymine [102]. The mutagenicity of the O^6-meG adduct is reflected by the fact that transgenic mice overexpressing AGT are protected from the development of thymic lympomas after exposure to N-methyl-N-nitrosourea (MNU) [103]. Conversely, mice deficient in AGT activity develop numerous lymphomas [104,105].

Methylation tolerance in MMR-deficient cells

Glickman *et al.* observed that the introduction of a mutation in *E. coli* MMR genes *mutS*, *mutL* or *mutH* abolishes the sensitivity of *E. coli dam recA* mutants to killing by the base analogue 2-aminopurine (2AP) [106,107]. Because of their defect in adenine methylation, *dam* mutants have lost the ability to discriminate between parental (methylated) and daughter (transiently undermethylated) strands and it has been postulated that the sensitivity of *dam* mutants to 2AP is caused by the fact that upon incorporation and replication, excision of mismatched 2AP would take place in both the parental and the newly synthesized strands. This would result in overlapping MMR excision tracts in both strands, thus introducing cytotoxic double strand breaks (DSBs; due to the additional RecA deficiency mutants are not able to repair these DSBs). In accordance with this, Glickman *et al.* observed that the resistance to 2AP is restored when a MMR defect is introduced in addition to the *dam* mutation. Based on these findings, Karran *et al.* examined the effect of MNNG toxicity on *dam mutS* and *dam mutL* mutants. They suggested that by analogy to the effect of 2AP on these strains, *dam* strains would be sensitive to MNNG because of attempted mismatch correction at basepairs in both parental and daughter strands containing O^6-meG [108]. In support of this, it was found that the hypersensitivity of *dam* mutants to the methylating agent MNNG was abolished by the introduction of either a *mutS* or a *mutL* mutation into *dam* strains. Soon thereafter, a link between the sensitivity to methylating agents and the presence of MMR was also proposed for eukaryotic cells. Goldmacher *et al.* isolated human cell lines tolerant to a high dose of MNNG. Despite the fact that no removal of O^6-meG adducts from DNA occurred in this cell line, no cytotoxicity was apparent. It was hypothesized that the tolerance of this mutant to MNNG was due to a non-functioning mismatch repair system, since the MNNG tolerance correlated with the absence of mismatch repair activity [109]. In addition, Branch *et al.* described human and hamster cell lines defective for a mismatch-binding activity that were tolerant to MNNG [110]. Duckett *et al.* showed that purified human MutSα binds to oligonucleotides containing both an O^6-meG:T mispair or an O^6-meG:C pair, with a slightly higher affinity for the O^6-meG:T mispair [111]. This observation was in support for a role for MMR proteins in the recognition of this mispair. Final proof for the role of MMR in the cytotoxicity of S_N1-methylating

agents in eukaryotic cells has come from De Wind *et al.* who generated *Msh2*-deficient mouse embryonic stem (ES) cells that were around 100 times less sensitive to MNNG in comparison to their wild type counterparts in the presence of an AGT inhibitor [9].

MMR-dependent cell cycle signalling upon treatment with methylation agents

Treatment of cells with methylating agents induces cell cycle signalling activity that is partially dependent on MMR. Goldmacher *et al.* showed that MNNG induced a G2-phase arrest in the second cell cycle after MNNG treatment in cells that are MMR-proficient, whereas this G2-phase arrest was absent in their MMR-deficient counterparts [109]. This was subsequently found by many other groups [112-115]. Kaina *et al*. were the first to show that the MNNG-induced cell death in MMR-proficient cells reflects activation of an apoptosis pathway, as measured by fluorescence activated cell sorting (FACS) analysis (appearance of apoptotic sub-G1-fraction) and DNA gel electrophoresis (showing DNA laddering indicative of apoptosis; [116]). The methylating agent-induced apoptosis was found to be both p53-dependent and p53-independent [117,118]. An extensive study with a cell line in which the MLH1 expression is tightly controlled by doxycyclin [114] showed that the MNNG-induced MMR dependent G2-phase arrest is mediated by the ataxia telangiectasia-and-rad3-related (ATR) pathway, since the MMR-dependent G2-phase arrest was abrogated by two inhibitors of this pathway, caffeine and UCN-01 [115]. In support of this, low dose MNNG treatment resulted in phosphorylation of the ATR-activated kinase CHK1 only in the MLH1-proficient cells. The ataxia telangiectasia mutated (ATM) kinase appeared to be dispensable for triggering the protein phosphorylation cascade and the G2-phase arrest [115]. In contrast with this, Adamson *et al.* showed that MNNG treatment does result in a MMR-dependent activation of ATM [119]. However, an important difference between both studies is that Adamson *et al.* used a much higher dose of MNNG. This dose is expected to result in BER induced DSBs, that can subsequently activate the ATM pathway [120].

Methylation induced mutagenesis in MMR-deficient cells

MMR-deficient cells are hypermutable by S_N1-methylating agents. This was elegantly shown *in vivo*: *Msh2*$^{-/-}$ mice carrying a transgenic *lacI* reporter system revealed striking increases in mutation frequency in response to MNU. The observed increases in mutation frequency were much higher than those observed in wild type and *Msh2*$^{+/-}$ animals [121]. The mutational spectrum of MMR-deficient cells treated with MNNG is dominated by GC>AT mutations [122]. These mutations also dominate the MNNG-induced mutational spectrum of both MMR-proficient and MMR-deficient cells *in vivo* [121] and probably reflects the propensity of O^6-meG to mispair with T [102]. The increased mutagenesis can be a consequence of the decreased sensitivity of MMR-deficient cells to methylating agents, of the absence of O^6-meG:T repair in MMR-deficient cells, or of a combination of both: in the absence of MMR, O^6-meG:T mispairs are not recognized, therefore no repair is induced, the mispair is fixed into a mutation in the subsequent round of replication and this mutant survives because no apoptosis is induced by the methylating agent in the absence of MMR.

1.4 The role of MMR in the cellular response to genotoxic agents: oxidative damage

Reactive oxygen species (ROS) arise in most cells as by-products of essential metabolic processes such as oxidative phosphorylation in the mitochondria [123]. They can cause damage to many cellular components, including genomic DNA. Examples of oxidative lesions induced in DNA are oxidized bases, broken DNA strands and DNA-protein crosslinks [2,124]. A lesion frequently induced by ROS is 8-hydroxyguanine (8-OH-G). 8-OH-G has a strong miscoding property, mainly resulting in 8-OH-G:A mispairs [125-127]. Different repair systems have evolved that deal with 8-OH-G lesions. The majority of them are parallel base excision repair (BER) pathways, but also MMR has been shown to play a role in the removal of 8-OH-G lesions (as will be discussed below).

In mammalian cells, 8-OH-G is substrate for the 8-oxoguanine glycosylase I protein (OGG1) in case 8-OH-G has paired with cytosine [128]. MutY homologue (MYH) addresses 8-OH-G:A mispairs [129-131] and is specifically directed to the mismatched adenine. The removal of misincorporated adenine followed by correct base pairing with cytosine results in an 8-OH-G:C pair that is, as described above, substrate for OGG1. Finally, MutT homologue 1 (MTH1) degrades 8-OH-dGTP from the nucleotide pool, thereby preventing the insertion of 8-OH-dGTP into DNA [132]. Colussi *et al.* showed that transfection of *hMTH1* brings about a significant reduction of DNA 8-OH-G levels in both mouse embryonic fibroblasts (MEFs) and mouse tumor cells indicating that the oxidized dNTP pool is a significant source of 8-OH-G present in the genome [133]. 2-Hydroxyadenine (2-OH-A) is another very mutagenic lesion induced by oxidative stress. It can mispair with adenine and cytosine [134] and is also substrate for hMTH1.

Oxidative damage resistance of MMR- deficient cells

A role for MMR in the repair of oxidative DNA damage was first postulated in yeast, in which it was found that the absence of MMR resulted in a dramatic increase of specific mismatches, an effect that could be reduced by growing the cells anaerobically [135]. A role of MMR in the cellular response to oxidative stress has also been demonstrated in *E. coli* [136]. Similar to the response

of *dam* mutants to S_N1-methylating agents, *dam* mutants are hypersensitive to the induction of oxidative damage in comparison to wild type cells and this sensitivity could be rescued by introducing a MMR mutation in addition to the *dam* mutation. In mammalian cells, different studies showed that MMR-deficient cells are slightly more resistant than MMR-proficient cells to ionizing radiation (IR) that induces oxidative damage in addition to DSBs [137-140]. However, other studies did not show a significant survival difference between MMR-proficient and MMR-deficient cells after IR [141-144]. Interestingly, the difference between the studies that did and did not show a significant difference in cellular survival after treatment with IR is that the studies in which a survival advantage for MMR-deficient cells was found used low IR dose-rates that are known to induce relatively more oxidative damage in addition to DSBs, whereas the other studies describe the cellular responses after a high acute dose of IR that induces relatively more toxic DSBs in addition to oxidative damage. This indicates that the role of MMR repair in the cellular response to oxidative damage can be obscured by the cellular response to DSBs when IR is used as a damage-inducing agent. In the case of low dose-rates of IR, the role of MMR in the cellular response to oxidative damage is more evident: the genomes of MMR-deficient cells show elevated levels of 8-OH-G after low dose IR that is accompanied by increased clonogenic survival and mutagenesis [137]. Similarly, Colussi *et al*. showed that the absence of hMutSα in mouse embryonic fibroblasts (MEFs) resulted in increased 8-OH-G levels compared to wild type MEFs after treatment with the oxidizing agent hydrogen peroxide. Their observations demonstrated a gene dosage effect: the levels of DNA 8-OH-G in $Msh2^{+/-}$ MEFs were intermediate between wild type and $Msh2^{-/-}$ cells [133]. Taking into account the fact that also MLH1-deficient cells show increased DNA 8-OH-G levels [133], it is likely that MMR plays a role in the removal of incorporated 8-OH-G from DNA, also in mammalian cells.

MMR dependent cell cycle signalling after oxidative stress

Oxidative damage can trigger MMR-dependent apoptosis in the absence of functional p53 [139]. In addition, it has been shown that in human cancer cell lines, treatment with IR results in a reduced and shorter G2-phase arrest in the absence of MMR [141,143]. Interestingly, the differences in G2-phase arrest were more

pronounced when cells were treated during S-phase. The observed differences in G2-phase arrest were accompanied by MMR dependent differences in CDC2 phosphorylation. Phosphorylation of CDC2 inhibits its kinase activity needed for cells to enter mitosis. Higher and prolonged CDC2 phosphorylation levels were observed in MMR-proficient cells after IR. Other proteins that play a role in the MMR-dependent phosphorlylation of CDC2 pathway still need to be identified. Interesting in this respect is the fact that Brown *et al.* [145] found that in human tumor cells, MSH2 interacts with CHK2, and MLH1 interacts with the ataxia telangiectasia mutated (ATM) protein and that this interaction is enhanced upon IR treatment. They also observed that MSH2- and MLH1-deficient tumor cell lines showed radio-resistant DNA synthesis (RDS) in response to different doses of IR similar to ATM deficient cells. The RDS in MMR-deficient cells coincided with aberrant phosporylation of CHK2 and CDC25A. It was subsequently postulated that the interaction of MSH2 and MLH1 with CHK2 and ATM at the site of oxidative damage induced by IR activates the ATM pathway resulting in activation of the S-phase checkpoint and inhibition of DNA synthesis. The authors proposed that recognition of IR-induced 8-OH-G:A by MSH2-MSH6 generates a molecular scaffold where different proteins, like ATM and CHK2 can interact, thereby activating signalling pathways that will induce an appropriate damage response. However, the validity of this model remains to be proven, also considering the fact that Cejka *et al.* did not find MMR-dependent induction of RDS and the coinciding MMR-dependent posttranslational modifications of CHK2 using the same MMR-proficient and deficient cell line pairs as Brown *et al.* [146].

Oxidative damage-induced mutagenesis in MMR-deficient cells

In *Saccharomyces cerevisiae* (*S. cerevisiae*), the importance of MMR in the removal of damage induced by oxidative stress was elegantly illustrated by experiments from Ni *et al*. [147]. They showed that inactivation of MMR in addition to OGG1 results in a synergistic increase of the mutation rate in *S. cerevisiae*. The increased mutations were found to be mainly GC>TA transversions, likely to be the consequence of misinsertions of adenine opposite 8-OH-dGTP bases that were abundantly present due to the OGG1 deficiency. The synergistic increase in mutagenesis upon the introduction of a MMR deficiency in an OGG1 mutant can

be explained by the fact that no homologues of MutT and MutY have been found in *S. cerevisae*, thus for the repair of 8-OH-G:A mispairs it has to rely entirely on MMR. In mammalian cells, the mutagenic effect of oxidative damage in MMR-deficient cells is illustrated by the fact that treatment with antioxidants partially reduces the mutator phenotype of MMR-deficient cells [148]. Also, it was shown that hMTH1 overexpression sufficient to reduce the steady-state level of DNA 8-OH-G in *msh2*$^{-/-}$ MEFs to background levels, strongly diminishes the mutator phenotype [133]. These observations indicate that a large part of the mutator effect associated with a MMR deficiency is a consequence of oxidative DNA damage. Accordingly, treatment of cells with oxidizing agents can induce MSI, a hallmark of MMR-deficient cells [149,150]. Russo *et al.* compared the spontaneous mutational spectra of mock-transfected MMR-deficient MEFs with *hMTH1*-transfected MMR-deficient MEFs. They observed a dramatic reduction of –G frameshifts after transfection of MMR-deficient MEFs with *hMTH1* [151]. This could indicate that polymerase slippage that occurs regularly during the replication of small sequence repeats is more prone to happen when one of the bases in the sequence repeat is oxidized. It was also shown that the AT>GC and the AT>TA base substitution rates dropped dramatically upon transfection with hMTH1. In addition, MTH1 transfection had a small reducing effect on the amount of AT>CG transversions. These data are in support of the hypothesis that (over)expression of MTH1 prevents incorporation of 2-OH-dATP and 8-OH-dGTP into DNA, thereby preventing direct mispairing with respectively guanine or adenine and -in the case of 2-OH-dATP- indirect mispairing with adenine or cytosine in the next round of replication. The strong suggestion that MMR repairs these mispairs remains however to be proven.

1.5 The role of MMR in the cellular response to genotoxic agents: cisplatin

cis-Diamminedichloroplatinum(II) (cisplatin) is a DNA crosslinking agent that has been successfully used in the treatment of cancer -most notably testicular cancer- for 30 years [152]. Cisplatin reacts preferentially with the N^7 atoms of adenine and guanine in DNA. It induces monoadducts, but mainly gives intrastrand cross-links of which the major adducts are 1,2-intrastrand cross-links (90%). Minor adducts are the 1,3-intrastrand cross-links (5-10%) and interstrand cross-links (ICLs, 1-3%) [153,154]. The ICLs require both nucleotide excision repair (NER) and recombinational repair (RR) pathways for their repair [155,156], whereas the cisplatin-induced intrastrand cross-links are mainly processed by NER [157-159]. Cells deficient in these repair pathways are hypersensitive to cisplatin. Finally, also MMR has been found to be a determinant for the cytotoxicity of cisplatin, albeit to a much lower extent than the above mentioned DNA repair pathways. In contrast to these repair pathways, the absence of MMR has been found to cause only a slight decrease in sensitivity to cisplatin. However, not in all studies performed to date could a role for MMR in the cytotoxicity of cisplatin be demonstrated (see below).

Cisplatin resistance of MMR-deficient cells

Several studies have shown that the MMR status of a cell is a factor that contributes to the cellular sensitivity to cisplatin. Fram *et al*. showed that loss of MMR renders *E. coli dam* mutants tolerant to cisplatin in a manner reminiscent of the acquisition of methylating agent tolerance in MMR-deficient *dam* mutants [160]. Recently, it has been shown that MutS is involved in suppressing recombination of platinated substrates in *E. coli* and it was proposed that the increased sensitivity of wild type over MMR-deficient cells to cisplatin treatment is caused by the abrogation of the cisplatin-induced recombination in wild type cells [161]. Also eukaryotic MMR-deficient cells have been shown to display higher resistance to cisplatin compared to wild type cells ([120] and references herein; [162-164]). However, Claij *et al.* showed that introduction of *Msh2* cDNA in an *Msh2*-deficient mouse ES cell clone that was shown to be 2-3 fold more resistant to cisplatin in comparison to wild type cells, did not restore cisplatin sensitivity. This

indicates that a distinct mutation in the MMR-deficient subclone had caused the cisplatin resistance [165]. This mutation could have arisen due to the mutator phenotype of MMR-deficient cells. To prevent this bias from occurring in a subsequently performed experiment with MMR-proficient and MMR-deficient cells, Claij *et al.* constructed a conditional *Msh2*$^{-/-}$ mouse ES line from a heterozygous *Msh2*$^{+/-}$ line. *De novo* inactivation of the *Msh2* allele did not result in decreased cisplatin sensitivity. However, Papouli *et al.* showed in a distinct isogenic system in which MMR can also be inactivated *de novo* that loss of MMR results in a small increase of resistance to cisplatin [144].

Interestingly, although mouse deletion mutants of *Msh2* and *Msh6* displayed a decreased sensitivity to cisplatin compared to wild type cells, point mutants of both proteins that lost MMR capabilities still showed an apoptotic response to cisplatin, similar to wild type cells [163,164]. This indicates that if MMR proteins play a role cisplatin toxicity, this role does not involve processing of the cisplatin-induced DNA lesions by the MMR pathway.

MMR-dependent cell cycle signalling upon treatment with cisplatin

Cisplatin treatment activates signalling pathways and some of these pathways depend on the presence of MMR proteins. It has been found that in mouse cells, cisplatin treatment leads to stabilization of p73, a process that is dependent on Mlh1 and c-Abl [166]. Stabilisation of p53 after cisplatin treatment was also observed, but this response appeared to be independent of Mlh1. Similar findings were obtained by Shimodaira *et al.* using human cells. They showed that PMS2 expression stabilized p73 after cisplatin treatment. In addition, a significant increase of the half-life of p73 was observed if p73 was coexpressed with PMS2 [167]. Finally, PMS2 and p73 were found to interact and this interaction was enhanced after cisplatin treatment. In their hands, the PMS2-induced stabilization of p73 could occur also in the absence of MLH1. The possible significance of the interaction of MMR proteins with p73 is illustrated by the fact that the methylating agents MNNG and temozolomide were shown to induce an Msh2-dependent apoptotic pathway that is independent of p53 [118]. The above-described findings for cisplatin indicate that this could possibly be an apoptotic pathway that is

mediated by p73. MMR has also been shown to play a role in the cisplatin-induced activation of the S-phase checkpoint. Lan *et al.* reported that cisplatin treatment results in MMR-dependent degradation of cyclin D1. Consequently, a reduced inhibition of replication after cisplatin treatment was observed in MMR-deficient cells [168].

Cisplatin-induced mutagenesis in MMR-deficient cells

Lin and Howell (1999) showed that in two MMR-deficient tumor lines, cisplatin generated significantly more cells that were resistant to different chemotherapeutic agents compared to their MMR proficient counterparts, suggesting that MMR is involved in the suppression of cisplatin-induced mutagenesis [169]. Recently, using a different assay in which the process of mutagenesis was studied by scoring the number of mutations at the *HPRT* gene, Lin *et al*. confirmed their previous findings by showing that in the absence of MMR significantly more mutations are induced at *HPRT* in comparison to wild type cells [162]. However, in both studies the observed increased mutagenesis in the absence of MMR could also have been the consequence of an additive effect of cisplatin to the spontaneous mutagenesis in MMR-deficient cells. Interestingly, in the latter study RNA interference-mediated knockdown of REV3, the catalytic subunit of translesion synthesis (TLS) polymerase ζ (polζ), reduced the mutagenicity of cisplatin significantly in MMR-deficient cells, suggesting a role for MMR in the removal of TLS-induced mismatches (as will be discussed in chapter two of this thesis). *In vivo* experiments of Sansom *et al.* showed that of different cisplatin doses tested, only the highest dose showed a protective effect of MMR on cisplatin-induced mutagenesis. Thus, no clear picture describing the role of MMR in counteracting cisplatin-induced mutagenesis has emerged yet [170].

1.6 The role of MMR in the cellular response to genotoxic agents: heterocyclic amines

Heterocyclic amines (HA) are produced during the cooking of meat and fish at high temperatures. They have been proposed to play a role in the etiology of diet-associated cancers [171-173]. 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) has been found to be the most abundant HA. It requires metabolic activation by the cytochrome P-450 enzymes and the N-O-acetyl transferase in order to be biologically active (reviewed in [174]). Upon activation, PhIP has been found to react with DNA mainly at guanines to form the N-(deoxyguanosin-8-yl)-2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (dG-C8-PhIP) adduct [175]. PhIP induced DNA lesions are substrate for NER [176]. Also the model compounds N-2-acetylaminofluorene (2-AAF) and the 2-AAF derivative N-acetoxy-2-AAF (AAAF) are HAs with biological activity. The major AAF adducts are N-(2'-deoxyguanosine-8-yl)-N-acetyl-2-aminofluorene (dG-C8-AAF) and the deacetylated dG-C8-AF adduct. These adducts are also substrates for NER [177].

HA resistance of MMR-deficient cells

Lower cytotoxicity levels after PhIP treatment in different MMR-deficient cell lines in comparison to their MMR-proficient counterparts have been observed in different studies [178-180]. A similar result was shown for AAAF: MSH6-deficient MT-1 cells showed a several fold higher resistance to AAAF than MMR-proficient TK-6 cells [181]. In the same study a similar increased resistance was observed for the MLH1-deficient cell line HCT116.

MMR dependent signalling upon treatment with HA

MMR-proficient TK-6 cells showed a several fold higher induction of p53 levels upon AAAF treatment than MMR-deficient MT-1 cells. Still, p53-deficient cells of the same origin as TK-6 and MT-1 cells were more sensitive to AAAF than MT-1 cells, indicative of both p53-dependent and p53-independent AAAF-induced apoptosis pathways mediated by MMR [181].

HA-induced mutagenesis in MMR-deficient cells

MMR-deficient cells have been found to be hypermutable after treatment with PhIP [178,179,182]. Also *in vivo* experiments showed that in *Msh2*$^{-/-}$ mice, PhIP induced significantly more mutations as compared to wild type and *Msh2*$^{+/-}$ mice [183]. In addition, Smith-Roe *et al.* found that the frequency of aberrant crypt foci (ACF), a preneoplastic biomarker for colon tumorigenesis, was significantly higher in Mlh1-deficient mice compared to their wild type littermates [184]. The mutational specificity of PhIP-induced mutations in both MMR-proficient and MMR-deficient cell lines includes a high percentage of GC>TA transversions. Additional mutations seen are frameshift mutations, specifically –G frameshifts in homopolymeric runs of guanine bases [81,182,185,186]. However, Zhang *et al.* did not observe an induction of –G frameshifts in the MMR-deficient mice upon PhIP exposure, thus questioning the role of MMR in the –G frameshift mutagenesis induced by PhIP. Still, the total amount of -1 frameshifts was significantly higher in MMR-deficient mice. In addition, PhIP was shown to induce a significant increase in GC>AT and GC>CG mutations in MMR-deficient mice, indicating that MMR may indeed be involved in counteracting PhIP induced mutagenesis [81,183].

1.7 The role of MMR in the cellular response to genotoxic agents: ultraviolet (UV) radiation

The UV spectrum is divided into three wavelength intervals: UVA (320-400nm), UVB (290-320nm) and UVC (200-290nm). UVA produces reactive oxygen species (ROS) in addition to photoproducts, but UVB and UVC have been shown to produce mainly photolesions [187]. The two primary photolesions caused by UV radiation are *cis-syn* cyclobutane pyrimidine dimers (CPDs) and (6-4)pyrimidine-pyrimidone photoproducts [(6-4)PPs]. These photolesions are generally well repaired by the nucleotide excision repair (NER) pathway. Two sub-pathways of NER have been identified: the global genome repair (GGR) pathway that eliminates photolesions from the whole genome and the transcription coupled repair (TCR) pathway that removes lesions only from the transcribed DNA strands [188]. The importance of NER is illustrated by the fact that xeroderma pigmentosum (XP) patients who carry mutations in NER protein encoding genes, are highly sensitive to sunlight and have an increased predisposition to develop skin cancer in sun-exposed areas of the body [189].

Resistance of MMR-deficient cells to UV damage

In recent years many studies have been performed in order to clarify a possible role of MMR in the cellular response to UV. However, the performed experiments have resulted in different outcomes, thus the role of MMR in the response to UV remains controversial. The first area of controversy concerns the question as to whether MMR plays a role in NER. Mellon and Champe found that TCR is lost completely in *E. coli* strains lacking MutS and MutL following UVC exposure [190]. In addition, Mellon *et al*. demonstrated a decrease in TCR of UVC-induced CPDs in MMR-deficient human cancer cell lines [191]. However, in a different study with *E. coli* using the same method as Mellon *et al*., it was shown that there is hardly any difference in TCR between UVC irradiated MMR-proficient strains and MMR-deficient *E. coli* strains [192]. In addition, the results obtained by Mellon *et al*. in human cells have not been confirmed: several different studies in mammalian cells showed that TCR of CPDs is independent of MMR [193]. Also, *S. cerevisiae* strains defective in different MMR proteins do not show

alterations in levels of either TCR or GGR of UVC-induced CPDs when compared to wild type cells [194]. Thus, most available data point in the direction of TCR being a repair process that can be executed independently of MMR.

The second area of controversy concerning the role of MMR in the UV-induced cellular response involves the difference in cellular survival of wild type and MMR-deficient cells after UV radiation. In transformed cells, a (marginally) increased sensitivity of MMR-deficient cells has been reported [191,195], but other studies showed a similar UV survival of MMR-deficient cells and wild type cells [196,197]. UVB treatment of primary Msh2- and Msh6-null MEFs showed an increased survival compared to isogenic wildtype cells [198-200].

MMR-dependent cell cycle signalling after UV irradiation

Van Oosten *et al*. showed that Msh2 played a role in the UVB-induced late S-phase arrest in mouse keratinocytes [201]. Msh2 deficiency significantly lowered the percentage of arrested cells *in vivo* (40-50 %) and *in vitro* (30-40%). The role of MMR in UV-induced cell signalling will be discussed in more detail in chapter 3.

UV- induced mutagenesis in MMR-deficient cells

Although MMR very likely does not play a role in TCR of UV-induced damage, it has been shown that in *E. coli* the absence of MMR results in a significant increase of UV-induced mutagenesis [202]. Support for a possible role of MMR in counteracting UV-induced mutagenesis in mammalian cells came from experiments with Chinese hamster ovary (CHO) cells in which a point mutation in Ercc1, a protein that plays an important role in NER, was combined with a MMR defect. The double mutant showed a several hundred-fold higher UV-induced mutation frequency compared to the single mutant with a NER defect only [197]. Still, it cannot be ruled out that other mutations were responsible for this effect, since the double mutants were obtained via random mutagenesis of *Ercc1* mutants with MNNG and subsequent selection for loss of MMR. Also, the very high mutation frequencies (up to $4x10^{-3}$) observed conflict with the small target site (25

nucleotides) of the ouabain mutation reporter used. Increased susceptibility to UV-induced mutagenesis of a MMR/NER double mutant compared to a single NER mutant has also been observed *in vivo*: *Xpc*$^{-/-}$ *Msh2*$^{-/-}$ mice appeared to be more prone to UVB-induced skin cancer than *Xpc*$^{-/-}$ mice [203], comparable to the increased UV induced tumorigenesis observed in *Xpa*$^{-/-}$ *Msh2*$^{-/-}$ mice relative to *Xpa*$^{-/-}$ mice [204]. However, this could also have been the consequence of the additive effects of NER on UVB mutagenesis and MMR on spontaneous mutagenesis. Shin *et al.* could not demonstrate an increase of mutations over background levels when *Pms2*$^{-/-}$ mouse kidney cells were exposed to UV [205]. Recently, using a site-specific reversion assay, the same group found again no increase of UV induced mutation frequencies over the spontaneous mutation frequency in MMR-deficient cells [206]. Still, a significant increase in the relative contribution of CC>TT tandem mutations was observed after UV irradiation specifically in the MMR-deficient cells, pointing at a role for MMR in counteracting UV-induced mutagenesis. A more detailed view on the role of MMR in UV induced mutagenesis will be given in chapter 2.

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Chapter 2

DNA mismatch repair mediates protection from mutagenesis induced by short-wave ultraviolet light

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Abstract

To investigate involvement of DNA mismatch repair in the response to short-wave ultraviolet (UVC) light, we compared UVC-induced mutant frequencies and mutational spectra at the *Hprt* gene between wild type and mismatch-repair-deficient mouse embryonic stem (ES) cells. Whereas mismatch repair gene status did not significantly affect survival of these cells after UVC irradiation, UVC induced substantially more mutations in ES cells that lack the MutSα mismatch-recognizing heterodimer than in wild type ES cells. The global UVC-induced mutational spectra at *Hprt* and the distribution of most spectral mutational hotspots were found to be similar in mismatch-repair-deficient and wild type cells. However, at one predominant spectral hot spot for mutagenesis in wild type cells, the UVC-induced mutation frequency was not affected by the mismatch repair status. Together these data reveal a major role of mismatch repair in controlling mutagenesis induced by UVC light and may suggest the sequence context-dependent direct mismatch repair of misincorporations opposite UVC-induced pyrimidine dimers.

2.1 Introduction

DNA mismatch repair (MMR) contributes significantly to the maintenance of genomic integrity. The primary function of MMR is to repair mismatched or misaligned (so-called insertion-deletion loops, IDLs) nucleotides resulting from replication errors [1]. In mammalian cells mismatches and short IDLs are recognized by MutSα, a heterodimer of MSH2 and MSH6. A minor complex, MutSβ, consisting of MSH2 and MSH3 also recognizes these small IDLs, in addition to larger IDLs. Binding of either recognition heterodimer is followed by recruitment of a second heterodimer, consisting of MLH1 and PMS2, that connects the mismatch recognition steps with the downstream repair steps, *i.e.* excision of the mismatch-containing strand, DNA resynthesis and sealing of the nick between the old and newly synthesized DNA [1]. Defects in MMR result in a spontaneous mutator phenotype reflected by microsatellite instability (MSI), a hallmark of cancer in patients with hereditary non-polyposis colorectal cancer (HNPCC) who carry a germ line mutation in one of four MMR genes, i.e. *MSH2*, *MSH6*, *MLH1*, or *PMS2* [2]. In recent years evidence has been obtained that MMR not only counteracts misincorporations by the replicative polymerases, but also plays a role in the cellular response to several genotoxic agents in mammalian cells. For instance, MMR-mediated toxicity and/or protection against mutagenesis has been observed after treatment with S_N1-methylating agents [3], thiopurine derivatives [4], oxidizing agents [5,6] and heterocyclic amines [7,8].

Ultraviolet light (UV) predominantly induces *cis-syn* cyclobutane pyrimidine dimers (CPDs) and (6-4) pyrimidine-pyrimidone photoproducts [(6-4)PPs] between adjacent bipyrimidines [9]. Data indicating that MMR may affect UV-induced mutagenesis in mammalian cells have come from Nara *et al.* [10] who found that the introduction of a MMR defect in Chinese hamster ovary (CHO) cells carrying a mutation in the nucleotide excision repair (NER, [11]) gene *Ercc1* dramatically increased the UV-induced mutation frequency. However, this study was performed with heavily mutagenized cell lines; therefore the risk of secondary mutations affecting the phenotype cannot be excluded. No mutational spectra were presented in this work to demonstrate that mutations were induced at bipyrimidine sites. In another recent paper, Shin-Darlak *et al.* [12] demonstrated

that specifically the frequency of UVB-induced tandem CC to TT reversions was increased in a MMR-deficient mouse kidney cell line. In this study, however, MMR status did not influence the UVB-induced single C to T transition frequency. The cell lines used contained the reversion reporter construct integrated at different genomic locations. Since e.g. replication direction or transcription [13] may affect mutagenesis and also since the efficiency of MMR may vary according the genomic location of the mismatch [14], this differential genomic location may have influenced UVB-induced lesion frequencies. In addition, since the MMR-deficient cell line was derived from an adult mouse and the cells were cultured for considerable time prior to the experiments, genetic drift consequent to the MMR deficiency cannot be excluded.

In this study we have used embryonic stem (ES) cell lines to investigate the role of MMR in UVC-induced cytotoxicity and mutagenesis. Since tumorigenesis is generally believed to initiate in undifferentiated stem cells [15,16], the response of these cells to genotoxic agents is of particular interest. In addition, since some of our ES cell lines were freshly derived, the effect of genetic drift on mutagenesis is minimized. We found that UVC-induced mutant frequencies at *Hprt* were six fold higher in MutSα-deficient ES cells than in wild type ES cells. Most spectral hotspots of UVC-induced mutations were identical between MutSα-proficient and MutSα-deficient cells. Although globally the mutation frequency at these spectral hotspots was increased in the MutSα-deficient cells, at one strong spectral hot spot in wild type cells the frequency of C to T transitions was not increased in the MMR-deficient cells, possibly due to inability of MMR to repair misincorporations opposite a pyrimidine dimer specifically at this site. Thus, our data are in agreement with sequence-context-dependent repair of misincorporations opposite UVC-induced pyrimidine dimers. However, at present we cannot exclude an alternative model in which UVC induces an MMR-dependent S-phase delay, allowing NER to remove the damage prior to mutagenic replication.

2.2 Materials and Methods

Cell lines and cell culture

129/OLA-derived primary diploid ES cell lines E14 (wild type), *Msh2*$^{-/-}$ line 9 (previously called dMsh2-9), *Msh3*$^{-/-}$, *Msh6*$^{-/-}$ and *Msh3*$^{-/-}$ *Msh6*$^{-/-}$ are described elsewhere [17,18]. *Msh2*$^{-/-}$ line 31 was independently derived from *Msh2*$^{+/-}$ ES cells (*sMsh2* line 42, [17]) as follows: 10^6 cells per gelatin-coated T90 culture dish were treated for two hours with 6-thioguanine (6TG, 40 μM). This (marginally mutagenic) treatment selectively induces apoptosis in MMR-proficient cells [4], selecting cells that have lost the wild type *Msh2* allele by loss of heterozygosity [19]. Of note, this procedure does not select for cells that have lost *Hprt* by mutational inactivation [19]. *Msh2*$^{-/-}$ line N4 was generated in the same fashion, but only after subcloning the *Msh2*$^{+/-}$ ES cells to purge the line from pre-existing *Msh2*$^{-/-}$ cells. Thus, *Msh2*$^{-/-}$ line 31, and particularly line N4, have freshly originated. This novel rederivation of *Msh2*$^{-/-}$ cell lines minimizes mutations at *Hprt* (particularly in line N4) and also mutations that might affect the cellular response to DNA damage that both may result from the spontaneous mutator phenotype inferred by the MMR deficiency. Early passages of line N4 line were used for the experiments described here. Genotyping of the 6TG-selected lines demonstrated loss of heterozygosity for *Msh2* in both lines 31 and N4, as expected (not shown). ES cells were cultured on irradiated mouse embryonic fibroblast (MEF) feeder cells using established procedures. Knockout DMEM (Life Technologies) was supplemented with 10% fetal calf serum, Glutamax, non-essential amino acids, penicillin, streptomycin, 0.1 mM β-mercaptoethanol and leukemia inhibitory factor (LIF); this medium is referred to as ES complete medium.

Determination of UVC-induced cytotoxicity and mutant frequencies at Hprt

Twenty hours before UVC irradiation, ES cells were seeded at a density of $5x10^6$ cells per gelatin-coated T90 culture dish and incubated in ES complete medium supplemented with 50% Buffalo rat liver (BRL) cell-conditioned medium (BRL complete medium). Prior to UVC irradiation [Philips TUV lamp, 254 nm], cells were washed with PBS. Cells were irradiated at a dose rate of 0.06 J/m^2/s after

removal of all PBS. After treatment, cells were trypsinized and seeded on gelatin-coated dishes in BRL complete medium at a density of 200-4000 cells per T60 dish (five dishes per dose) to determine cell survival. The survival curves are the result of at least three independent experiments per cell line.

Induction of apoptosis was measured using the Apofluor® Green Caspase detection kit (ICN Biomedicals, Inc.); processing, staining and fixing of the cells were performed according the protocol supplied by the manufacturer. Approximately 40 hours after treatment for two hours with 6TG (40 μM) or with UVC (0.5 J/m^2), cells were collected by trypsinization, added to the culture supernatant (comprising floating cells) and incubated for 1 hour at 37°C with the fluorescent Apofluor® Green reagent that irreversibly binds to activated caspases. After incubation, fluorescence levels were measured by flow cytometric analysis.

For the determination of UVC-induced mutant frequencies at *Hprt*, $5x10^6$ (mock treatment) or $10x10^6$ (UVC treatment) ES cells were seeded one day prior to treatment on gelatin-coated T90 culture dishes with BRL complete medium. Cells were irradiated as above, trypsinized and propagated for 6 days on feeder cells in ES complete medium to allow loss of wild type Hprt mRNA and protein. Subsequently, $4x10^5$ ES cells were seeded per gelatin-coated T90 dish in BRL complete medium containing 30 μM 6TG (five dishes per dose) to select for Hprt-deficiency. In parallel, cloning efficiencies were determined by seeding 250 cells of each population in a T60 dish (three dishes per dose) in BRL complete medium (without 6TG). After 9 days, clones were washed with NaCl (0.9% w/v), fixed and stained using methylene blue solution (0.15% w/v in methanol) and counted. Mutant frequencies at *Hprt* were determined by correcting the number of 6TG-resistant colonies in the mock-treated and UVC-treated dishes for the cloning efficiencies. UVC-induced mutant frequencies at *Hprt* were obtained by subtracting the spontaneous mutant frequencies from the mutant frequencies found after UVC treatment for each ES cell line. Mutation experiments have been performed at least three times per cell line.

Isolation of Hprt-mutant clones, RNA extraction and Hprt cDNA synthesis

To determine UVC-induced mutational spectra at *Hprt*, 6TG-resistant clones from independently UVC-treated populations of wild type and *Msh2*$^{-/-}$ ES cells were picked and transferred to 24-well tissue culture plates. This procedure assures independence of UVC-induced mutations. Upon confluency, RNA was isolated employing TRIzol reagent (Invitrogen) using the protocol from the manufacturer. For cDNA synthesis, the RNA pellet was resuspended in 18 µl of anneal buffer (10 mM Tris-HCl [pH 8.3], 250 mM KCl, 1 mM EDTA) and to 9 µl of this suspension 1µl (20pmol) *Hprt*-cDNA primer (GCAGCAACTGACATTTCTAAA) was added. This was added to 17.6 µl cDNA buffer (20.5 mM Tris-HCl [pH 8.3], 13.6 mM $MgCl_2$, 6.8 mM dithiothreitol [DTT], 0.34mM dNTPs) followed by addition of 2.5U AMV reverse transcriptase (Promega) and subsequent incubation for 1h at 37°C.

Amplification and sequencing of Hprt cDNA

1.5 µl of synthesized cDNA was used to amplify the *Hprt* coding region in a total volume of 25.7 µl containing 5 µl of 5x PCR+Mg^{2+} buffer (0.5 vol. 10x Hot Goldstar PCR buffer [Eurogentec] + 0.5 vol. 16.7 mM $MgCl_2$), 0.63 µl of a mix of 2.5 mM of each dNTP, 5 pmol of each of the PCR primers hprt-mus1 (TTTTTGCCGCGAGCCGACC) and san2m13 (CGACGTTGTAAAACGACGGCCAGTGCAGATTCAACTTGCCGCTC) and 0.25U of Hot Goldstar polymerase (Eurogentec). After an initial denaturation/activation step of 10 min at 94°C, 35 cycles of PCR were performed (30 sec at 94°C, 30 sec at 55°C, 1 min 30 sec at 72 °C), followed by a final extension step of 10 min at 72°C. Automated DNA sequence analysis of the crude PCR products was performed using the M13 primer (TTGTAAAACGACGGCCAGT) on Applied Biosystems equipment.

Analysis of UVC-induced mutations

For the wild type cells the frequency of UVC-induced single nucleotide substitutions at bipyrimidines was calculated as follows: we multiplied the fraction of UVC-induced single nucleotide substitutions at bipyrimidines, as determined by sequence analysis (see Supplementary Table 2), with the total mutant frequency found (see Figure 3). We applied a correction for the spontaneous (*i.e.* after mock treatment) mutations (caused by the MMR deficiency) for each individual *Msh2*$^{-/-}$ ES cell line used. We first calculated the spontaneous single nucleotide substitution frequencies at bipyrimidines as above. This background frequency of single nucleotide substitutions at bipyrimidines was then subtracted from the frequency of single nucleotide substitutions at bipyrimidines in UVC-treated *Msh2*$^{-/-}$ ES cells, calculated as above (see Supplementary Tables 1 and 2). These corrections resulted in the net frequencies of UVC-induced single nucleotide substitutions at bipyrimidines in *Msh2*$^{-/-}$ ES cells.

Frequencies of each of the UVC-induced six nucleotide substitutions at bipyrimidines were calculated in a similar fashion by multiplying the total frequency of single nucleotide substitutions at bipyrimidines, as calculated above, with the relative fraction of each possible nucleotide substitution. Again, in case of the *Msh2*$^{-/-}$ cells, we corrected these numbers with the frequencies of spontaneous substitutions at bipyrimidines found in mock-treated cells (see Supplementary Tables 1 and 2).

For assessing the distribution of mutations between the four different bipyrimidines in the different genotypes all mutations at multimer pyrimidine runs were excluded in case the original location of the dimer could not be assessed (e.g. in case the substitution was found in the middle of a multimer pyrimidine run). We employed the Chi-square test to determine whether there were significant differences, between wild type and *Msh2*-deficient cells, in the number of single nucleotide substitutions derived from lesions at the transcribed or non-transcribed strand and in the number of single nucleotide substitutions induced at the different bipyrimidines, or at the 5′ versus the 3′ nucleotide of the bipyrimidine.

Spectral hotspots of mutations were defined by the presence of four or more single substitution mutations at the same nucleotide taking into account the presence of 354 bipyrimidines in both strands of the *Hprt* coding sequence ($p \leq 0.05$). p was calculated using the Poisson distribution on the assumption that all the observed mutations are independent. We initially considered correction of the hotspots found after UVC treatment for spontaneous single nucleotide substitutions at these sites in the *Msh2*$^{-/-}$ cells, taking into account that the frequency of substitutions at bipyrimidines was increased five fold after UVC treatment in these cell lines. However, as no UVC-induced hotspots were found that were specific for the *Msh2*$^{-/-}$ lines (Supplementary Table 2) this correction ultimately was not necessary.

2.3 Results

Near-isogenic ES cell lines with targeted disruptions of the MutSα (Msh2 and Msh6 subunits) and the MutSβ (consisting of the Msh2 and Msh3 subunits) mismatch-binding dimers are excellent tools to investigate cellular phenotypes caused by defects in each of the MMR sub-pathways. In this study we investigated the involvement of each sub-pathway in survival and mutagenic responses after the induction of DNA damage by UVC light. We used wild type reference line E14; *Msh2*$^{-/-}$ lines 9 [17], 31, and N4 (this line was freshly established from an *Msh2*$^{+/-}$ ES cell line [17] to minimize the consequences of genetic drift associated with MMR deficiency), *Msh3*$^{-/-}$ ES cells, *Msh6*$^{-/-}$ ES cells and *Msh3*$^{-/-}$ *Msh6*$^{-/-}$ doubly-deficient ES cells [18].

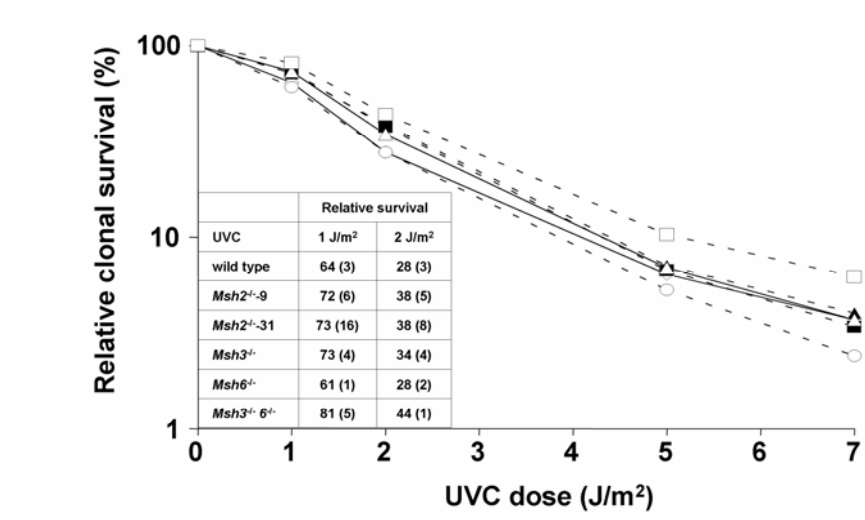


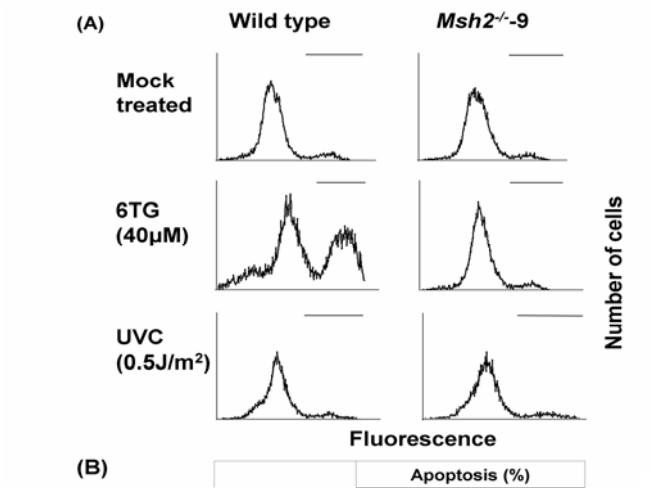
Figure 1. Relative clonal survival of *Msh*-deficient ES cell lines after UVC treatment. Dashed lines: MutSα-deficient lines. Wild type (diamonds), *Msh3*$^{-/-}$ (MutSβ-deficient, open triangles), *Msh2*$^{-/-}$ line 9 (closed triangles), *Msh2*$^{-/-}$ line 31 (closed squares), *Msh6*$^{-/-}$ (circles) and *Msh3*$^{-/-}$ *Msh6*$^{-/-}$ (open squares) lines. Insert: relative survival after 1 and 2 J/m² UVC, between parentheses: standard deviations, n ≥ 3.

MMR status does not affect the sensitivity to -or induction of apoptosis after- UVC radiation of mouse ES cells

Msh2$^{-/-}$ primary mouse embryonic fibroblasts (MEFs) show a slightly increased survival, and reduced apoptosis, upon UVB treatment [20,21]. We therefore investigated whether MMR affects clonal survival of ES cells after UVC treatment using ES cell lines carrying a targeted disruption of both alleles of one, or two, *Msh* genes. To further exclude the possibility that the phenotype of the MMR gene mutants was due to a secondary mutation introduced as a consequence of the mutator phenotype of MMR-deficient cells, several independently obtained *Msh2*$^{-/-}$ ES cell lines were studied. We found no consistent correlation between the MMR gene status and the survival to UVC light (Figure 1). In agreement, the kinetics of induction of apoptosis by UVC light (not shown) and the total number of induced apoptotic cells (Figure 2) are not affected by the MMR status. These results demonstrate that in ES cells MMR is not an important mediator of UVC-induced cytotoxicity. In contrast, high levels of apoptosis were invariably induced by 6TG in wild type ES cells as expected (Figure 2, [4,19]).

MutSα-deficient ES cells are hypermutable by UVC light

We subsequently determined the frequency of spontaneous and UVC-induced mutants at *Hprt* in the wild type and MMR-deficient ES cell lines. As expected, ES cell lines with *Msh2* and *Msh6* deficiencies displayed elevated spontaneous mutant frequencies compared to the wild type ES cell line (Figure 3A). The relatively lower spontaneous mutant frequency of the *Msh6*$^{-/-}$ ES cell line likely is due to the redundant repair of small IDLs by MutSβ. In line with the minor role of Msh3 in MMR, the *Msh3*$^{-/-}$ ES cells did not display a spontaneous mutator phenotype at *Hprt*. As expected, the *Msh3 Msh6* doubly-deficient ES cells displayed a similar spontaneous mutation frequency as the *Msh2*$^{-/-}$ ES cells. *Msh2*$^{-/-}$ line N4 (dark grey bars in Figure 3A) has accumulated less spontaneous mutations at *Hprt* likely because it was freshly established.



(B)

	Apoptosis (%)	
	Wild type	*Msh2*$^{-/-}$-9
Untreated	8	7
6TG (40μM)	40	8
UVC (0.5J/m²)	6	6

Figure 2. Apoptosis of *Msh2*$^{-/-}$ ES cells at 40 hours after mock, UVC (0.5J/m²) or 6TG (40μM, 2 hrs) treatment. Horizontal bars: apoptotic fraction. Left panels: wild type ES cells, right panels: *Msh2*$^{-/-}$ ES cells. Bottom panel: quantification of the apoptotic fractions.

Next, we calculated UVC-induced mutant frequencies in all ES cell lines by subtracting the spontaneous mutant frequencies at *Hprt* from the mutant frequencies found after UVC treatment (Figure 3B). To our surprise, the UVC-induced mutant frequencies in all MutSα-deficient ES cell lines (shades of grey, black and cross-hatched bars in Figure 3B) were substantially higher than those in wild type (white bars) and *Msh3*$^{-/-}$ (dotted bars) ES cells (*i.e.* between 250 x10^{-6} and 350x10^{-6} in the different MutSα-deficient ES cell lines opposed to 53x10^{-6} in wild type ES cells after treatment with 2J/m² UVC, Figure 3B). Importantly, a significant increase of the frequency of UVC-induced mutants was also found at a lower UVC dose of 0.5 J/m² in *Msh2*$^{-/-}$ line 9 ES cells, compared to wild type cells (Figure 3B). At this UV dose survival was high (Figure 1) and no significant levels of apoptosis were induced in either cell line (Figure 2). These data indicate that the difference in UVC-induced mutant frequencies is not related to selective elimination of wild type cells containing a heavy burden of pyrimidine dimers. In summary, we

demonstrate an important, survival-independent, role for MutSα-, but not MutSβ-dependent MMR in counteracting UVC-induced mutagenesis in ES cells.

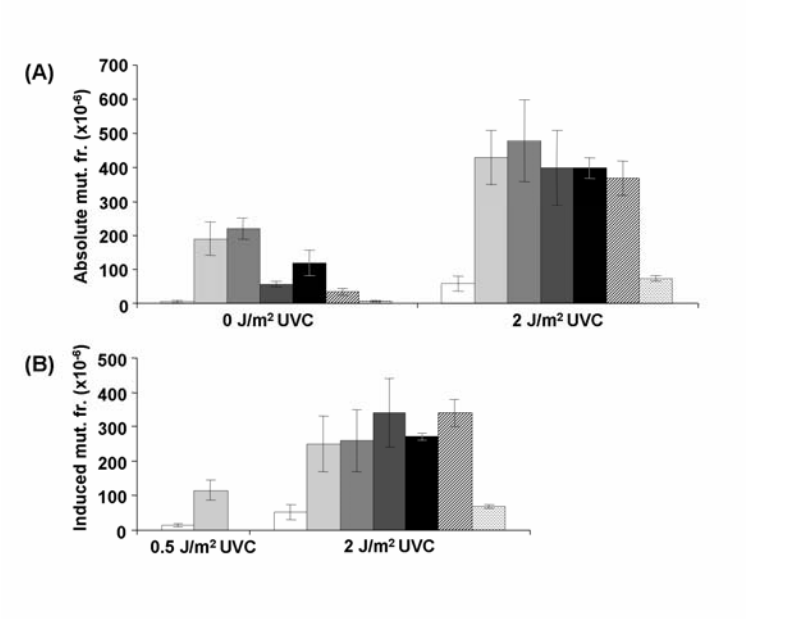


Figure 3. *Hprt* mutant frequencies. (A) Mutant frequencies after mock and UVC (2J/m²) treatment. (B) UVC (0.5J/m² and 2J/m²)-induced mutant frequencies obtained by subtracting the spontaneous mutant frequencies from the mutant frequencies found after UVC treatment. ES lines: wild type (clear bars), *Msh2*$^{-/-}$ line 9 (light grey bars), *Msh2*$^{-/-}$ line 31 (middle grey bars), *Msh2*$^{-/-}$line N4 (dark grey bars), *Msh3*$^{-/-}$ *Msh6*$^{-/-}$ (black bars), *Msh6*$^{-/-}$ (cross-hatched bars), *Msh3*$^{-/-}$ (dotted bars). Bars represent means ±SD (n ≥ 3).

UVC-induced mutational spectra at Hprt are similar between wild type and Msh2$^{-/-}$ *ES cells*

MutSα and its *Escherichia coli* homologue MutS have been shown to directly bind to mismatches opposite a pyrimidine dimer (called compound lesions). Binding affinities frequently are in the same order of magnitude as the affinities for normal mismatches, depending on the specific compound lesion and the sequence context [22,23,24]. We envisioned two possible mechanisms for the MMR-dependent suppression of UVC-induced mutagenesis in ES cells. Firstly, an MMR-dependent enhancement of the UVC-induced delay at the S-phase of the cell cycle ([25]; Borgdorff *et al.*, in preparation; see chapter 3) that might allow NER to

remove DNA photoproducts prior to misincorporation. Alternatively, MMR might directly counteract UVC light-induced mutagenesis by recognizing and removing misincorporations opposite pyrimidine dimers.

To investigate these possibilities we determined the UVC-induced spectra, and the spectral distribution, of UVC-induced mutations within the *Hprt* gene in wild type and *Msh2*$^{-/-}$ ES cells. Two independently obtained *Msh2*$^{-/-}$ ES cell lines were used in this experiment (line 9 and newly generated line N4). The complete mutational spectra are available as Supplementary Table 2. Since the mutational spectra for both *Msh2*$^{-/-}$ lines were similar, we pooled the spectra obtained from these lines. From non-irradiated *Msh2*$^{-/-}$ ES cells, 35 independent mutant *Hprt* alleles were sequenced as a negative control. From the UVC-treated cell lines, independent mutant *Hprt* alleles were sequenced from 104 *Msh2*$^{-/-}$ clones and from 28 MMR-proficient 6TG-resistant clones, augmented with 56 previously sequenced mutant *Hprt* alleles from MMR-proficient clones [26], totaling 84 *Hprt* mutants. Although the UVC-induced mutant frequency is increased six fold in the *Msh2*$^{-/-}$ ES cell lines, a similar number of mutants isolated after UVC treatment was analyzed from each genotype for practical reasons. Mutants bearing tandem, and multiple independent, substitutions were excluded from the analysis, as were base substitutions not at bipyrimidines, frameshifts, deletions and insertions. This was done since these types of mutations could not unambiguously be related to mutagenic translesion synthesis of pyrimidine dimers. Of note, in contrast to UVB-irradiated MMR-deficient murine kidney cells [12], in the *Msh2*$^{-/-}$ ES cells no specific increase (compared to single nucleotide substitutions) was found in the (low) frequency of UVC-induced tandem CC to TT transitions (Supplementary Table 2). Thus, in UVC-treated wild type cells, 60 of the 84 mutants contained single nucleotide substitutions at bipyrimidine sites within *Hprt* (71% of all mutants). In the UVC-treated *Msh2*$^{-/-}$ ES cells, 71 of 104 (68%) of all mutants contained single nucleotide substitutions at bipyrimidine sites, whereas only 13 of 35 (37%) of the *Hprt* mutants in mock-treated cells contained single nucleotide substitutions at bipyrimidine sites; most spontaneous mutations in mock-treated cells were frameshifts at short mononucleotide repeats (see Supplementary Table 2 and Supplementary Figure 1).

Frequencies of UVC-induced single nucleotide substitutions at bipyrimidine sites were calculated by multiplying the total mutant frequencies after UVC treatment by the fraction of single nucleotide substitutions found at bipyrimidine sites. In case of the *Msh2*$^{-/-}$ ES cells, we corrected for the frequency of spontaneous single nucleotide substitutions at bipyrimidine sites. Thus, the frequency of single nucleotide substitutions at bipyrimidine sites induced by 2J/m^2 UVC in wild type cells was calculated to be 42x10^{-6}, in the *Msh2*$^{-/-}$ ES cells the (corrected) frequency was 226x10^{-6} (Supplementary Table 1). From these data it is obvious that the higher frequency of UVC-induced mutations in *Msh2*$^{-/-}$ ES cells occurred specifically at bipyrimidine sites. This demonstrates a role of MMR in the suppression of mutagenesis specifically at UVC-induced pyrimidine dimers.

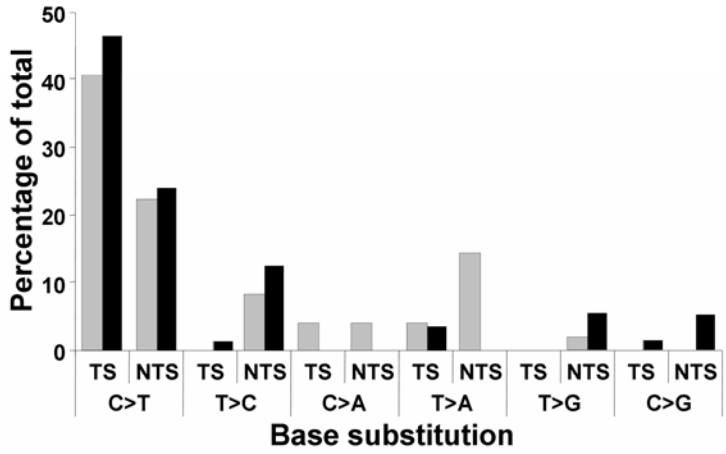


Figure 4. UVC-induced (2J/m^2) mutational spectrum at *Hprt*. Percentages of single nucleotide substitutions found at bipyrimidines in either the transcribed strand (TS) or the non-transcribed strand (NTS) of wild type (grey bars) and *Msh2*$^{-/-}$ *Hprt* mutants (black bars) are indicated.

We also calculated, in a very similar fashion, the relative contributions and frequencies of all six possible base nucleotide substitutions, induced at bipyrimidines, in wild type and *Msh2*$^{-/-}$ ES cells (Supplementary Table 1 and Figure 4). No obvious differences in the relative contributions of each of the substitutions were found between wild type and *Msh2*-deficient ES cells (Figure 4). This indicates that MMR suppresses different UVC-induced single nucleotide substitutions to a similar extent. Furthermore, we did not find a significant

difference between both genotypes in the relative contributions of single nucleotide substitutions derived from lesions at the transcribed DNA strand compared to the non-transcribed strand (p = 0.69, Supplementary Table 1; see also Figure 4 and Supplementary Table 2). In addition, we did not observe significant differences in the overall single nucleotide substitution frequencies induced at the different bipyrimidines (CC, TC, CT and TT, p = 0.26; Table 1). In addition, no significant difference in the overall proportions of substitutions at the 5′ versus the 3′ nucleotide at the bipyrimidine sites was detected between both genotypes (p= 0.98, Table 1). The only significant (p = 0.004) difference between both genotypes is a reduction in the relative frequency of specific T to A transversions at the 3′ nucleotide of TT dimers in the UVC-treated *Msh2*$^{-/-}$ cells (Table 1, in bold).

Table 1. Mutation spectrum at *Hprt* analyzed for single nucleotide substitutions on all four bipyrimidines[a]

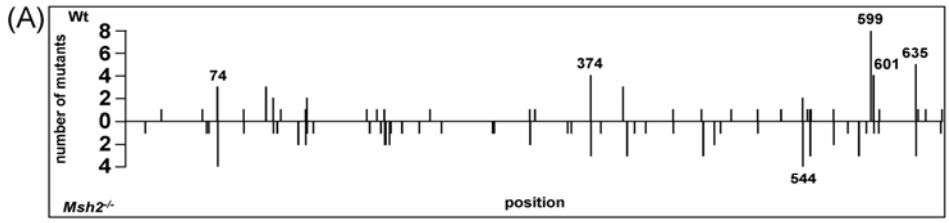
TO FROM	CA	CC	CG	CT	AC	GC	TC	TA	TG	TT	AT	GT
CC	0[A],0[B](0[C])	0,0(0)	0,0(0)	2,2(0)	0,1(0)	0,0(0)	0,2(1)	0,0(0)	0,0(0)	0,0(0)	0,0(0)	0,0(0)
CT	0,0(0)	0,5(2)	0,0(1)	0,0(0)	0,0(0)	0,0(0)	0,0(0)	0,0(0)	0,0(0)	2,2(1)	0,1(0)	0,0(0)
TC	0,0(0)	0,0(0)	0,0(0)	0,0(0)	0,0(0)	0,0(0)	0,0(0)	3,0(0)	0,1(0)	6,13(0)	0,0(0)	0,0(0)
TT	0,0(0)	0,0(0)	0,0(0)	0,0(3)	0,0(0)	0,0(0)	1,5(1)	**7,1(0)**	0,1(0)	0,0(0)	4,1(1)	0,2(0)

[a]Only substitutions at bipyrimidines are counted as well as at longer multimer pyrimidine runs when the position of the dimer is unambiguous, *i.e.* when the nucleotide substitution is at the extreme 5′ or 3′ of the pyrimidine run. Of note, the hotspot at position 599 is excluded for this reason.
[b]Wild type (2J/m^2 UVC): 19 substitutions at 3′ pyrimidine; 6 at 5′ pyrimidine
[c]*Msh2*$^{-/-}$ (2J/m^2 UVC) 28 substitutions at 3′ pyrimidine; 9 at 5′ pyrimidine
[d]*Msh2*$^{-/-}$ (0J/m^2 UVC): 10 substitutions

A UVC-induced mutational hotspot is specific for wild type ES cells

Analysis of the distribution of the UVC-induced single nucleotide substitutions at bipyrimidines in the *Hprt* gene in both genotypes revealed that the mutations were not randomly distributed (Figure 5A, Supplementary Figure 1). We defined spectral hotspots as four or more substitutions at one single bipyrimidine sequence, taking into account the presence of 354 bipyrimidines in the *Hprt* coding sequence. Thus, only when, at a given pyrimidine dimer, the

difference between the number of substitutions is four or more, a specific effect of MMR on mutagenesis at this position is considered significant ($p \leq 0.05$; Supplementary Figure 1). A similar non-random distribution of UVC-induced mutations was previously described for other cell types [24]. No significant differences were found between both genotypes for most spectral hotspots (Figure 5A and B). Nevertheless we did observe one highly significant difference between both genotypes. A very strong spectral hotspot ($p = 9 \times 10^{-9}$) at position 599 in a penta-pyrimidine stretch in the wild type ES cells appears absent in the *Msh2*$^{-/-}$ ES cell lines (Figure 5C; Supplementary Figure 1). Since the actual UVC-induced mutation frequency is six fold higher in the *Msh2*$^{-/-}$ cell lines whereas similar numbers of mutations at *Hprt* were analyzed from both genotypes, we assume that the absolute nucleotide substitution frequencies at this spectral hotspot may be similar in both genotypes. This suggests that UVC-induced mutagenesis specifically at position 599 is not controlled by MMR.



(B)

position hotspot	number of mutants in wt	number of mutants in *Msh2*$^{-/-}$
74	3	4
374	4	3
544	2	4
599	8	0
601	4	1
635	5	3

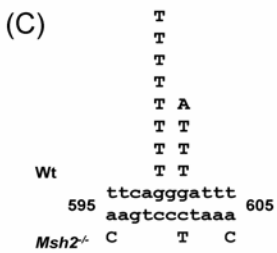


Figure 5. Location of UVC (2J/m²)-induced single nucleotide substitutions at bipyrimidines at *Hprt* of (A) 60 wild type and 71 *Msh2*-deficient ES cells (B) Number of nucleotide substitutions found at nucleotide hotspots in wild type and *Msh2*-deficient ES cells (C) Apparent spectral hotspot of mutations at position 599 of *Hprt* in the wild type ES cells. Nucleotide substitutions in wild type cells are indicated above the *Hprt* sequence, the nucleotide substitutions found in the *Msh2*$^{-/-}$ lines (lines 9 and N4) below the sequence. Wt: wild type.

2.4 Discussion

MMR plays a pivotal role in the maintenance of genomic integrity, as is illustrated by the elevated spontaneous mutant frequencies of MutSα-deficient ES cells. Here we show that MMR not only prevents spontaneous mutagenesis due to replicative polymerase errors, but also suppresses UVC-induced mutagenesis. Thus, compared to wild type cells, UVC-induced mutant frequencies were increased on the average six fold in the MutSα-, but not in the MutSβ, deficient ES cell lines. Higher frequencies of UV-induced mutants were also found in CHO cells carrying combined defects in the NER gene *Ercc1* and in MMR, compared to *Ercc1* single-mutant CHO cells [10]. However, the small target site of the Ouabain mutation reporter used (a few nucleotides), [27] is difficult to reconcile with the very high induced mutation frequencies (up to $4x10^{-3}$) observed. Prior to these experiments CHO cells were mutagenized extensively to induce loss of *Ercc1*- and MMR, respectively. Therefore a secondary phenomenon induced by immortalization of the cells or the subsequent mutagenic treatments may underlie the observed phenotype.

It was found by others that purified MutSα, and its *E. coli* homologue MutS, bind all three mismatches opposite a dithymidine CPD or (6-4)PP, albeit in a sequence context-specific fashion [22,23,24]. It therefore is probable that binding of MutSα to these compound lesions after mutagenic translesion synthesis is a key intermediate in MMR-dependent suppression of UVC-induced mutagenesis. A number of possible mechanisms can explain the involvement of MutSα in suppressing mutagenesis by UVC light after binding to compound lesions. We investigated the possibility of selective, MMR-dependent, elimination of heavily UVC-damaged cells resulting in loss of the cells that are most prone to mutations. Although previous studies did show a slightly increased survival of primary *Msh2*- and *Msh6*-deficient MEFs after exposure to UVB light [20,21], sensitivity of ES cell lines to UVC treatment was not dependent on MMR status. Furthermore, no consistent differences in UVC-induced apoptosis that might explain the differences in UVC-induced mutant frequencies were found between the genotypes, in contrast to previous results with MEFs [21]. Thus, these data do not support the option that survival or apoptotic differences underlie the difference in UVC-

induced mutant frequencies.

It was demonstrated recently that Msh2 plays a role in the UVB-induced S- phase delay in NER-deficient mouse keratinocytes [25]. We have found that the UVC-induced S-phase delay in NER-proficient ES cells is also partially MutSα-dependent (Borgdorff *et al*., in preparation; see chapter 3). We therefore considered the option that enhanced NER, enabled by the stronger S-phase delay in wild type cells, is responsible for the suppression of UVC-induced mutagenesis in these cells. However, in cultured rodent cells, including ES cells, CPD dimers (that underlie most UVC-induced mutations [28]) generally are believed to be refractory to NER at the non-transcribed DNA strand [13,29,30,31]. For this reason, mutagenesis originating from CPDs at this strand is not likely affected by differences in S-phase progression. Only prereplicatively mispaired CPDs that are a good substrate for NER *in vitro* [22], are expected to be less mutagenic consequent to a prolonged MMR-dependent S-phase delay in wild type cells. Cytosine in CPD dimers can deaminate prereplicatively; the resulting U within the compound dimer may lead to a C to T transition if not repaired by NER before replication [32]. Indeed, for UVB-induced CC CPDs a strong effect of prereplicative deamination on the double transition frequency to TT was demonstrated. This effect was much weaker for single C to T transitions [33] indicating that at least part of the single C to T transitions may not have originated from deamination of the cytosine but from mutagenic translesion synthesis [34] of a cytosine imino tautomer within the dimer incorporating an adenine [35]. In mouse kidney cells deficient for the *Pms2* MMR gene an increased frequency of UVB-induced tandem CC to TT transitions (but not of single C to T transitions) was found at the non-transcribed strand of a transfected *Aprt* gene [12]. Therefore this result is consistent with accelerated S-phase progression in *Pms2*-deficient cells resulting in reduction of prereplicative NER specifically of U<>U/GG CPD dimers. In contrast, we found that in *Msh2*$^{-/-}$ ES cells the frequency of UVC-induced tandem transitions at CC bipyrimidines was not increased beyond that of single nucleotide substitutions, including those substitutions that are not at cytosines (Figure 4, Supplementary Table 2, also see Table 1). This result suggests that the MMR-dependent enhanced S-phase delay, enabling prereplicative NER of (doubly or singly) deaminated cytosine within CPDs, may only play a limited role in reducing UVC-induced mutagenesis in ES

cells. Supporting this notion, complete abolishment of the UVC-induced S-phase delay in both wild type and *Msh2*$^{-/-}$ ES cells does not significantly alleviate the hypermutability by UVC of MMR-deficient ES cells (Borgdorff *et al*., in preparation; see chapter 3).

We infer that our results are most consistent with MMR directly reversing translesion synthesis-induced misincorporations opposite pyrimidine dimers, as was proposed previously [23,36]. Evidence for such a direct role of MMR in repairing mismatches within compound photoproducts in *Escherichia coli* exists [36] although in mammalian cell extracts MMR of compound photoproducts was not detected [37].

The sequence context dependence of MutSα-binding to mispaired pyrimidine dimers predicts that in some sequence contexts misreplicated pyrimidine dimers may be a poor substrate for direct repair by MMR. At these positions, in wild type cells a spectral hotspot of UV-induced mutations is expected at which the absolute mutation frequency is independent of MMR status. The spectral hotspot for C to T transitions at a pyrimidine stretch at position 599 of *Hprt*, at which the mutation frequency is not increased by *Msh2* deficiency, may be an example of such a site.

Exclusion of the spectral hotspot at position 599 of *Hprt* from the analysis of the UVC-induced overall nucleotide substitution pattern reveals a moderate increase in the relative frequency of single C to T transitions in the *Msh2*$^{-/-}$ cells (31% in wild type cells versus 47% in *Msh2*$^{-/-}$ cells, see also Table 1). This may indicate (relatively) efficient MMR-mediated removal of adenines within compound C:A mismatches, possibly originating from mutagenic translesion synthesis of a cytosine tautomer within the CPD [35]. However, we cannot formally exclude that prereplicative deamination of single C residues within a CPD followed by misincorporation of A underlies some of these C to T substitutions (but see above). Conversely, the significant paucity of T to A transversions at TT dimers in the *Msh2*$^{-/-}$ cells (Table 1 in bold, Supplementary Table 1) may indicate that T<>T/AT compound CPD lesions are poorly repaired by MMR.

Based on the MMR-dependent six fold reduction of UVC-induced mutation frequencies we estimate that MMR might repair the misincorporation in more than 80% of compound lesions, depending on the specific compound lesion and the sequence context. This is considerably less efficient than the efficiency of repair of 'normal' mismatches, which is higher than 99% [38]. If direct repair of compound mismatches indeed proves to underlie the MMR-mediated reduction of UVC-induced mutagenesis this would set a paradigm for a novel role of MMR in the postreplicative removal of misincorporations opposite damaged nucleotides, and thereby in avoiding DNA damage-induced mutagenesis.

The role of MMR in counteracting UV-induced mutagenesis may also have practical implications. Since cancer is believed to originate from stem cells, this finding may be of relevance in understanding the role of MMR in suppressing DNA damage-induced cancer. As an example, the observation that mice, doubly deficient for NER and MMR develop UVB-induced skin cancer with extremely short latency [39,40] might be explained by excessive UV-induced mutagenesis due to the absence of the anti-mutagenic effect of MMR. HNPCC patients carry a heterozygous defect in one allele of one of the MMR genes. Stochastic loss of the wild type allele is believed to result in the emergence of MMR-deficient cells [19]. Therefore, exposure of HNPCC patients to sunlight may predispose these patients to skin cancer. In support, patients with Muir-Torre syndrome, a subgroup of HNPCC, develop skin cancer at high frequency [41].

Acknowledgements

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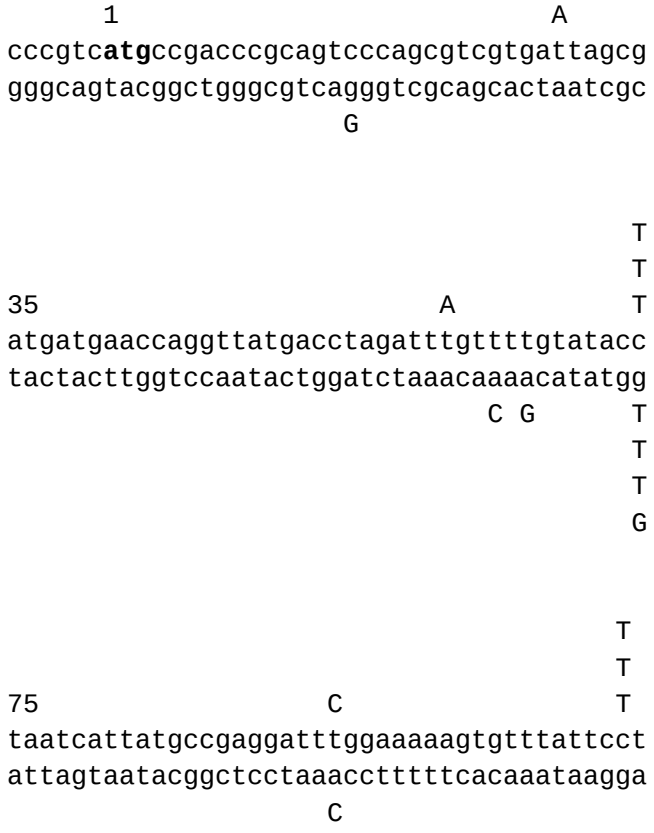
Supplementary figures

Supplementary Figure 1. Mutation spectrum at bipyrimidines at *Hprt*

Above sequence: wild type 2 J/m^2 UVC (n=60)
Below sequence: *Msh2*$^{-/-}$ 2 J/m^2 UVC (n=71)
Below sequence between brackets: *Msh2*$^{-/-}$ mock treated (n=13)



```
    T                            C
115 T     A                     TC
catggactgattatggacaggactgaaagacttgctcgag
gtacctgactaatacctgtcctgactttctgaacgagctc
    T  C                T     TC (T)T
                        T     T


155                                    C
atgtcatgaaggagatgggaggccatcacattgtggccct
tacagtacttcctctaccctccggtagtgtaacaccggga


195    T     T
ctgtgtgctcaaggggggctataagttctttgctgacctg
gacacacgagttccccccgatattcaagaaacgactggac
 C         C  TT  TA        G
               TT  T
                  (T)

235       A
ctggattacattaaagcactgaatagaaatagtgatagat
gacctaatgtaatttcgagacttatctttatcactatcta
 C                 C
                  (C)
                  (C)


275
ccattcctatgactgtagattttatcagactgaagagcta
ggtaaggatactgacatctaaaatagtctgacttctcgat
                    CG


315       T   A
ctgtaatgatcagtcaacgggggacataaaagttattggt
gacattactagtcagttgccccctgtattttcaataacca
          T
          T
```

```
                  A
                  A
                  A
355               A
ggagatgatctctcaactttaactggaaagaatgtcttga
cctctactagagagttgaaattgacctttcttacagaact
T  T              C       A
                  C
                  A
     T
     T
395  T
ttgttgaagatataattgacactggtaaaacaatgcaaac
aacaacttctatattaactgtgaccattttgttacgtttg
        T     A        T
        T
        T

435  C                     T
tttgctttccctggttaagcagtacagccccaaaatggtt
aaacgaaagggaccaattcgtcatgtcggggttttaccaa
 (C) C                      T        G
                            T        G
                            T
                           (A)

475         G                    T
aaggttgcaagcttgctggtgaaaaggacctctcgaagtg
ttccaacgttcgaacgaccacttttcctggagagcttcac
   A           (G)      (A)      T
   (C)
```

```
                              A
515         T                 T   A AT
ttggatacaggccagactttgttggatttgaaattccaga
aacctatgtccggactgaaacaacctaaactttaaggtct
                 (C)          T     T
                              T     T
                              T     T
                              T  (A)


555           T
caagtttgttgttggatatgcccttgactataatgagtac
gttcaaacaacaacctacacgggaactgatattactcatg
              T          T        T
              T                   T
                                  G  A

    T
    T
    T
    T
    T A
    T T
    T T
595 T T    A
ttcagggatttgaatcacgtttgtgtcattagtgaaactg
aagtccctaaacttagtgcaaacacagtaatcactttgac
C     T   C       (C) A
                  (C)
635
T
T
T
T
T A     A            A
gaaaagccaaatacaaagcctaaga
ctttcggtttatgtttcggatt
T                   C
T
T
```

Supplementary Table 1. Calculation of single nucleotide substitution frequencies at bipyrimidine sites at *Hprt*[a]

Wild type							*Msh2*$^{-/-}$					
Transitions	Strand	# of UVC-induced substitutions	Frequency of UVC-induced substitutions	% of UVC-induced substitutions	Transitions	Strand	# of substitutions after UVC	Frequency of substitutions after UVC[c]	# of substitutions (mock treatment)	Frequency of substitutions (mock treatment)[c]	Frequency of UVC-induced substitutions[c]	% of UVC-induced substitutions[c]
C>T	TS[b]	24	17×10^{-6}	40	C>T	TS	28	110×10^{-6}	1	4×10^{-6}	106×10^{-6}	47
	NTS[b]	13	9×10^{-6}	22		NTS	15	59×10^{-6}	1	4×10^{-6}	55×10^{-6}	24
T>C	TS	0	0	0	T>C	TS	1	4×10^{-6}	0	0	4×10^{-6}	2
	NTS	5	4×10^{-6}	8		NTS	14	55×10^{-6}	7	30×10^{-6}	25×10^{-6}	11
C>A	TS	3	2×10^{-6}	5	C>A	TS	1	4×10^{-6}	1	4×10^{-6}	0	0
	NTS	2	1×10^{-6}	3		NTS	1	4×10^{-6}	1	4×10^{-6}	0	0
T>A	TS	3	2×10^{-6}	5	T>A	TS	2	8×10^{-6}	0	0	8×10^{-6}	4
	NTS	9	6×10^{-6}	15		NTS	1	4×10^{-6}	1	4×10^{-6}	0	0
T>G	NTS	1	1×10^{-6}	2	T>G	NTS	4	16×10^{-6}	1	4×10^{-6}	12×10^{-6}	5
C>G	TS	0	0	0	C>G	TS	1	4×10^{-6}	0	0	4×10^{-6}	2
	NTS	0	0	0		NTS	3	12×10^{-6}	0	0	12×10^{-6}	5
Total		60	42×10^{-6}	100	Total		71	280×10^{-6}	13	54×10^{-6}	226×10^{-6}	100

[a] Calculated by multiplying the proportion of each single nucleotide substitution (Supplementary Table 2) with the UVC-induced mutant frequencies at *Hprt* (Figure 3). In case of the *Msh2*$^{-/-}$ cells corrections were applied for the substitution and mutation frequencies in mock-treated cells (see main text). The percentage of all single nucleotide substitutions is also shown in Figure 4.

[b] TS: Transcribed DNA strand (template strand), NTS: Non-transcribed DNA strand (coding strand).

[c] Overall frequencies of single nucleotide substitutions in the *Msh2*$^{-/-}$ cells were based on weighted averages of frequencies obtained from *Msh2*$^{-/-}$ lines N4 and 9.

Supplementary Table 2. Mutational spectra at *Hprt*

Wild type ES cells, 2 J/m² UVC

Transitions

Position	Mutation	Strand	Target seq. (NTS)
51	T>C	ND[a]	TTA (T) GAC
74	C>T	NTS	TAC (C) TAA
74	C>T	NTS	TAC (C) TAA
74	C>T	NTS	TAC (C) TAA
95	T>C	NTS	ATT (T) GGA
113	C>T	NTS	TTC (C) TCA
113	C>T	NTS	TTC (C) TCA
113	C>T	NTS	TTC (C) TCA
119	C>T	TS	ATG (G) ACT
119	C>T	TS	ATG (G) ACT
145	C>T	NTS	AGA (C) TTG
146	T>C	NTS	GAC (T) TGC
146	T>C	NTS	GAC (T) TGC
194	T>C	NTS	CCC (T) CTG
202	C>T	NTS	GTG (C) TCA
208	C>T	TS	AAG (G) GGG
325	C>T	NTS	GAT (C) AGT
400	C>T	TS	GTT (G) AAG
400	C>T	TS	GTT (G) AAG
400	C>T	TS	GTT (G) AAG
440	T>C	NTS	TGC (T) TTC
463	C>T	NTS	AGC (C) CCA
508	C>T	NTS	TCT (C) GAA
527	C>T	NTS	GGC (C) AGA
544	C>T	TS	TTT (G) AAA
551	C>T	NTS	TTC (C) AGA
569	C>T	TS	TTG (G) ATA

599	C>T	TS	TCA (G) GGA
599	C>T	TS	TCA (G) GGA
599	C>T	TS	TCA (G) GGA
599	C>T	TS	TCA (G) GGA
599	C>T	TS	TCA (G) GGA
599	C>T	TS	TCA (G) GGA
599	C>T	TS	TCA (G) GGA
599	C>T	TS	TCA (G) GGA
601	C>T	TS	AGG (G) ATT
601	C>T	TS	AGG (G) ATT
601	C>T	TS	AGG (G) ATT
635	C>T	TS	CTG (G) AAA
635	C>T	TS	CTG (G) AAA
635	C>T	TS	CTG (G) AAA
635	C>T	TS	CTG (G) AAA
635	C>T	TS	CTG (G) AAA

Transversions

Position	Mutation	Strand	Target seq. (NTS)
29	T>A	NTS	TGA (T) TAG
62	T>A	NTS	ATT (T) GTT
125	T>A	NTS	TGA (T) TAT
197	C>A	ND	TCT (G) TGT
197	C>A	ND	TCT (G) TGT
245	T>A	NTS	ACA (T) TAA
329	C>A	NTS	AGT (C) AAC
374	T>A	NTS	CTT (T) AAC
374	T>A	NTS	CTT (T) AAC
374	T>A	NTS	CTT (T) AAC
374	T>A	NTS	CTT (T) AAC
487	T>G	NTS	AGC (T) TGC
544	C>A	TS	TTT (G) AAA
548	T>A	NTS	AAA (T) TCC

550	C>A	NTS	ATT (C) CAG
565	C>A	ND	GTT (G) TTG
584	T>G	ND	ACT (A) TAA
601	C>A	TS	AGG (G) ATT
606	C>A	TS	TTT (G) AAT
637	T>A	TS	GGA (A) AAG
643	T>A	TS	GCC (A) AAT
656	T>A	TS	CCT (A) AGA

Tandem mutations

Position	Mutation	Strand	Target seq. (NTS)
74	C>T	NTS	TAC (CT) AAT
75	T>C	NTS	TAC (CT) AAT
207	C>G	TS	CAA (G) GGG
208	C>T	TS	AAG (G) GGG
391	T>A	NTS	GTC (TT) GAT
392	T>C	NTS	GTC (TT) GAT
568	C>T	TS	GTT (GG) ATA
569	C>T	TS	GTT (GG) ATA
600	C>T	TS	CAG (GG) ATT
601	C>T	TS	CAG (GG) ATT

Multiple Independent Mutations

Position	Mutation	Strand	Target seq. (NTS)
183	C>T	ND	TCA (C) ATT
185	-T		ACA (T) TGT
245	T>A	NTS	ACA (T) TAA
247	T>G	TS	ATT (A) AAG
296	T>C	NTS	ATT (T) TAT
325	C>T	NTS	GAT (C) AGT
361	C>A	TS	GAT (G) ATC
368	C>A	NTS	TCT (C) AAC
382	T>C	TS	GGA (A) AGA
384	C>T	TS	AAA (G) AAT
601	C>T	TS	AGG (G) ATT
603	T>A	NTS	GGA (T) TTG
632	C>T	NTS	AAA (C) TGG
634	C>A	TS	ACT (G) GAA

Deletions/Insertions

49	-T	NTS	GGT (T) ATG
346-349	-GTTA	ND	AAA (GTTA) TTG
442	-T	NTS	CTT (T) CCC
del 17bp			
del 54bp			
del 54bp			
del 54bp			

***Msh2*$^{-/-}$ ES cells 2 J/m² UVC**

Bold = *Msh2*$^{-/-}$ line N4 mutants

Italic = Msh2$^{-/-}$ line 9 mutants

Transitions

Position	Mutation	Strand	Target seq. (NTS)
65	**T>C**	**NTS**	**TGT (T) TTG**
74	**C>T**	**NTS**	**TAC (C) TAA**
74	**C>T**	**NTS**	**TAC (C) TAA**
74	*C>T*	*NTS*	*TAC (C) TAA*
95	**T>C**	**NTS**	**ATT (T) GGA**
119	**C>T**	**TS**	**ATG (G) ACT**
122	**T> C**	**NTS**	**GAC (T) GAT**
139	**C> T**	**TS**	**ACT (G) AAA**
139	**C> T**	**TS**	**ACT (G) AAA**
145	**C>T**	**NTS**	**AGA (C) TTG**
145	**C>T**	**NTS**	**AGA (C) TTG**
146	**T>C**	**NTS**	**GAC (T) TGC**
151	**C>T**	**NTS**	**GCT (C) GAG**
196	**T>C**	**NTS**	**CTC (T) GTG**
200	**T>C**	**ND**	**GTG (T) GCT**
205	**T>C**	**TS**	**CTC (A) AGG**
208	**C>T**	**TS**	**AAG (G) GGG**
208	*C>T*	*TS*	*AAG (G) GGG*
209	**C>T**	**TS**	**AGG (G) GGG**
209	**C>T**	**TS**	**AGG (G) GGG**
212	**C>T**	**TS**	**GGG (G) CTA**
212	**C>T**	**TS**	**GGG (G) CTA**
236	**T>C**	**NTS**	**TGC (T) GGA**
254	*T>C*	*NTS*	*CAC (T) GAA*
295	**T>C**	**NTS**	**GAT (T) TTA**
325	**C>T**	**NTS**	**GAT (C) AGT**

325	*C>T*	*NTS*	*GAT (C) AGT*
355	**C>T**	**TS**	**GGT (G) GAG**
358	**C>T**	**TS**	**GGA (G) ATG**
374	*T>C*	*NTS*	*CTT (T) AAC*
374	**T>C**	**NTS**	**CTT (T) AAC**
403	**C>T**	**TS**	**GAA (G) ATA**
403	**C>T**	**TS**	**GAA (G) ATA**
403	*C>T*	*TS*	*GAA (G) ATA*
418	**C>T**	**TS**	**ACT (G) GTA**
440	**T>C**	**NTS**	**TGC (T) TTC**
464	*C>T*	*NTS*	*GCC (C) CAA*
464	**C>T**	**NTS**	**GCC (C) CAA**
464	**C>T**	**NTS**	**GCC (C) CAA**
508	**C>T**	**NTS**	**TCT (C) GAA**
544	**C>T**	**TS**	**TTT (G) AAA**
544	**C>T**	**TS**	**TTT (G) AAA**
544	**C>T**	**TS**	**TTT (G) AAA**
544	*C>T*	*TS*	*TTT (G) AAA*
550	**C>T**	**NTS**	**ATT (C) CAG**
550	**C>T**	**NTS**	**ATT (C) CAG**
550	*C>T*	*NTS*	*ATT (C) CAG*
569	*C>T*	*TS*	*TTG (G) ATA*
569	**C>T**	**TS**	**TTG (G) ATA**
580	*C>T*	*TS*	*CTT (G) ACT*
589	**C>T**	**TS**	**AAT (G) AGT**
589	**C>T**	**TS**	**AAT (G) AGT**
595	*T>C*	*NTS*	*TAC (T) TCA*
601	*C>T*	*TS*	*AGG (G) ATT*
605	**T> C**	**NTS**	**ATT (T) GAA**
635	C>T	TS	CTG (G) AAA
635	**C>T**	**TS**	**CTG (G) AAA**
635	**C>T**	**TS**	**CTG (G) AAA**
655	*T>C*	*NTS*	*GCC (T) AAG*

Transversions

Position	Mutation	Strand	Target seq. (NTS)
16	**C>G**	**NTS**	**AGT (C) CCA**
67	**T>G**	**NTS**	**TTT (T) GTA**
74	**C>G**	**NTS**	**TAC (C) TAA**
85	*C>G*	*ND*	*TAT (G) CCG*
105	*C>A*	*ND*	*AGT (G) TTT*
109	*T>A*	*ND*	*TTT (A) TTC*
109	**T>A**	**ND**	**TTT (A) TTC**
109	**T>A**	**ND**	**TTT (A) TTC**
213	*C>A*	*NTS*	*GGG (C) TAT*
215	*T>G*	*ND*	*GCT (A) TAA*
215	**T>G**	**ND**	**GCT (A) TAA**
222	**C>G**	**NTS**	**GTT (C) TTT**
296	**T>G**	**NTS**	**ATT (T) TAT**
374	*T>A*	*NTS*	*CTT (T) AAC*
382	**T>A**	**TS**	**GGA (A) AGA**
409	*T>A*	*TS*	*ATA (A) TTG*
473	*T>G*	*NTS*	*TGG (T) TAA*
473	**T>G**	**NTS**	**TGG (T) TAA**
478	**C>A**	**TS**	**AAG (G) TTG**
589	*C>G*	*TS*	*AAT (G) AGT*
593	**T>A**	**ND**	**AGT (A) CTT**
617	**C>A**	**ND**	**TTT (G) TGT**

Tandem mutations

Position	Mutation	Strand	Target seq. (NTS)
108	**T>C**	**NTS**	**GTT (T) ATT**
109	**T>A**	**ND**	**TTT (A) TTC**
171	**C>T**	**TS**	**GAT (GG) GAG**
172	**C>T**	**TS**	**GAT (GG) GAG**
202	**C>T**	**NTS**	**GTG (CT) CAA**
203	**T>C**	**NTS**	**GTG (CT) CAA**
206	**T>A**	**TS**	**TCA (AG) GGG**
207	**C>T**	**TS**	**TCA (AG) GGG**
473	*T>G*	*NTS*	*TGG (TT) AAG*
474	*T>A*	*NTS*	*TGG (TT) AAG*
477	*C>A*	*TS*	*TAA (GG) TTG*
478	*C>T*	*TS*	*TAA (GG) TTG*
538	**C>T**	**TS**	**GTT (GG) ATT**
539	**C>T**	**TS**	**GTT (GG) ATT**
594	*C>T*	*NTS*	*GTA (CT) TCA*
595	*T>A*	*NTS*	*GTA (CT) TCA*

Multiple Independent Mutations

Position	Mutation	Strand	Target seq. (NTS)
107	*T>A*	*NTS*	*TGT (T) TAT*
109	*T>A*	*ND*	*TTT (A) TTC*

98-102	**+A**	**ND**	**AAAAA**
113	**C>T**	**NTS**	**TTC (C) TCA**
123	*C>T*	*TS*	*ACT (G) ATT*
125	*T>A*	*NTS*	*TGA (T) TAT*
187	*C>T*	*ND*	*ATT (G) TGG*
194	*T>A*	*NTS*	*CCC (T) CTG*
207	*C>T*	*TS*	*CAA (G) GGG*
214	*+C*	*ND*	*GGC (+C) TAT*

Deletions/Insertions

Position	Mutation	Strand	Target seq. (NTS)
207-212	*-G*	*TS*	*GGGGGG*
207-212	*-G*	*TS*	*GGGGGG*
208	*+T*	*TS*	*CAA (+T) GGG*
208	*+T*	*TS*	*CAA (+T) GGG*
403	*+AAG*	*TS*	*GAA (+AAG) GAT*
421-424	*-A*	*TS*	*GGT (AAAA) CAA*
450	**+T**	**ND**	**GTT (+T) AAG**
462-465	**-C**	**ND**	**AG (CCCC) AA**
540	*+A*	*TS*	*TGG (+A) ATT*
580	*+T*	*NTS*	*CTT (+T) GAC*

[a]ND: Mutation is a substitution not at a bipyrimidine

***Msh2*$^{-/-}$ ES cells 0 J/m² UVC**

Bold = *Msh2*$^{-/-}$ line N4 mutants

Italic = *Msh2*$^{-/-}$ line 9 mutants

Transitions

Position	Mutation[a]	Strand	Target seq. (NTS)[a]
149	*C>T*		*TTG (C) TCG*
212	**C>T**		**GGG (G) CTA**
254	**T>C**		**CAC (T) GAA**
254	*T>C*		*CAC (T) GAA*
437	*T>C*		*CTT (T) GCT*
479	*T>C*		*AGG (T) TGC*
533	*T>C*		*ACT (T) TGT*
574	**C>T**		**TAT (G) CCC**
574	*C>T*		*TAT (G) CCC*
614	*T>C*		*ACG (T) TTG*
614	**T>C**		**ACG (T) TTG**

Transversions

69	*T>A*	*TTG (T) ATA*
464	*C>A*	*GCC (C) CAA*
491	*T>G*	*TGC (T) GGT*
500	**C>A**	**AAA (G) GAC**
548	*T>A*	*AAA (T) TCC*

Deletions/Insertions

16-17	*+C*	*CCC (+C) AGC*
29-30	*+T*	*ATT (+T) AGC*
60-62	*+T*	*TTT (+T) GTT*
98-102	*+A*	*AAAAA*
207-212	**-G**	**GGGGGG**

207-212	*-G*	*GGGGGG*
207-212	*-G*	*GGGGGG*
208	*+T*	*CAA (+T) GGGGGG*
208	*+T*	*CAA (+T) GGGGGG*
333-337	**-G**	**GGGGG**
334	*+T*	*ACG (+T) GGGG*
462-465	**-C**	**AG(CCCC)AA**
462-465	**-C**	**AG(CCCC)AA**
466-469	*-A*	*AAA (-A) TGG*
496-499	*+A*	*AAAA*
496-499	*+A*	*AAAA*
496-499	**+A**	**AAAA**
496-499	**+A**	**AAAA**
614-617	*+T*	*TTT (+T) GTG*

[a] Although no UVC-induced DNA damage has been present, the mutated pyrimidine is shown by default

Chapter 3

The UVC-induced S/G2-phase delay in mouse ES cells is partially dependent on MutSα

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Abstract

Here we describe that in mouse ES cells, UVC treatment induced an S/G2-phase delay that was partially dependent on MutSα. The UVC-induced cell cycle delay coincided with the appearance of phosphorylated Chk1 of which the amount was significantly higher in MutSα-proficient cells compared to MutSα-deficient cells. Importantly, the observed differences in checkpoint activation between MutSα-proficient and MutSα-deficient ES cells did not account for the increased UVC-induced mutagenesis in MutSα-deficient cells, since abolition of the UVC-induced S/G2-phase delay by the phosho-Chk1 inhibitor UCN-01 did not reduce the difference in UVC-induced mutation frequencies between MutSα-proficient and MutSα-deficient mouse ES cells.

3.1 Introduction

Short wave UV (UVC) light is a known inducer of replication blocks due to its induction of helix distorting photolesions mainly consisting of *cis-syn* cyclobutane pyrimidine dimers (CPDs) and (6-4) pyrimidine-pyrimidone photoproducts [(6-4)PPs] [1]. We have shown that in the absence of the mismatch recognition dimer MutSα (consisting of Msh2 and Msh6) the number of mutations at pyrimidine dimer sites significantly increased upon UVC irradiation (see chapter 2). Interestingly, van Oosten *et al.* showed that in an $Xpc^{-/-}$ background Msh2 also plays a role in UVB-induced S-phase checkpoint activation [2]. Here we describe that in mouse ES cells, proficient for NER, UVC induces an S/G2-phase delay that is reduced in MutSα-deficient cells in comparison to wild type cells.

A central regulator of DNA damage checkpoints in mammalian cells is ATR (ATM and Rad3 related). ATR has been shown to regulate responses to a broad range of DNA damaging agents amongst which there is UV. ATR activation results in phosphorylation and activation of its downstream kinase Chk1 [3]. We observed that the UVC-induced S/G2-phase delay coincided with Chk1 phosphorylation and that the level of phosphorylation was clearly reduced in MutSα-deficient cells. We were able to abolish the S/G2-phase delay by (pre-)treating the cells with the phospho-Chk1 inhibitor 7-hydroxystaurosporine (UCN-01). Treatment of wild type and MutSα-deficient cells with UCN-01 did not reduce the difference in UVC-induced mutation frequencies between both genotypes. This indicates that the MMR-induced cell cycle delay does not contribute to the reduction of UVC-induced mutagenesis after UVC treatment. These results are in favour of a direct role for MMR proteins in the repair of UVC-induced lesions. We hypothesize that MutSα-dependent ATR-mediated signalling after UVC treatment is induced by the presence of single-stranded MMR intermediates.

3.2 Materials and Methods

Cell lines and cell culture

129/OLA-derived primary diploid mouse embryonic stem (ES) cell line E14 (wild type), *Msh2*$^{-/-}$ line 9 (previously called dMsh2-9) and *Msh6*$^{-/-}$ cells are described elsewhere [4,5]. *Xpa*$^{-/-}$ *Msh6*$^{-/-}$ ES were obtained by targeting of *Xpa*$^{-/-}$ mouse ES cells with an *Msh6* targeting construct and subsequent selection of *Msh6*$^{-/-}$ cells by treatment of the cells with 40 μM 6TG during two hours, as described [6]. ES cells were cultured on irradiated mouse embryonic fibroblast (MEF) feeder cells using established procedures. Knockout DMEM (Life technologies) was supplemented with 10% fetal calf serum, Glutamax, non-essential amino acids, penicillin, streptomycin, 0.1 mM β-mercaptoethanol and leukemia inhibitory factor (LIF); this medium is referred to as ES complete medium.

Mutagen treatments and chemicals

Twenty hours before chemical treatment and/or UVC-irradiation, ES cells were seeded at a density of 1.5 x 10^6 (control) or 3.0 x 10^6 (cells to be treated) cells per gelatin-coated T90 culture dish and incubated in ES complete medium supplemented with 50% Buffalo rat liver (BRL) cell-conditioned medium (BRL complete medium). For UVC irradiation in the presence of UCN-01 (provided in powder form by the Drug Synthesis and Chemistry Branch, National Cancer Institute [NCI], USA; reconstituted in DMSO to a final concentration of 2.1 mM and stored at -80°C), cells were incubated with 300nM or 600nM UCN-01 for 50 minutes prior to treatment. Subsequently, UCN-01 medium was collected, cells were washed with PBS, irradiated with 0, 0.5 or 1 J/m^2 at a dose rate of 0.08 J/m^2/s UVC light [Philips TUV lamp, 254 nm] and incubated in recycled UCN-01 medium.

Flow cytometric cell cycle analysis

For the determination of cell cycle profiles cells were trypsinized, washed twice with PBS and fixed in 70% ethanol. Analysis of BrdU incorporation and DNA content using flow cytometry was performed as described previously [7]. Data analysis was performed as described before [8] using WinMDI 2.8 (free software).

Western blotting

Cell lysates were made at the indicated time points after UVC treatment by washing the cells three times with PBS and lysing the cells in 2x protein sample buffer (Maniatis). Proteins were separated on 10% SDS-PAGE gels and transferred to Hybond C+ membranes. Membranes were blocked for two hours in PBS containing 0.1% Tween, 0.1% BSA and 5% dried milk. Subsequently, membranes were incubated with antibodies against phospho-Chk1-Ser317 (Bethyl laboratories), actine (Oncogene) or Msh6 (made in-house) diluted in PBS containing 0.1% Tween, 5% dried milk and 0.5% BSA. After washing, blots were incubated with HRP-conjugated secondary antibodies, washed and developed.

Determination of UVC- induced mutation frequencies at Hprt in the presence of UCN-01

After UVC irradiation and incubation in UCN-01-containing medium for 12 hours, cells were propagated for 6 days on feeder cells in ES complete medium. Subsequently, $4x10^5$ ES cells were seeded per gelatin-coated T90 dish in BRL complete medium containing 30 μM 6TG (five dishes per dose). In parallel, cloning efficiencies were determined by seeding 250 cells per T60 dish (three dishes per dose) in BRL complete medium without 6TG. After 9 days, clones were washed with NaCl (0.9% w/v), fixed, stained using methylene blue solution (0.15% in methanol) and counted. Mutation frequencies at *Hprt* were determined by correcting the number of 6TG-resistant colonies in the mock-treated and UVC-treated dishes for the cloning efficiencies. UVC-(with or without UCN-01) induced mutation frequencies at *Hprt* were obtained by subtracting the spontaneous mutation frequencies or the mutation frequencies found after treatment with UCN-01 alone from the mutation frequencies found after UVC treatment (with or

without UCN-01) for each ES cell line. Mutation experiments have been performed three times per cell line.

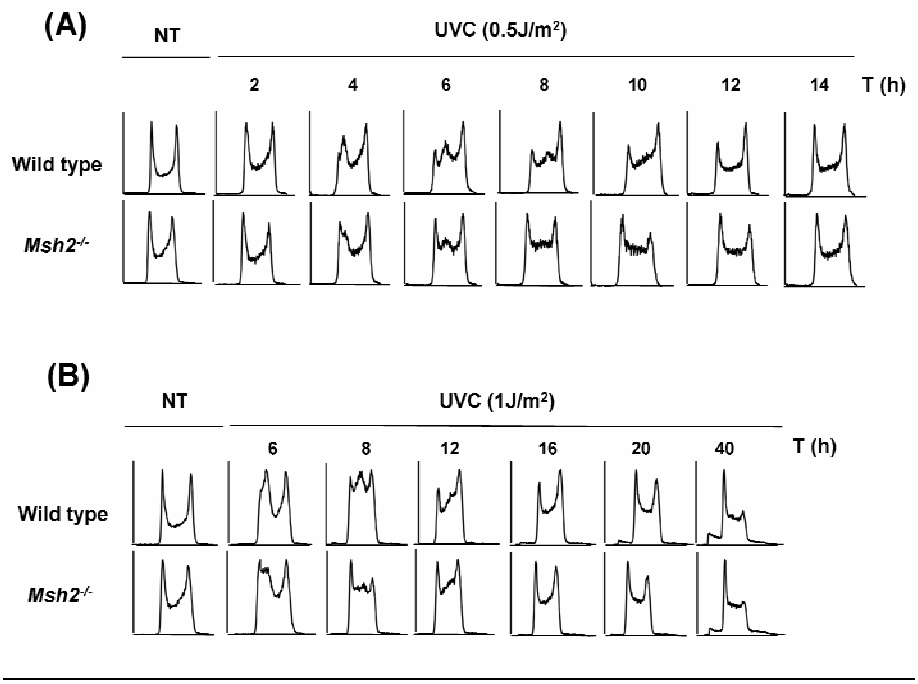
Determination of treatment induced apoptosis

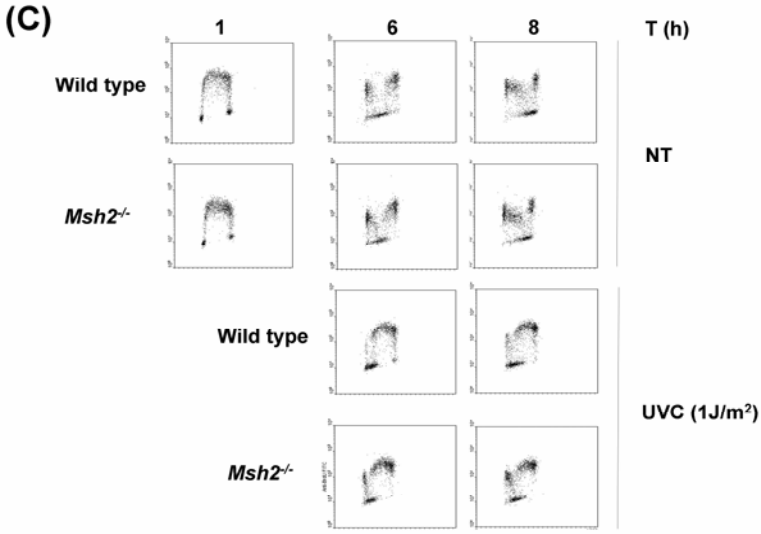
12 and 38 Hours after UCN-01/UVC treatments, cells (including the floating cells from the supernatant) were collected and incubated for 1 hour at 37°C with the Apofluor® Green reagent (ICN Biomedicals, Inc.) that irreversibly binds to activated caspases. After the incubation period, cells were fixed and fluorescein levels were measured by flow cytometric (FACS) analysis.

3.3 Results

MutSα-deficient cells show a reduced S/G2-phase delay after UVC treatment

To investigate whether Msh2 is involved in the UVC-induced inhibition of cell cycle progression in NER-proficient mouse ES cells, we compared cell cycle profiles of wild type and *Msh2*-deficient ES cells after UVC treatment. As can be seen in Fig. 1A, UVC (0.5 J/m^2) caused a delay in cell cycle progression in both genotypes. However, wild type cells were more delayed in S/G2-phase than *Msh2*$^{-/-}$ cells, a finding also reflected by the reduced G1-phase peak in wild type cells compared to *Msh2*$^{-/-}$ cells at 4 to 12 hours after UVC treatment (Fig. 1A). A dose of 1 J/m^2 induced an increased cell cycle delay in comparison to 0.5 J/m^2 in both genotypes, but also at this dose the late S/G2-phase delay was more pronounced in wild type cells than in *Msh2*$^{-/-}$ cells (Fig. 1B). The difference in S/G2-phase progression between wild type and *Msh2*$^{-/-}$ cells after UVC treatment became quantifiable when we pulse-labeled the cells with BrdU immediately after UVC (1 J/m^2) irradiation and quantified the BrdU-positive G1-phase populations at 6 and 8 hours after UVC treatment (Fig. 1C and 1D). At six hours after UVC irradiation 4% of BrdU positive cells were in G1-phase in the wild type population whereas 20% of the BrdU positive *Msh2*$^{-/-}$ cells were already in G1-phase at this time point. At 8 hours post UVC treatment the difference between wild type and *Msh2*$^{-/-}$ cells had become smaller, due to the fact that also the wild type cells were clearly entering G1-phase at this time point. Thus, UVC treatment results in an S/G2-phase delay in both wild-type and *Msh2*$^{-/-}$ cells, but in the absence of MMR this delay is reduced, illustrated by the appearance of a clear G1-phase population at an earlier time point after UVC treatment in *Msh2*$^{-/-}$ cells. Cell cycle analysis in *Msh6*$^{-/-}$ cells showed that these cells behave in a similar way as *Msh2*$^{-/-}$ cells after UVC irradiation (data not shown), indicating that the mismatch recognition heterodimer MutSα (consisting of Msh2 and Msh6) is partially responsible for the induction of an S/G2-phase delay after UVC irradiation.


(A)
NT
UVC (0.5J/m²)
2
4
6
8
10
12
14
T (h)
Wild type
Msh2-/-
(B)
NT
UVC (1J/m²)
6
8
12
16
20
40
T (h)
Wild type
Msh2-/-


(C)
1
6
8
T (h)
Wild type
Msh2-/-
NT
Wild type
Msh2-/-
UVC (1J/m²)

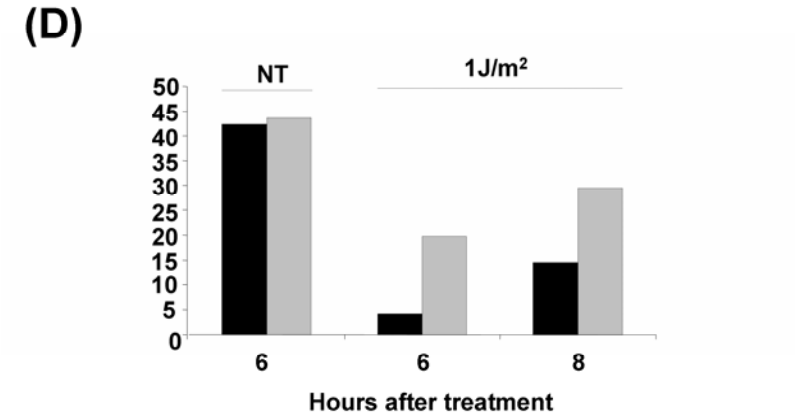


Figure 1. Cell cycle profiles of wild type and *Msh2*$^{-/-}$ cells after mock (NT) or UVC treatment (0.5 or 1 J/m²). Wild type and *Msh2*$^{-/-}$ cells were irradiated with 0.5 J/m² (A) or 1 J/m² (B) and stained with propidium iodide at the indicated time points after UVC irradiation. C) FACS analysis of wild type and *Msh2*$^{-/-}$ cells that were pulse-labeled with BrdU directly after UVC treatment and incubated for the indicated time intervals. D) Quantification of BrdU-positive G1-phase cells present at 6 and 8 hours after UVC (1J/m²) treatment. Black bars: wild type; grey bars: *Msh2*$^{-/-}$.

UVC induces MutSα-dependent Chk1 phosphorylation

UVC-induced checkpoint activation is a result of ATR signalling. ATR transduces the DNA damage-induced signal by phosphorylation of Chk1 [3]. To know whether the decreased S/G2-phase delay in MutSα-deficient cells after UVC treatment coincided with reduced checkpoint signalling, we analysed phosphorylated Chk1 levels at different time points after UVC irradiation. As can be seen in figure 2A, Chk1 phosphorylation could already be observed at 0.5 hour after UVC treatment. Interestingly, in a nucleotide excision repair (NER) deficient background, MutSα deficiency resulted in significantly lower levels of phosphorylated Chk1 compared to wild type cells. Similar observations were made in NER proficient *Msh2*$^{-/-}$ cells (data not shown). These results show that UVC treatment induces rapid Chk1 activation (as measured by its phosphorylation) and that the activation of Chk1 is partially dependent on the presence of MutSα.

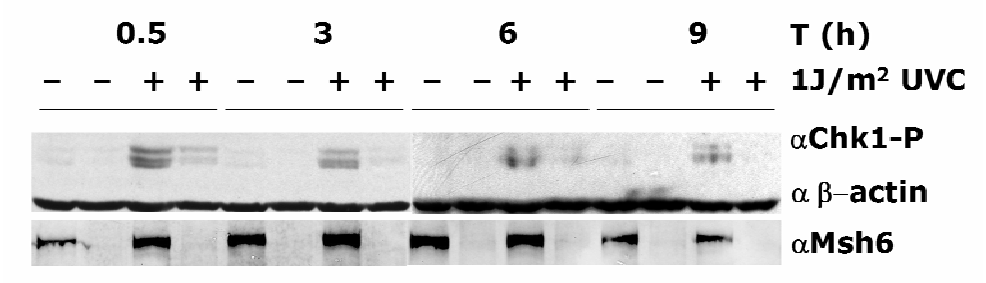


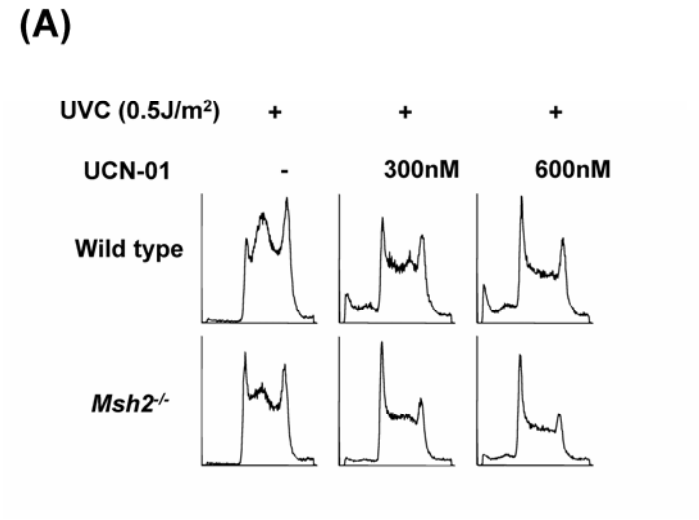
Figure 2. Western blot analysis of wild type and *Xpa*$^{-/-}$ *Msh6*$^{-/-}$ cells treated with UVC (1J/m²). Lysates were made at the indicated timepoints after UVC treatment and Western blot analysis was performed using the indicated antibodies.

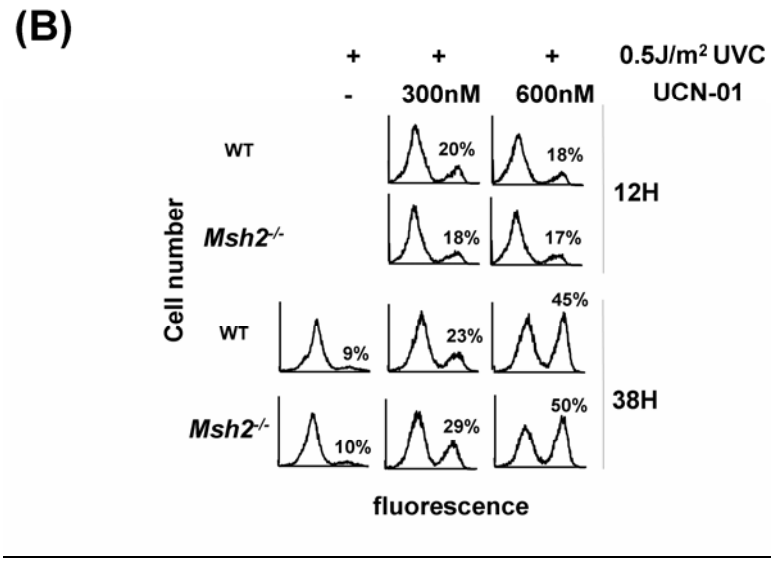
Difference in UVC mutagenicity between MutSα-proficient and -deficient cells is not reduced by abolition of the Chk1-dependent S/G-phase delay

We have shown that the absence of MutSα results in an approximately six-fold higher UVC-induced mutation frequency in MutSα-deficient cells compared to wild type cells (see chapter 2). We hypothesized that this difference in induced mutation frequency by UVC could be related to the fact that wild type cells have extra time to repair the UVC-induced DNA damage before (mutagenic) replication due to the induction of a MutSα-dependent late S/G2-phase delay. To test this hypothesis, we treated wild type and *Msh2*$^{-/-}$ ES cells with the phospho-Chk1 inhibitor 7-hydroxystaurosporine (UCN-01) in order to abolish the UVC-induced S/G2-phase delay and subsequently determined the UVC-induced mutation frequencies in both lines. As can be seen in figure 3A, UCN-01 treatment resulted in a dose-dependent reduction of the UVC-induced S/G2-phase delay. In some experiments the sub-G1 peak was stronger in wild type cells than in *Msh2*$^{-/-}$ cells, indicative of a difference in apoptosis between both genotypes. However, a higher sub-G1 peak was not consistently observed in wild type cells. Moreover, quantification of apoptosis by determining activated caspase levels did reproducibly not show a difference between both genotypes (Fig. 3B).

Interestingly, when we measured the UVC-induced mutation frequencies in both lines in the presence of 600nM UCN-01, no reduction of the difference in mutagenicity between both lines was found (Fig. 3C). From these results we

concluded that the UVC-induced, MutSα-dependent S/G2-phase delay -and possibly associated enhanced repair events- is not the major cause for the decreased UVC-induced mutagenesis in wild type cells compared to MutSα-deficient cells.





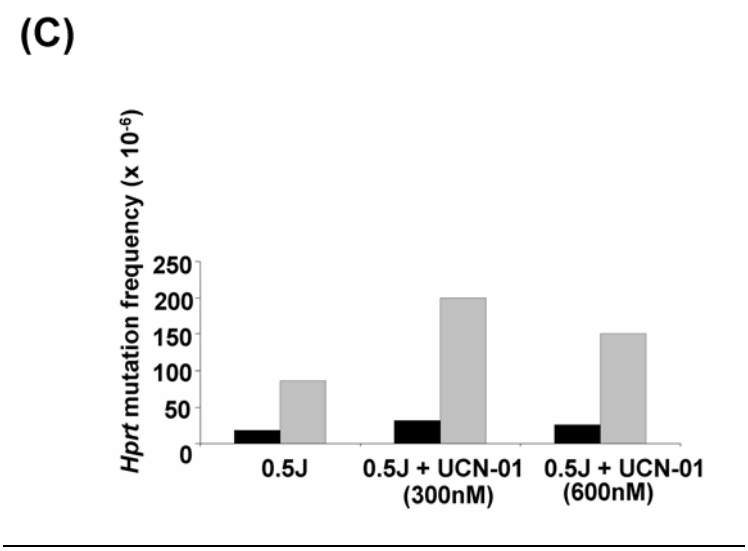


Figure 3. Cellular response of wild type and *Msh2*$^{-/-}$ cells after UVC treatment in the presence of UCN-01. A) FACS analysis of wild type and *Msh2*$^{-/-}$ cells stained with propidium iodide 6 hrs after UVC/UCN-01 treatment. B) Quantification of apoptosis in wild type and *Msh2*$^{-/-}$ cells at the indicated time points after UVC/UCN-01 treatments. C) UVC-induced *Hprt* mutation frequencies in wild type and *Msh2*$^{-/-}$ cells in the presence of UCN-01. Black bars: wild type; grey bars: *Msh2*$^{-/-}$.

3.4 Discussion

UV light, although beneficial to us with regard to some aspects (e.g. the production of vitamin D in our skin), can be very noxious to us. The deleterious effect of UV light is caused by the fact that UV is absorbed by cellular DNA resulting in the induction of different photolesions. The majority of these UV-induced DNA lesions consists of CPD and (6-4)PPs [1]. If not properly repaired, the UV-induced DNA photoproducts can result in mutations in cellular DNA after error-prone replication of the damaged DNA template. If this occurs in growth-controlling genes this can result in malignant transformation of the cell, a process reflected by the carcinogenic properties of UV light [9].

The cell is equipped with various defense mechanisms against the deleterious effects of UV [10-12]. Firstly, the versatile nucleotide excision repair (NER) system is able to efficiently repair UV-induced photolesions (with the exception of CPDs in the non-transcribed strand in rodent cells as has been discussed in chapter 2). In case of a high damage load, a second defence mechanism comes into play, *i.e.* the induction of a cell cycle arrest. A transient arrest of the cell cycle allows the cell to repair the induced DNA damage, after which cell cycle progression can be resumed [12]. Finally, cells that are too heavily damaged can be removed by the induction of apoptosis.

In addition to the conservation of genomic integrity, the removal of the UV-induced photolesions is of vital importance for the continuation of RNA transcription. UV-induced DNA photolesions block the progression of RNA polymerase, thereby inhibiting transcription. To cope with this, eukaryotes are equipped with a specialised NER pathway that specifically removes lesions from the transcribed strand [11]. Mouse *Xpa*$^{-/-}$ cells that have a defect in this transcription coupled repair (TCR) system (in addition to their defect in global genome repair [GGR]) fail to enter S-phase upon UV irradiation in G1-phase and undergo apoptosis [13]. *Xpc*$^{-/-}$ cells that have a functional TCR system but are dysfunctional in GGR, are able to enter S-phase after UVB irradiation but they arrest in late S-phase [13]. This is likely due to the presence of unrepaired lesions in these cells. Van Oosten *et al.* found that the late S-phase arrest observed in *Xpc*$^{-/-}$ mouse

keratinocytes could be partially rescued by an additional defect in MMR: *Xpc* $^{-/-}$ *Msh2*$^{-/-}$ cells showed 40% (*in vivo*) and 30% (*in vitro*) reduced late S-phase arrest when compared to *Xpc*$^{-/-}$ cells [2].

In order to get more insight into the mechanism of this UV-induced Msh2-dependent late S-phase arrest we studied UVC-induced cell signalling in cells proficient for NER. Using wild type and MutSα-deficient mouse ES cells, we found that UVC induces a late S/G2-phase delay in mouse ES cells proficient for NER and this delay was shown to be markedly reduced in the absence of the mismatch recognition dimer MutSα. In our experiments we could not make a distinction between S-phase cells or G2-phase cells, thus it remains to be determined whether the observed MutSα-dependent delay involved only the S-phase or also the G2- phase.

To investigate the signalling pathway involved in the induction of the MutSα-dependent cell cycle delay, we studied Chk1 activation. Chk1 is part of the ATR signalling pathway [3]. We focussed on the ATR pathway since ATR has been found to be the major player in cell signalling caused by replication blocks [3]. We have shown here that the UVC-induced cell cycle delay coincided with Chk1 phosphorylation, in support of findings by Heffernan *et al.* [14]. Interestingly, the level of Chk1 phosphorylation was significantly higher in MutSα-proficient cells compared to MutSα-deficient cells. This indicates that UVC induces checkpoint signalling that is partially dependent on MutSα.

MutSα has been shown to bind to mismatched photoproducts [15,16]. We hypothesized that the MutSα-dependent S/G2-phase delay and coinciding Chk1 phosphorylation is induced by signalling of MutSα to the cell cycle machinery upon binding of MutSα to a mismatched photoproduct. The thus induced MutSα-dependent S/G2-phase delay would serve to give the NER system extra time to repair UVC-induced photolesions. CPDs are induced by UV with higher frequencies than 6-4PP and are a poor substrate for GGR [17-20], especially in rodent cells that lack the UV-damaged DNA-binding protein (UV-DDB) -a protein complex important for GGR of CPDs [21]. For these reasons CPDs underlie most UV-induced mutagenesis in mouse cells. We infer that the moderately extended

S-phase delay in wild type cells at best moderately affects the repair of 6-4PPs, that are only poorly mutagenic [19] and, uniquely in the transcribed strand, affects the repair of the highly mutagenic CPDs. However, we did not observe a difference in the ratio of mutations induced in the non-transcribed strand versus the transcribed strand between wild type and MutSα-deficient cells. In addition we found that abolishment of the UV-induced S-phase delays in wild type and MMR-deficient cells did not result in diminution of the difference in UV-induced mutation frequencies. For these reasons we infer that the extended S-phase delay is likely not the cause of the higher UVC-induced mutation frequency in MutSα-deficient cells (see chapter 2 for a more extended discussion on this matter).

Our results are in favour of a direct role rather than an indirect role for MMR in the removal of mismatches in UVC-induced photolesions. We hypothesize that the lower UVC-induced mutagenesis observed in MutSα-proficient cells is a consequence of MutSα-mediated repair of UVC-induced compound lesions that arose due to error-prone replication of DNA containing photolesions. In this case, the increased S/G2-phase delay together with the increased level of phosphorylated Chk1 that we observed in wild type cells could be explained by the presence of single stranded DNA repair intermediates, since these intermediates have been found to be bound by the regulatory protein of ATR, ATR interacting protein (ATRIP), thereby activating the ATR kinase [22,23]. Activation of the ATR kinase would in turn result in CHK1 activation. In chapter 5 this model will be discussed in more detail.

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Chapter 4

Spontaneous and mutagen-induced loss of DNA mismatch repair in *Msh2*-heterozygous mammalian cells

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Abstract

We have developed a simple procedure that enables the efficient selection of cells that are deficient for DNA mismatch repair (MMR). This selection procedure was used to investigate the frequency of fortuitous MMR-deficient cells in a mouse embryonic stem cell line, heterozygous for the MMR gene *Msh2*. We found a surprisingly high frequency ($3x10^{-4}$) of *Msh2*-deficient cells. The wild type *Msh2* allele was almost invariably lost by loss of heterozygosity. Single treatments with the genotoxic agents ethylnitrosourea, UVC radiation and mitomycin C resulted in a further increase of the number of $Msh2^{-/-}$ cells in the heterozygous cell line. This increase was not only due to induced loss of the wild type allele but also to a selective growth advantage of preexisting $Msh2^{-/-}$ cells to ethylnitrosourea and UVC. Mitomycin C, in contrast to ENU and UVC, uniquely induced loss of heterozygosity at *Msh2*. These mechanistically different ways of loss of the wild type *Msh2* allele reflect the different repair pathways processing these different types of damage. Heterozygous germ line defects in one of the MMR genes underlie the hereditary non-polyposis colorectal cancer (HNPCC) syndrome. Based on the results described here we hypothesize that mutagen-induced loss of MMR in the intestine of these patients contributes to the tissue specificity of carcinogenesis in HNPCC patients.

4.1 Introduction

Loss of replicational fidelity results in the rapid accumulation of genomic mutations in replicating cells, a condition that underlies genetic disease, including cancer. DNA mismatch repair (MMR) is a pathway, conserved in prokaryotic and eukaryotic lineages, that recognizes and removes misincorporations by the replicative polymerases [1,2]. MMR is initiated by binding of a mismatched nucleotide(s) by one of two heterodimeric proteins. In eukaryotic cells, the heterodimer of Msh2 and Msh6 proteins that is also called MutSα, is able to bind to base-base mismatches and small insertion-deletion loops (IDLs), resulting from slippage of the replicative polymerase while replicating simple sequence repeats. A minor and partially redundant mismatch binding heterodimer (MutSβ) consists of the Msh2 and Msh3 proteins and binds to larger IDLs. Binding of either MutSα or MutSβ to misincorporations results in recruitment of MutLα, a heterodimeric protein consisting of the Mlh1 and Pms2 proteins, or of one of two minor heterodimers that consist of the Mlh1 and either Pms1 or Mlh3 proteins. These ternary complexes trigger a canonical process that results in excision of a stretch of the newly synthesized DNA strand that encompasses the misincorporation, followed by resynthesis of the excised single-stranded DNA fragment by polymerase ∂. Loss of MMR results in a strong spontaneous mutator phenotype, a consequence of the persistence of replication errors. An inherited heterozygous defect in *Msh2*, *Msh6*, *Mlh1* or *Pms2* underlies the common cancer predisposition hereditary nonpolyposis colorectal cancer (HNPCC) syndrome [3]. In HNPCC patients, loss of the wild type allele of the germ line-mutated MMR gene results in the rapid accumulation of mutations in genes involved in regulating cellular proliferation which underlies the accelerated development of (mainly) colorectal cancer. In addition to its indispensable role in removing misincorporations, MMR is involved in determining cellular sensitivity to a number of DNA damaging agents, including S_N1-methylating agents, the nucleoside analog 6-thioguanine (6TG) and cisplatin [4].

Here we have exploited the acquired tolerance of MMR-deficient cells to 6TG to investigate the frequency and mechanism of spontaneous- and mutagen-induced loss of the wild type MMR gene allele in cultured mouse embryonic stem

(ES) cells, heterozygous for *Msh2*. We found a high frequency of spontaneous loss of the wild type *Msh2* allele, likely by a chromosome loss-duplication event. Loss of the wild type *Msh2* allele could substantially be increased by a single treatment with DNA damaging agents of various classes. Based on these results we hypothesize that exposure to DNA damaging agents in the intestine, via induction of loss of MMR, may be an important etiological factor in the development of colorectal cancer in HNPCC patients.

4.2 Materials and methods

Cell lines and culture, selection for MMR-deficient cells

129/OLA-derived primary diploid mouse embryonic stem ES cell lines E14 (wild type), sMsh2-42 (*Msh2*$^{+/-}$) and dMsh2-9 (*Msh2*$^{-/-}$) are described elsewhere [5]. ES cells were cultured on irradiated feeder cells using established procedures. Knockout DMEM (Life technologies) was supplemented with 10% fetal calf serum, Glutamax, non-essential amino acids, penicillin and streptomycin, 0.1 mM β-mercaptoethanol, and leukemia inhibitory factor (LIF). For selection of *Msh2*-deficient cells, 2x10^6 ES cells were seeded per gelatin-coated T90 dish in medium supplemented with 50% Buffalo rat Liver (BRL) cell-conditioned medium. Next day, medium was replaced by the same medium containing 40 μM 6TG for two hrs, followed by washing with phosphate buffered saline (PBS) after which fresh medium (without 6TG) was added. This procedure was repeated at day seven. Surviving colonies were counted and picked between days twelve and fourteen. To control for the presence of *Hprt* mutants, that also may be selected by the brief 6TG treatment, we plated 2x10^6 *Msh2*$^{+/-}$ ES cells per P90 dish in medium containing 30 μM 6TG for 12 days. Using these selective conditions, only *Hprt* mutants are selected whereas *Msh2*$^{-/-}$ and wild type ES cells are killed. All experiments were performed at least three times.

Treatment with mutagens and selection for loss of MMR

ES cells were seeded at a density of 0.5x10^6 cells per 10 cm^2 well on irradiated feeder cells. Next day, cells were treated with genotoxic agents as follows: ethylnitrosourea (ENU 0.2 mg/ml) or mitomycin C (MMC, 0.3 μM) was added to the medium in the indicated concentrations. After one (ENU) or two (MMC) hrs of incubation, cells were washed with PBS and fresh medium was added. Prior to treatment with short-wave UVC light [Philips TUV lamp, 254 nm] with a total dose of 4 J/m^2, at a dose rate of 0.2 J/m^2/s, cells were washed with PBS. Immediately after treatment, medium was added to the cells. The dose of each mutagen used was based on an approximate survival of 30%. Two and six days after mutagen treatment cells were trypsinized, counted and reseeded. 6TG

selection for *Msh2*-deficiency was initiated at least seven days after mutagen treatment. To enable to control for differential sensitivity of preexisting *Msh2*$^{-/-}$ cells to the mutagens, resulting in an over- or underestimation of induced MMR loss, as well as to control for the efficiency of 6TG selection, we performed parallel control experiments. In these experiments $2x10^3$ *Msh2*$^{-/-}$ cells were mixed with $2x10^6$ wild type cells before mutagen treatment. In addition we controlled for the induction of *Hprt* mutants by mutagen treatment, as described above. All experiments were performed at least three times.

Genotyping, Western blotting and hypoxanthine aminopterin thymidine (HAT) selection.

Loss of heterozygosity (LOH) at *Msh2* was assessed by a multiplex PCR. To this purpose, selected colonies were lysed for 1 hr at 60°C in 20 μl lysis buffer (300 μg/ml proteinase K in 10 mM TRIS/HCl, pH 8.0, 50 mM KCl, 0.045% (v/v) Tween-20 and 0.045% (v/v) Nonidet P-40), followed by 10 min incubation at 95°C to inactivate the enzyme. This DNA was used in a multiplex PCR reaction containing primers P1 (CGGCCTTGAGCTAAGTCTATTATAAGG), P2B (GTGGGGGTGGGATTAGATAAATGC), and P3 (CCAAGATGACTGGTCGTACATAAG) using Hot Goldstar TAQ polymerase (Eurogentec Belgium). Primers P1 and P2B, diagnostic for the mutated allele, amplify a 205 base pair fragment encompassing the poly(A) signal from the inserted *hyg* marker and flanking *Msh2* genomic sequences. Primers P1 and P3 amplify a 164 base pair fragment from the wild type allele only (modified from [6]). PCR fragments were separated on 3% agarose gels. For Western blotting, cells were washed with PBS and lysed in protein sample buffer. Proteins were separated on 10% denaturing polyacrylamide gels and blotted to nitrocellulose membrane. Hybridizations with anti-Msh2 [7] and detection by enhanced chemoluminescence were performed according to established procedures. HAT medium which is toxic to *Hprt* mutants, but not to *Msh2* mutants, was prepared by adding 100 μM hypoxanthine, 1μM aminopterin and 20 μM thymidine to medium containing 50% BRL-conditioned ES cell medium.

4.3 Results

High frequency of Msh2$^{-/-}$ cells in an Msh2$^{+/-}$cell line

The MMR-mediated toxicity of S_N1-methylating agents, provides a paradigm for the role of MMR in eliciting cellular responses to damaged DNA [4,8]. The thiopurine 6-thioguanine (6TG), incorporated into DNA as 6-deoxythioguanine, provokes a similar MMR-dependent toxicity as S_N1-methylating agents. This is caused by its efficient methylation to 6-methylthioguanine by endogenous methylating agents. Replication of 6-methylthioguanine results in mispairing with thymine and processing by MMR, resulting in cell killing as described above. In keeping with this, MMR-deficient ES cells have acquired a 20 to 100-fold tolerance to S_N1-methylating agents [5], and 6TG [9]. Based on this we have developed a procedure that enables selection of MMR-deficient ES cells from an MMR-proficient population. Thus, whereas dual brief treatments of $2x10^6$ wild type ES cells with 6TG did not result in the emergence of clones (not shown), *Msh2*$^{-/-}$ cells, mixed with $2x10^6$ wild type ES cells, were selected after dual brief selection with 6TG with an efficiency of, on the average, 36% (Table 1). The genotype of these clones was verified using multiplex PCR and in all cases was found to be *Msh2*$^{-/-}$ (not shown). This experiment validates the quantitative selection of *Msh2*$^{-/-}$ cells from a population of MMR-proficient cells.

When $2x10^6$ *Msh2*$^{+/-}$ cells were subjected to dual brief selection with 6TG, surviving colonies were recovered at high frequency (Table 1). Based on the 36% efficiency of recovery for this procedure we estimate that the frequency of fortuitously *Msh2*-deficient cells in an *Msh2*-heterozygous cell line is 3.1×10^{-4}. Multiplex PCR on 68 of these clones revealed that nearly all clones had lost the wild type *Msh2* allele (Figure 1). To control for the presence of clones that have survived the brief 6TG selection as a consequence of a mutation of the *Hprt* gene, rather than from loss of MMR, we subjected all cultures also to continuous 6TG selection. This resulted in the selection of *Hprt*-deficient clones at only a very low frequency (Table 1). This confirms that all clones, selected by the dual brief 6TG treatment, represent *Msh2*$^{-/-}$ cells.

Table 1. Selection of fortuitously *Msh2*-deficient clones from an *Msh2*$^{+/-}$ cell line

Experiment	Frequency of 6TG-tolerant clones[a]	*Hprt* Mutation Frequency[b]	Selection Efficiency (%)[c]	Frequency of fortuitous *Msh2* deficiency
1	110 x 10^{-6}	<0.5 x 10^{-6}	38	280 x 10^{-6}
2	110 x 10^{-6}	0.5 x 10^{-6}	48	240 x 10^{-6}
3	100 x 10^{-6}	<0.5 x 10^{-6}	39	260 x 10^{-6}
4	130 x 10^{-6}	0.5 x 10^{-6}	29	450 x 10^{-6}
5	79 x 10^{-6}	<0.5 x 10^{-6}	33	240 x 10^{-6}
6	55 x 10^{-6}	1.5 x 10^{-6}	41	130 x 10^{-6}
7	120 x 10^{-6}	<0.5 x 10^{-6}	26	480 x 10^{-6}
8	140 x 10^{-6}	<0.5 x 10^{-6}	37	380 x 10^{-6}
Mean	110 x 10^{-6} ± 30 x 10^{-6}	3 x 10^{-6} ± 0.5 x 10^{-6}	36.4 ± 7.0	310 x 10^{-6} ± 120 x 10^{-6}

[a] Obtained by dual brief 6TG selection of Msh2$^{+/-}$ cells.

[b] Obtained after continuous selection with 6TG.

[c] Selection efficiency for Msh2-deficient cells was determined by mixing 2x10^3 Msh2$^{-/-}$ cells with 2x10^6 wild type cells followed by dual brief 6TG selection.

Induction of MMR loss by genotoxic agents

We wanted to investigate whether exposure to genotoxic agents induces loss of MMR in heterozygous cells. To this aim we have tested three model genotoxic treatments: (1) mitomycin C (MMC), a chemotherapeutic drug used in the treatment of bladder cancer [10]. MMC induces intra- and interstrand crosslinks, the latter are highly toxic and can be repaired by homology-dependent recombination [11]; (2) ethylnitrosourea (ENU), a drug that induces ethyl adducts at purine bases (mainly O^6-ethylguanine, N^3-methyladenine and N^7-ethylguanine, substrates for base- and nucleotide excision repair [12]); (3) UVC light, specifically inducing TT pyrimidine (6-4) pyrimidone photoproducts [(6–4)PPs] or TT *cis-cyn*

cyclobutane pyrimidine dimer (CPD) photoproducts between adjacent pyrimidines. Both photoproducts confer helical distortion to the DNA and can be removed by nucleotide excision repair [13]. The dose of the genotoxic agents used was based on an average survival of each cell line of approximately 30% (not shown) *Msh2*$^{+/-}$ ES cells were treated once with each agent, followed by at least one week of culture to allow expression of MMR deficiency. Subsequently, 2x10^6 cells were seeded and selected for selection for tolerance to dual brief 6TG treatments. Tolerant clones were counted and isolated. We corrected for the presence of *Hprt* mutants by using prolonged 6TG treatment (Table 2, see also above). In all genotoxin-treated *Msh2*$^{+/-}$ cultures we found significant enhancement of the number of colonies that were tolerant to the dual brief 6TG selection. *Hprt*-mutant clones were almost not found after long-term 6TG selection (Table 2). Thus, a single exposure to moderately toxic does of genotoxic agents from different classes results in the emergence of a large number of MMR-deficient ES cells in an *Msh2*-heterozygous culture. Treatment with higher mutagen doses resulted in a dose-dependent increase in the number of 6TG-tolerant clones (not shown).

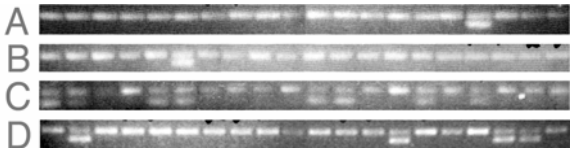

Figure 1. Multiplex PCR on 6TG-tolerant clones selected from an *Msh2*$^{+/-}$ cell line. The upper band represents the *Msh2* allele, disrupted by gene targeting, the lower allele represents the untargeted wild type allele. A: Fortuitous 6TG-tolerant clones display generalized LOH at *Msh2*. B: 6TG-tolerant clones selected after treatment of the *Msh2*$^{+/-}$ cell line with MMC almost uniquely display LOH at Msh2. C: 6TG-tolerant clones selected after treatment of the *Msh2*$^{+/-}$ cell line with ENU, frequently displaying conservation of heterozygosity. D: 6TG-tolerant clones selected after treatment of the *Msh2*$^{+/-}$ cell line with UVC, frequently displaying conservation of heterozygosity.

We investigated whether this increased number of 6TG-tolerant clones after mutagen treatment might be a consequence of differential survival of the preexisting *Msh2*$^{-/-}$ cells in the *Msh2*$^{+/-}$ ES cell line to the mutagen. This was done by mixing 2x10^3 *Msh2*$^{-/-}$ cells with 2x10^6 *Msh2*$^{+/+}$ cells. These control cultures were mock treated or treated with all three mutagens followed by selection for 6TG tolerance in an identical fashion as the *Msh2*$^{+/-}$ cell line. As shown in Table 2, *Msh2*$^{-/-}$ cells indeed displayed a slight survival advantage after treatment with ENU and UVC whereas *Msh2*$^{-/-}$ cells were marginally sensitive to the crosslinker

MMC. These minor differences in mutagen sensitivity were confirmed by performing survival assays using the three cell lines used in this study (not shown). We corrected the number of *Msh2*$^{-/-}$ cells that were selected from the mutagen-treated *Msh2*$^{+/-}$ cell line for the growth advantage or disadvantage of the preexisting *Msh2*$^{-/-}$ cells to the mutagens. This allowed us to define the fraction of cells in which loss of MMR was induced in the heterozygous cell line by the mutagen (Table 2).

Table 2. Mutagen-induced loss of Msh2

Agent	# of 6TG-tolerant clones[a]	# of *Hprt* mutants[b]	# of *Msh2* mutants[c]	# of preexisting *Msh2*$^{-/-}$ cells[d]	Relative survival of *Msh2*$^{-/-}$ cells[e]	Selection efficiency (%)[f]	Frequency of preexisting *Msh2*$^{-/-}$ cells[g]	Induced mutation frequency[h]
MMC	815± 174	1.3 ± 0.58	814± 174	256 ± 75	0.75 ± 0.21	36 ± 7	270 ± 51 x 10^{-6}	840±240 x 10^{-6}
ENU	541 ± 97	30 ± 21	511± 118	181 ± 86	1.30 ± 0.17	37 ± 4	320 ± 130 x 10^{-6}	380 ± 56 x 10^{-6}
UVC	532 ± 97	12 ± 6	520 ± 96	231 ± 28	1.34 ± 0.17	39 ± 9	440 ± 218 x 10^{-6}	280±150 x 10^{-6}

[a] Number of clones obtained by dual brief 6TG treatment of 2x10^6 Msh2$^{+/-}$ cells treated with the indicated mutagen.
[b] Obtained after continuous selection with 6TG of 2x10^6 Msh2$^{+/-}$ cells treated with the indicated mutagen.
[c] Obtained by subtracting the number of Hprt mutants from the number of clones after dual brief 6TG selection.
[d] Obtained by parallel dual brief 6TG selection of 2x10^6 Msh2$^{+/-}$ cells in the same experiments.
[e] Survival (dis)advantage of Msh2$^{-/-}$ cells after single treatment with the indicated mutagen. Obtained by mixing wild type cells with a predefined number of Msh2$^{-/-}$ cells followed by mutagen treatment and dual brief 6TG selection.
[f] Selection efficiency for Msh2$^{-/-}$ cells by dual brief 6TG selection.
[g] Frequency of the preexisting Msh2$^{-/-}$ cells in the Msh2$^{+/-}$ cell line, corrected for the relative survival of Msh2$^{-/-}$ cells to the mutagen treatment.
[h] Frequency of de novo Msh2$^{-/-}$ clones, induced by single treatment with the indicated mutagen. This includes LOH and non-LOH events.

Mechanism of loss of Msh2

Allele-specific PCR of 6TG tolerant clones derived from different genotoxin-treated *Msh2*$^{+/-}$ ES cells demonstrated significant differences in the frequency of LOH at the wild type *Msh2* allele. Thus, whereas in virtually all MMC-induced *Msh2*$^{-/-}$ clones the wild type allele was lost by LOH, this was much

less so in the *Msh2*$^{-/-}$ clones that were induced by ENU and UVC (Figure 1, Table 2). To confirm that *Msh2* was lost in the ENU-induced 6TG tolerant clones that did not display LOH at *Msh2*, we investigated 97 of these clones for Msh2 expression by Western blotting. Indeed, we found reduced Msh2 expression or (partial) truncation of the protein in 70 of these clones (Figure 2). We assume that these clones carry an ENU-induced mutation at *Msh2* that results in destabilization of the protein. Nevertheless, normal expression of Msh2 was found in 27 clones (Figure 2).

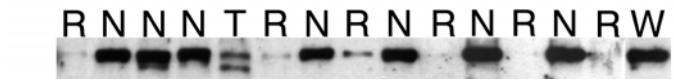

Figure 2. Western blot analysis of Msh2 from clones surviving dual brief 6TG treatment, after treatment with 0.2 mg/ml ENU, that did not display LOH at *Msh2*. R: reduced expression or absence of the protein, N: (near)-normal expression, T: (Partial) truncation of the protein, W: *Msh2*$^{+/-}$ cell lysate.

To investigate whether these clones were true Msh2 mutants or had acquired 6TG tolerance due to mutation at *Hprt* these clones were grown in HAT-containing medium that is toxic to *Hprt* mutants. Sixteen of the clones survived the HAT treatment; we infer that these carry a point mutation at *Msh2* that inactivates protein activity. We conclude that ENU, and by inference also UVC, induce LOH at *Msh2* as well as mutations that result in loss of protein expression or inactivate protein activity.

Table 3. Mutagen-induced loss at *Msh2* other than LOH

Agent	% non-LOH events (#PCR)[a]	% induced *Msh2*$^{-/-}$ clones[b]	% of induced non-LOH events[c]
None	1 (68)	N.A.	N.A.
MMC	3 (63)	76 ± 20	4 ± 1 (3/76)
ENU	36 (275)	54 ± 7	67 ± 8 (36/54)
UVC	20 (310)	39 ± 16	51 ± 21 (20/39)

[a] Between parentheses the total number of clones analyzed by multiplex PCR for each treatment.

[b] Derived from the frequency of preexisting versus de novo (mutagen-induced) clones (Table 2). N.A.: not applicable.

[c] Indicates percentage of mutagen-induced loss of Msh2 other than by LOH.

4.4 Discussion

Here we describe a procedure for the selection of *Msh2*$^{-/-}$ cells that are present in an MMR-proficient primary diploid ES cell line. The procedure relies on the established cross-tolerance of MMR-deficient cells to S_N1-methylating agents and 6TG. Thus, two brief treatments with 6TG resulted in the recovery of 36% of the *Msh2*$^{-/-}$ cells that were mixed in a predefined ratio with wild type ES cells. Almost all selected clones that were investigated by multiplex PCR-based genotyping had the correct genotype (*Msh2*$^{-/-}$). In addition, the background of clones that have acquired tolerance to 6TG consequent to fortuitous mutation of the *Hprt* gene is very low. For these reasons we conclude that the selection procedure not only is efficient but also results in very few false-positive clones.
Using this selection procedure we have determined the frequency of spontaneous *Msh2*-deficient ES cells in an *Msh2*$^{+/-}$ ES cell line to be 3.1 x 10^{-4}. This is a very high, but not unexpected, frequency since the exposure of ES cell clones carrying a mono-allelically inserted *neo* resistance marker to high G418 concentrations resulted in the emergence of cells, homozygous for the *neo* marker, with a frequency of between 10^{-3} and 10^{-4}. In contrast, spontaneous loss of the wild type *Aprt* allele in *Aprt*$^{+/-}$ ES cells is over two orders lower [14]. Thus, frequencies of spontaneous LOH may be dependent on the particular locus or chromosome. Alternatively, fortuitous MMR-deficient cells might have a very slight growth advantage, resulting in their gradual accumulation in an *Msh2*$^{+/-}$ cell line. In virtually all *Msh2*$^{-/-}$ clones, originating from the *Msh2*$^{+/-}$ ES cell line, the wild type allele of *Msh2* was not lost by mutation but rather by LOH. In concordance with this result, uniparental disomy was found to be a major cause of spontaneous LOH in ES cells [15]. Of note, the increased spontaneous mutation frequency in diploid yeast strains heterozygous for *MSH2* or *MLH1* was found to be caused by LOH at *MSH2* or *MLH1*, respectively [16].

We investigated whether exposure to mutagens induces loss of the wild type *Msh2* allele in heterozygous cells. The mutagens we used induce different classes of DNA damage, enabling to assess whether the induction of MMR-deficient cells is dependent on the DNA damage type. Single exposure to MMC induced *Msh2*$^{-/-}$ cells at a very high rate in the heterozygous clone. Interestingly,

we found a slightly increased sensitivity of the *Msh2*$^{-/-}$ ES cells to MMC. Enhanced toxicity of MMC towards MMR-deficient cells was also described by others [17] although for other cell lines this was not seen [18]. We investigated the mechanism of loss of the wild type *Msh2* allele in the induced *Msh2*$^{-/-}$ clones and almost uniquely found LOH events (Figure 1, Table 3). In agreement, MMC also induces LOH *in vivo* [19].

Similarly to MMC treatment, treatment of *Msh2*$^{+/-}$ ES cells with ENU resulted in an enhanced number of *Msh2*$^{-/-}$ cells in the heterozygous cell line. Part of this enhancement was caused by a slight growth advantage of preexisting *Msh2*$^{-/-}$ cells in the *Msh2*$^{+/-}$ cell line to the ENU treatment. A similar slight tolerance of *Msh2*$^{-/-}$ ES cells to ENU was recently found by others [20]. Multiplex PCR on selected clones revealed LOH at *Msh2* in the majority of clones. However, after correcting for the preexisting spontaneous *Msh2*$^{-/-}$ cells that all have undergone LOH (Figure 1, Table 2) it was obvious that, in contrast to treatment with MMC, most of the ENU-induced *Msh2*$^{-/-}$ clones have not arisen through induction of LOH (Table 3). Since ENU is a strong point mutagen this is not an unexpected finding. We investigated Msh2 expression in these clones by Western blotting and found (partial) truncation of Msh2 or partial or complete loss of signal in most clones. In the remaining clones, Msh2 expression appeared normal or near-normal (Figure 2). We infer that ENU mainly induces point mutations that interfere with antibody recognition, Msh2 protein expression or stability, with protein function, or alternatively, intragenic deletions resulting from collapsed replication forks at the site of the DNA damage.

Not only ENU exposure but also UVC exposure confers a slight growth advantage to *Msh2*$^{-/-}$ cells indicating a role of MMR in processing UV-induced pyrimidine dimers *in vivo*. Previously, some MMR-deficient cells were found to display enhanced survival after treatment with UVB light (reviewed in [21]). However, since UVB induces oxidative DNA damage in addition to pyrimidine dimers, enhanced survival might have been caused here by MMR-dependent responses to oxidative DNA damage [22,23]. Similarly to induction of *Msh2*-deficient cells with ENU, the majority of UVC-induced *Msh2*$^{-/-}$ cells did not display LOH at *Msh2*, supporting the notion that UVC acts as a point mutagen (Table 3).

HNPCC patients are heterozygous for a mutation in one of the MMR genes. Tumorigenesis in these patients is accompanied by loss of the wild type allele by mutation or by LOH [24-26]. Based on our findings described here we hypothesize that in somatic tissues of HNPCC patients spontaneous loss of MMR is a ubiquitous event, resulting in a population of somatic cells with a mutator phenotype. In the intestine, which is continuously exposed to endogenous (like nitrosamines in bile) and exogenous (like food-derived polycyclic aromatic hydrocarbons [PAH]) mutagens, additional MMR-deficient cells may be induced. The acquired slight tolerance of MMR-deficient cells to mutagens of different classes may further contribute to increasing the frequency of MMR-deficient cells in the intestine. In agreement with the latter hypothesis, crypts of the small intestine of MMR-deficient mice display reduced apoptosis after treatment with PAH [27] and with nitrosamines [28]. The enhanced induction of mutations in the MMR-deficient mouse intestine by these agents [28-30] suggests that mutagen exposure of MMR-deficient cells in the colon of a HNPCC patient will further accelerate carcinogenesis. We hypothesize that this combination of spontaneous and mutagen-induced loss of MMR in the intestine, together with enhanced survival and enhanced induction of mutations in MMR-deficient cells are major determinants of the tissue specificity and etiology of cancer in HNPCC.

Acknowledgements

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Chapter 5

General conclusions and discussion

5.1 MMR: caretaker of our genome

Throughout the last two decades it has become clear that MMR proteins can be seen as caretakers of our genome. One does not have to be religious to acknowledge the fact that this is beautifully symbolized by the shape of the prokaryotic mismatch recognition dimer MutS: in the presence of a suitable DNA substrate, MutS adapts the form of two praying hands where one of the thumbs -symbolizing one of the MutS monomers and corresponding to Msh6 in eukaryotes- makes contact with the mismatch (figure 1; [1,2]). As described in chapter 1, the caretaking role of MMR extends beyond the monitoring of replication fidelity and MMR plays an important role in the cellular response to a variety of DNA damaging agents. As a consequence, absence of an intact MMR system results in a reduced sensitivity upon treatment with a variety of DNA damaging agents and/or increased DNA damage-induced mutagenesis (see chapter 1). The results described in chapter 2 and 3 of this thesis confirm the important role of MMR in counteracting mutagenesis induced by DNA damage, and the results of our studies show that short-wave UV (UVC) is an important member of the group of DNA damaging agents that, upon exposure to these agents, require an intact MMR system in order to suppress damage-induced mutagenesis.

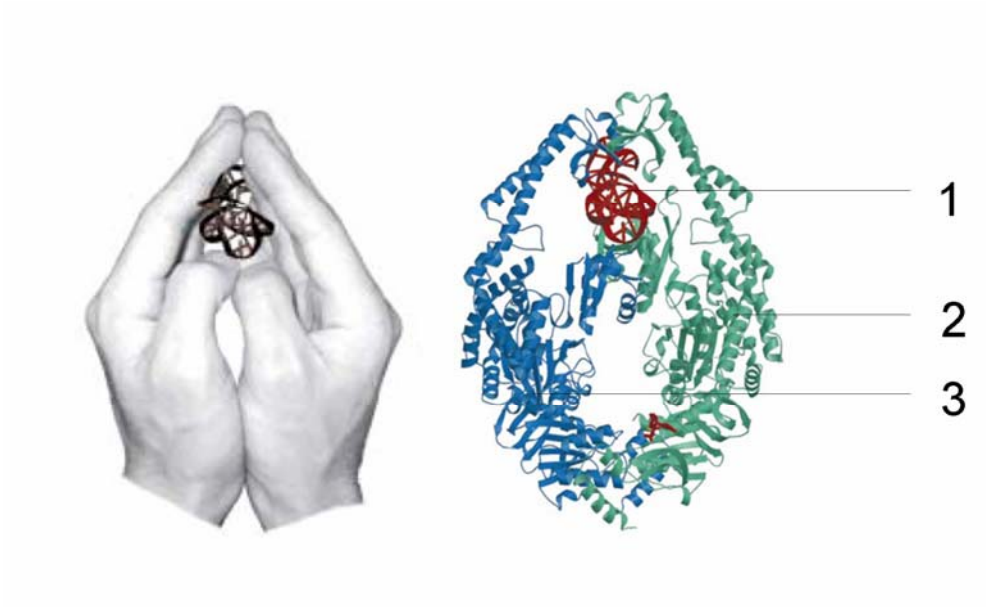

Figure 1: The crystal structure of the *E. coli* MutS dimer is similar to a pair of praying hands. 1=DNA; 2=mismatch binding monomer (corresponding to Msh6 in eukaryotes); 3=second monomer (corresponding to Msh2 in eukaryotes) (adapted from [1] and [3]).

5.2 Role of MMR in the prevention of damage-induced mutagenesis

Exactly how MMR plays a role in the reduction of DNA damage-induced mutagenesis has not been elucidated yet. The main question to be answered in this respect is whether MMR counteracts mutagenesis by taking part in removal of the mismatch within the induced DNA compound lesion that resulted from error-prone translesion synthesis (TLS) or if MMR rather reduces mutagenesis by creating the circumstances suited for other repair proteins to remove the damage. In the latter case, MMR proteins could have a damage sensing and signalling role by activating cell cycle checkpoint proteins upon the recognition of DNA damage. The induced cell cycle arrest would give proteins of other repair pathways time to repair the induced DNA damage, thereby obviating error-prone TLS that would give rise to mutations. Alternatively, MMR proteins could signal to apoptosis effector proteins upon sensing of DNA damage thereby contributing to the elimination of damaged cells that are at risk to accumulate mutations. It is also possible that MMR proteins are engaged in both functions.

Support for both the signalling function of MMR proteins and for the removal of mismatches incorporated opposite adducted bases upon the infliction of DNA damage can be found throughout the literature (reviewed in [4]). A lot of data has been gathered by studying the DNA damage response of MMR-proficient and MMR-deficient cells upon treatment with S_N1-methylating agents like MNNG. As described in chapter 1, treatment with MNNG causes apoptosis only in the presence of MMR and MMR-deficient cells are hypermutable by S_N1-methylating agents. From several studies with S_N1-methylating agents two models have emerged, one model favouring a signalling role for MMR in the cellular response to S_N1-methylating agents, the other a role for MMR in the removal of the base incorporated opposite the methylated guanine, as will be described in the following two paragraphs.

5.3 Role for MMR proteins in DNA damage signalling: 'direct signalling model'

Support for a role of MMR in damage signalling in response to treatment with S_N1-methylating agents has come from Gradia *et al*. [5]. They observed that O^6-meG lesions when paired with cytosine or thymine were able to stimulate exchange of ADP for ATP in the MSH2-MSH6 (MutSα) mismatch recognition dimer thereby inducing a conformational change in the protein complex. Based on similarity of this process to the RAS GTPase which adapts an inactive conformation in the GDP-bound form and an active conformation in the GTP-bound form, MutSα was envisioned to be a 'molecular switch' that signals to apoptosis effector proteins in the active, ATP-bound form. A critical observation pointing against a signalling function of MutSα alone has come from the work of Cejka et al., who showed that cells deficient for MLH1 or PMS2, but proficient for MSH2, fail to induce MNNG-related cell death [6]. This finding indicates that at least a partial assembly of the MMR 'repairosome' is required in order for apoptotic signalling to occur.

An important observation conflicting with the direct signalling model is the fact that although O^6-meG:T mispairs are already present during the first round of replication after treatment, apoptosis has been observed to be induced only in the second S-phase [6-8], possibly indicative of the necessity for a repair

intermediate to be generated that upon replication induces apoptosis (see below). However, Lin *et al*. found that mouse cells bearing a point mutation in the ATPase domain of Msh2 resulting in impaired MMR, showed an apoptotic response similar to wild type cells after treatment with cisplatin [9]. This indicates that processing of the (compound) lesion is not a prerequisite for apoptosis to be induced upon treatment with DNA damaging agents.

5.4 Role for MMR proteins in the removal of mismatches in compound lesions: 'futile cycling model'

Support for a role of MMR in the damage removal of mismatches from compound lesions has been provided by Ceccotti *et al*., who found that O^6-methylguanine residues elicit DNA repair synthesis by MMR in human cell extracts [10]. Thus, the 'futile cycling' model has been proposed to explain the S_N1-methylating agent-induced apoptosis in MMR-proficient cells [11]. According to this model apoptosis is caused by futile MMR events targeted to the cytosine or the thymine bases that are preferentially incorporated opposite the O^6-methylated guanine during DNA replication. The futility of the repair events originates from the fact that repair will result in the initial situation, i.e. an O^6-meG:T mispair or a O^6-meG:C pair, since the methyl adduct will not have been removed (of note: the MNNG experiments are generally performed in the absence of the methyl-guanine methyl transferase (MGMT) protein which would otherwise remove the methyl-group from the O^6-guanine position [12-15]). Consequently, new repair events are repeatedly triggered, thus resulting in futile cycling of MMR. The repeated MMR excision events lead to the continuous presence of single stranded DNA (ssDNA) which results in double strand breaks (DSBs) upon replication and these are a strong signal for the induction of apoptosis [16]. Importantly, as is illustrated by the increased frequency of GC> AT mutations upon MNNG treatment in cells proficient for MMR [17], not all O^6-meG:T pair results in futile repair.

Additional support for the 'futile cycling model' has come from Kaina *et al*., who found that MNNG treatment resulted in the appearance of chromosomal rearrangements such as sister chromatid exchanges (SCEs) during the second cell cycle after treatment. The SCEs were hypothesized to be the result of

recombination events initiated from the DNA gaps caused by continuous removal of the cytosine or the thymine incorporated opposite the O^6-methylated guanine [7]. In support of this, it has recently been demonstrated in Saccharomyces cerevisiae (*S. cerevisiae*) that inactivation of the homologous recombination pathway –essential for the repair of DNA breaks- sensitizes *S. cerevisiae* to the toxic effects of MNNG [18]. In addition, Stojic *et al.* recently showed that the G2 arrest induced by the S_N1-methylating agent MNNG and coinciding MMR-dependent phosphorylation of CHK1 (a downstream target of ATR and an important player in cell cycle checkpoint activation) is mainly observed after the second S-phase post-treatment, with only a small amount of CHK1 phosphorylation being visible at 6 hours after MNNG treatment. From this, it was concluded that processing of some kind had to take place for the ATR activation to occur, a conclusion favoring a role for MMR proteins in the removal of mismatches in compound lesions such as the O^6-meG:T mispair [8].

5.5 Validity of 'direct signalling' or 'futile cycling' model as general concept for the role of MMR in the cellular response to UVC

Although direct recognition of correctly paired damaged nucleotides might be sufficient for the induction of MMR-dependent direct signalling, it is most likely that recognition of mismatches in the context of damaged nucleotides underlies the MMR-dependent suppression of DNA damage-induced mutagenesis. As mentioned in the preceding chapters this has been proven to be true for several types of compound lesions induced by different DNA damaging agents, including photolesions (see chapter 1). In case of UV damage, affinities of MutSα for photoproduct/base mismatches have been found to be lower than for base/base mismatches, but the affinity for these compound lesions is supposed to be sufficient for MMR–dependent signaling or repair [19,20].

5.6 Reduction of UVC-induced mutagenesis by direct signalling of MSH2-MSH6

As described above, MNNG has been found to induce Chk1 phosphorylation mainly after the second S-phase after treatment. Interestingly, we clearly observed Chk1 phosphorylation already as early as 0.5 hour after UVC-

treatment, and the level of phosphorylation was significantly higher in MutSα-proficient cells compared to MutSα-deficient cells. Since the induction of photoproducts by UVC occurs instantly and the majority of cells in a mouse ES cell population consist of S-phase cells, it is very likely that at 0.5 hour after UVC treatment UVC-induced compound lesions are already present. Thus the observed early Chk1 phosphorylation can be the result of either MMR-dependent signalling after compound lesion recognition or a consequence of removal of the mismatch in the compound lesion by MMR.

Brown *et al.* found that ionizing irradiation (IR) induces association of MSH2 with CHK2 and binding of MLH1 to ATM, an observation in support of the MMR dependent signalling hypothesis [21]. The association of the MMR proteins with the proteins of the ATM signalling pathway was found to induce a replication arrest and MMR-deficient cells displayed radio-resistant DNA synthesis (RDS). However, the induction of RDS specifically in MMR-deficient cells could not be reproduced by Cejka *et al.* using several MMR proficient and deficient cell line pairs [22].

In the absence of proper S-phase checkpoint activation, as is the case during RDS [23], cells continue replicating their DNA despite the presence of DNA damage. In the case of IR, a potent inducer of double strand breaks and oxidized bases, RDS can be mutagenic, e.g. radiation induced 8-OH-G lesions will give rise to GC > TA transversions upon error-prone TLS (see paragraph 1.5). Similarly we observed that MutSα-deficient cells displayed a reduced late S/G2-phase delay upon treatment with UVC and this coincided with increased UVC-induced mutagenesis in the MutSα-deficient cells. As mentioned in chapter 2, a MMR-induced S-phase arrest could give NER time to remove UVC-induced photoproducts prior to (error-prone) replication. However, since abolition of the S/G2-phase delay in both MMR-proficient and MMR-deficient cells by the phospho-Chk1 inhibitor UCN-01 did not result in a diminution of the difference in UVC-induced mutation frequencies between both genotypes, the MutSα-induced S/G2-phase delay at most plays a marginal role in the reduction of UVC-induced mutagenesis. This prompted us to hypothesize that the removal of the mismatches in the UVC-induced compound photolesions by MMR would play a more

important role in the reduction of MMR-dependent UVC-induced mutagenesis (see below).

5.7 Reduction of UV mutagenesis by direct non-futile MMR of UVC-induced compound lesions

As described in paragraph 5.6, the inactivation of signalling downstream of ATR by UCN-01 resulting in a similar S/G2-phase delay in both wild type and MMR-deficient cells did not result in a reduced difference in UVC-induced mutation frequencies between wild type and MMR-deficient cells (see also chapter 3). Thus we studied the possibility of direct mismatch removal by MMR of bases misincorporated opposite photolesions. For this purpose we examined the UVC-induced mutational spectra at *Hprt* in both MMR-proficient and MMR-deficient mouse ES cells. We observed sequence context-dependent differences in mutation induction in both genotypes (chapter 2). Most sites in the Hprt gene where increased mutation induction occurred after UVC treatment overlapped between wild type and MutSα-deficient cells. However, the frequency of mutation induction at these sites was markedly higher in the MutSα-deficient cells. In addition, we observed one clear hotspot of mutations in the wild type cells that did not coincide with a higher mutation frequency in the MutSα cells, suggestive of a hotspot of mutation that is refractory to MMR-dependent repair. The above mentioned observations are in support of a role for sequence context-dependent recognition and removal of mismatches incorporated opposite adducted bases as it was hypothesized in the 'futile repair model' described in paragraph 5.4.

However, if a model similar to the 'futile repair model' for MMR in the removal of MNNG-induced compound lesions applies to the UVC-induced compound lesions, how can we explain that the apoptotic differences between wild type and MMR-deficient cells are not significant after UVC treatment whereas they are pronounced after MNNG treatment? To answer this question we need to realize that in contrast to the situation in S_N1-methylating agent-treated cells (in which the methyl moiety will not be removed from O^6-guanine), futile MMR after UVC treatment will be prevented by the action of nucleotide excision repair (NER) that has a relatively high affinity for mispaired photoproducts, including CPDs

[24]. Thus, even if MMR of the compound lesion would result in an introduction of a new mismatch by error-prone MMR, futile cycling (and the related induction of apoptosis) of MMR will be prevented because sooner or later the photoproduct in the compound lesion will be removed by NER.

5.8 Checkpoint signalling by MMR intermediates

If the MMR proteins are rather involved in the removal of mismatches opposite UVC-induced photolesions than involved in signalling to checkpoint proteins, the question remains how the observed MutSα-induced ATR signalling after UVC treatment can be explained. Evidence from both in vivo and in vitro studies have revealed that RPA-coated single stranded DNA (ssDNA), a general intermediate of DNA repair pathways recruits ATRIP, the regulatory partner of ATR. Recently, it was shown that RPA is essential for MMR [25]. The association of ATRIP and RPA was found to be required for the recruitment of ATR to sites of DNA damage [26-28]. Thus, the UVC-induced Chk1 phosporylation that we observed specifically in wild type cells might be explained by binding of the RPA protein present on ssDNA intermediates (that were created during MMR of the UVC-induced photolesions) to the ATRIP-ATR complex and the subsequent phosphorylation of Chk1 (figure 2). Similarly, it was shown that in budding yeast, damage processing by NER of UV lesions was required for checkpoint activation [29]. Interestingly Dodson et al., using RNA interference-mediated suppression of RPA, found that neither RPA1 nor RPA2 were essential for hydroxyurea-or UV-induced phosporylation of CHK1, results that are at first sight in contrast with our proposed model [30]. However the experiments of Dodson et al. were performed in HEK 293T cells which are MMR-deficient due to promoter methylation of the MLH1 gene [31]. In this case the observed CHK1 phosphorylation is caused by MMR-independent (and thus, according to our model, RPA-independent) signalling.

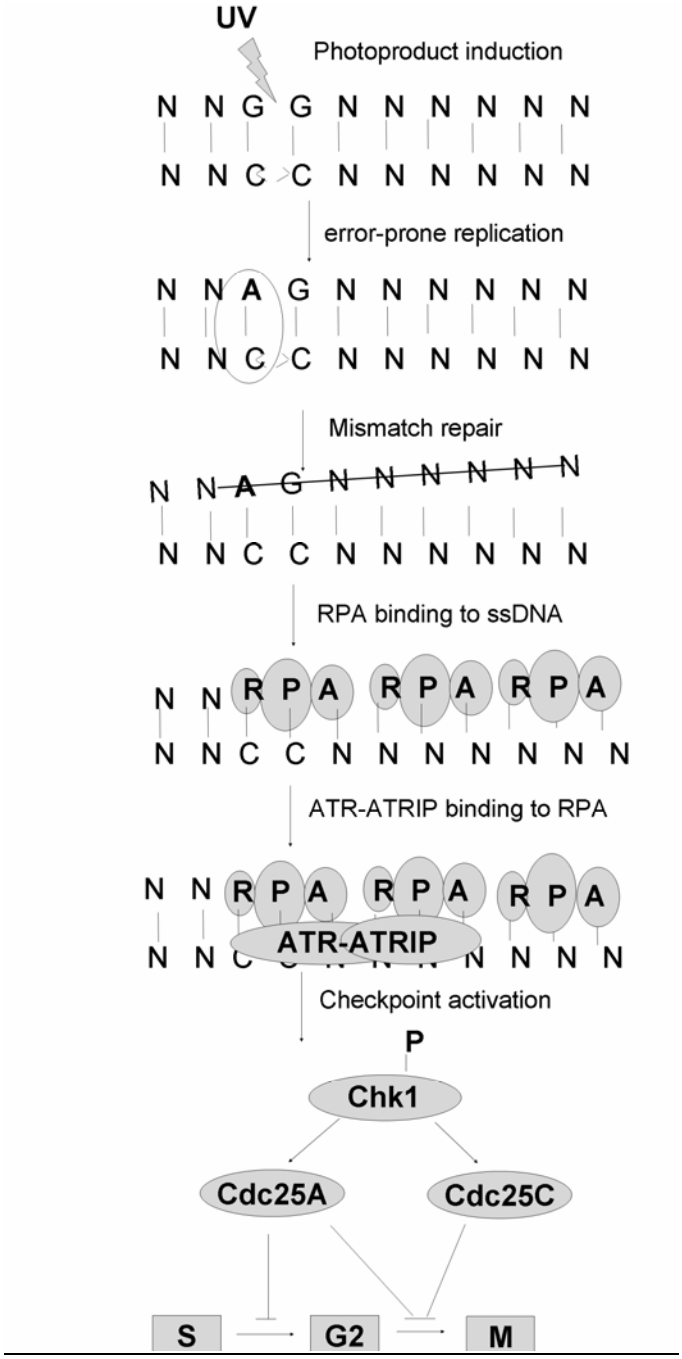

Figure 2: Hypothesis for MMR-dependent checkpoint activation after UVC treatment (adapted from [26-28])

5.9 Difference in checkpoint activation after UVC and MNNG

If we assume that both the O^6-meG lesions and the UVC-induced photolesions are recognized and processed by MMR, why are the kinetics of checkpoint activation and subsequent induction of cell cycle arrest different between UVC and MNNG treatments (see paragraph 5.6 and figure 3)? Importantly, when we compare the cell cycle profiles of mouse ES cells after UVC and MNNG treatments, the kinetics of the transient cell cycle arrests are only partially different. As can be seen in figure 3, both genotoxic agents induce a late S/G2-phase arrest during the first cell cycle after treatment (Fig. 3, 6H), in addition to the S-phase arrest induced in the UVC-treated cells, caused by stalling of the replication fork (O^6-meG lesions generally cause less stalling of the replication fork [32]). According to the model presented in figure 2, this would reflect Chk1 activation mediated by single-stranded MMR intermediates. Of note, Stojic *et al.* did show some Chk1 phosphorylation at six hours after MNNG treatment (see paragraph 5.4 and [8]) which may be caused by signalling of the RPA-coated ssDNA repair intermediates like we hypothesize to occur after UVC treatment, or by direct signalling of MMR proteins after binding to MNNG-induced compound lesions. To distinguish between both mechanisms, it would be interesting to study the MNNG-induced CHK1 phosporylation after transfection of wild type and *Msh2*-deficient cells with siRNA targeting RPA. If processing of the lesion and associated RPA coating of the ssDNA intermediates is required in order to activate CHK1, phosphorylation levels of CHK1 will be reduced/absent after treatment (depending on the knock-down efficiency of the siRNA in the presence of siRNA targeting RPA).

The S-phase arrest and the apoptosis that is visible at 18 hours after MNNG treatment can be explained by the conversion of single strand breaks that are the result of continuous MMR on the O^6-meG:C or O^6-meG:T lesions into double strand breaks during this second S-phase, a phenomenon that is absent in the UVC-treated cells due to the non-futile character of the repair (see paragraph 5.7).

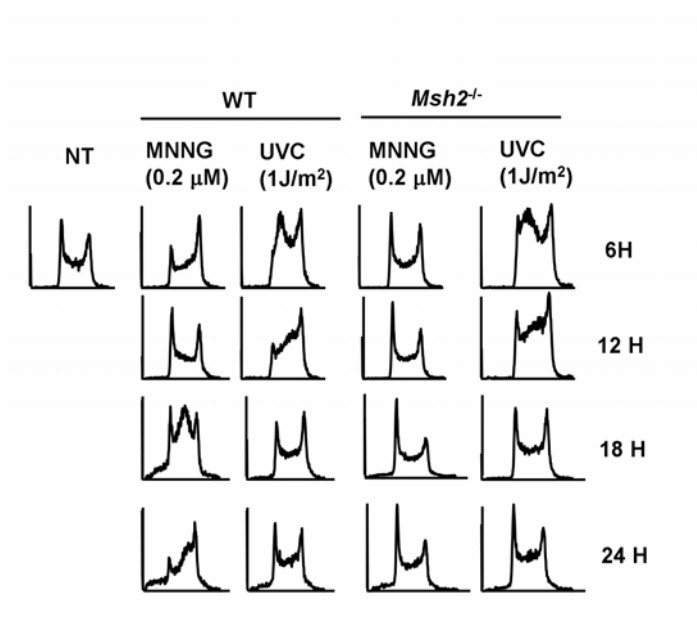

Figure 3: Cell cycle profiles of wild type and *Msh2*$^{-/-}$ mouse ES cells at different time points after mock (NT), MNNG or UVC treatment (own experiments)

5.10 HNPCC patients and UV

An intriguing question deriving from the data presented in this thesis is whether individuals carrying a germline mutation in one of the MMR genes and thus being at risk to develop HNPCC should limit themselves in the exposure to UV. As we have shown in chapter 4, UVC induces loss of the wild type allele in *Msh2*$^{+/-}$ mouse ES cells thereby inducing cells that are completely MMR-deficient. Consequently, these cells will display a hypermutable phenotype upon exposure to UVC, a process that could result in carcinogenicity at the level of an organism. Is this a process likely to occur in HNPCC patients as well? One important thing to mention here is that although our studies have been performed with UVC, a wavelength that has been filtered out from the sunlight by the ozone layer once the light reaches the earth, this is still an important question since UVA and UVB that do reach the earth are also potent inducers of photolesions and both have carcinogenic properties [33]. Interestingly, a sub-syndrome within the HNPCC syndrome, called Muir-Torre syndrome (MTS) is characterized by the development

of cutaneous and sebaceous neoplasms in association with one or more of the visceral malignancies as were described in chapter 1 [34]. Although these cutaneous and sebaceous neoplasms are relatively rare, they do develop typically in sun-exposed areas, such as the head and the neck. In addition, Shpitz *et al.* observed that in a subset of both dysplastic nevi and cutaneous melanomas a partial or complete loss of MSH2 or MLH1 had occurred [35].

Support for a role of MMR in the prevention of skin cancer has also come from studies with mice. Young *et al.* showed that MMR-deficient mice develop skin tumours at a lower level of cumulative UVB exposure and at an earlier time of onset than wild-type mice [36]. In addition, *Xpc*$^{-/-}$*Msh2*$^{-/-}$ mice and *Xpa*$^{-/-}$*Msh2*$^{-/-}$ mice appeared to be more prone to UVB-induced skin cancer than *Xpc*$^{-/-}$ mice and *Xpa*$^{-/-}$ mice respectively [37,38]. However, the accelerated UV-induced skin carcinogenesis in the NER and MMR doubly-deficient mice can also be the result of the additive effect of the MMR defect (that causes a mutator phenotype) to the NER defect (responsible for the UV-mutagenesis). Thus, no final conclusions can be drawn from these latter experiments on a role for MMR in counteracting UV-induced carcinogenesis.

When we combine our UV-data with the data on the response of MMR-deficient cells to other classes of DNA-damaging agents described in chapter 1, it is tempting to speculate that the observed increased mutagenesis after exposure to the listed genotoxic agents in MMR-deficient cells is the result of the absence of the repair of mismatches in compound lesions that arose as a result of error-prone TLS in a similar way as we describe for UVC. As described in chapter 1.1, we are continuously exposed to endogenous and exogenous DNA damaging agents and cells in HNPCC patients that have lost the wild type allele of the germ line-mutated MMR gene will display a hypermutable phenotype to these agents. As described earlier, this may be the reason why the major site of tumorigenesis in HNPCC patients is the intestine, a site where many food-borne genotoxic compounds circulate ([39]; see chapter 1, paragraph 3).

Another source of exogenous genotoxic compounds to which HNPCC patients might be hypermutable are chemotherapeutic agents used in cancer

treatment. The adducts induced by cisplatin for example, a widely used chemotherapeutic agent, has been shown to be bypassed in an error-prone way [40] creating compound lesions that could be substrate for MMR. In support of this, knockdown of the catalytic subunit of TLS polymerase pol ζ eliminated the enhanced mutagenicity of cisplatin in MMR-deficient cells [41]. The second generation cisplatin analogue oxaliplatin has been found to be bypassed in a relatively error-free manner [40], thus oxaliplatin would be a more suitable chemotherapeutic agent for HNPCC patients. Oxaliplatin is frequently used in the treatment of colorectal cancer [42].

5.11 Future perspectives

Although the results presented in this thesis combined with the results of MutSα binding studies are in strong support of a role of MMR in the removal of UVC-induced photolesions, direct demonstration of the removal by MMR of mismatches opposite photolesions *in vivo* remains to be awaited. In a recent study, Wang *et al.* checked for 3' nick-directed MMR excision of T[CPD]T/AG and T[6-4]T/AG substrates in vitro in sequence contexts that had previously been shown to induce substantial binding by MutSα. Both substrates failed to provoke excision while the CPD substrate, pre-treated with *E. coli* photolyase to reverse the CPD and create a TT/AG mispair did result in excision [43]. One explanation for this may be the fact that the right conditions for excision of a mismatch from a compound lesion are different from the conditions required for mismatch excision from a simple mispair and these conditions may have not been met yet in vitro. In this respect and given the fact that the most frequent mutations induced by UV are C>T transitions, particularly in the MMR-deficient cells (when the hotspot at position 599 at *Hprt* is excluded, chapter 2, figures 4 and 5), it would be best using a substrate containing a C:A mismatch, such as C[CPD]C/GA. A difficulty in studying these substrates however is that CPDs containing cytosine deaminate rapidly [44].

To investigate whether or not the MMR-dependent S/G2-phase delay enhances NER and thereby reduces mutagenesis after UVC treatment, UVC-induced mutation frequencies of NER-deficient cells could be compared with the

UVC-induced mutation frequencies of MMR/NER double mutants. If MMR exerts its anti-mutagenic effects by enhancing NER, the mutation frequencies of the double mutants should not significantly differ from the UVC-induced mutation frequency in the NER single mutant.

From our data we can not conclude whether the enhanced UVC-induced mutagenesis is a result of (non-repair) of compound CPDs or compound (6-4)PPs. A way to distinguish amongst the mutagenicity of these photolesions in MMR-deficient cells is to create MMR-deficient cells expressing either CPD photolyase or 6-4PP photolyase. These enzymes are capable of reversing either lesion with high specificity in a reaction that is light dependent [45]. By determining the mutation frequencies in MMR-deficient cells stably transfected with either the CPD photolyase or the 6-4PP photolyase it will be clear which of the two photoproducts has the highest mutagenic capacity in MMR-deficient cells.

Finally, to get a better picture of the MMR-dependent signalling pathway induced after UVC, the powerful siRNA technology could be used. Chemical ATR checkpoint kinase inhibitors such as Wortmannin and caffeine have a great disadvantage of inhibiting more than one target and this can confound the experiment. siRNA oligos that specifically target the mRNA of one protein can be of use in determining which checkpoint proteins are important for the establishment of the MMR-dependent S/G2-phase arrest after UVC irradiation.

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Summary

Growth, maintenance and injury-related healing of a multicellular organism require that cells making up an organism divide regularly. Prior to cellular division, the cell duplicates its DNA such that the resulting daughter cells will both contain a copy of the genome. Since DNA encodes the proteins that give the cell a specific function in the organism, error-free DNA duplication is essential for the conservation of proper cellular functioning of the daughter cells and subsequent generations. Thus, in addition to highly accurate replicative DNA polymerases (polymerase δ and ε in eukaryotic cells), cells are equipped with DNA mismatch repair (MMR) machinery that specifically repairs errors that arise during erroneous DNA replication. Typical replication errors are mismatches and insertion/deletion loops (IDLs). Cells that are deficient for MMR display a mutator phenotype as a result of the non-repair of mismatches and the contraction/extension of small repeated DNA sequences (microsatellites) due to the absence of IDL repair. The latter process is designated as microsatellite instability (MSI).

The proteins that make up the MMR pathway have been highly conserved during evolution, illustrating the importance of the pathway in organisms ranging from bacteria to mammals. In **chapter 1** the different steps of a MMR event are discussed and the proteins responsible for every step in both prokaryotic and eukaryotic MMR described. The experiments that form the core of this thesis have been performed with eukaryotic cells, *i.e.* mouse embryonic stem (ES) cells. In mouse cells recognition of replication errors takes place by one of two heterodimers (respectively called MutSα and MutSβ): a dimer of Msh2 and Msh6 is responsible for the recognition of mismatches and small IDLs, whereas the Msh2-Msh3 dimer is responsible for recognition of both small and larger IDLs. Recognition of the replication error is followed by excision of part of the error-containing strand, resynthesis of the excised DNA strand and ultimately ligation of the newly synthesized DNA to the pre-existing DNA strand.

The importance of the MMR pathway is further illustrated in **chapter 1** by the description of the disease hereditary non-polyposis colorectal cancer (HNPCC), a hereditary cancer syndrome associated with a germ line defect in one of the

genes encoding the MMR pathway. HNPCC is a disease with high penetrance (hence designated a dominant inherited disease despite the fact that it is a recessive disorder at the genetic level, *i.e.* both alleles need to be inactivated in order for the disease to develop). Patients develop cancer at an early stage, usually during the third or fourth decade of their life. This frequently involves cancer of the colon, but patients also present with extra-colonic cancers such as cancer of the endometrium, small intestine and stomach.

In the second half of **chapter 1** a more recently discovered role for MMR proteins is discussed, *i.e.* a role in the cellular response to genotoxic agents. As can be read, MMR status is a determining factor for the toxicity and mutagenicity of methylating agents, oxidative damage-inducing agents, cisplatin, heterocyclic amines and ultra-violet (UV) light. The general trend is that MMR-deficient cells are hypermutable by genotoxic agents such as the compounds described above. This property of MMR-deficient cells frequently coincides with a decreased sensitivity to the particular compound. In addition, it has been found that upon treatment with some genotoxic agents (e.g. UV), an S-phase delay is induced that is partially dependent on functional MMR.

The anti-mutagenic properties of MMR in response to exposure to DNA damaging agents have so far not been studied in great detail, apart from the hypermutable phenotype of MMR-deficient cells after treatment with S_N1-methylating agents described in paragraph 1.4. In **chapter 2** the mutagenic properties of short wave UV (UVC) in mouse ES cells proficient and deficient for MMR are further explored. It is shown that treatment of mouse ES cells with UVC induced six times more mutations in cells deficient for the MMR recognition dimer Msh2-Msh6 (MutSα), whereas cells without Msh2-Msh3 (MutSβ) show mutation levels equal to wild type cells. The increased mutagenicity of UVC in MutSα-deficient cells could not be accounted for by increased levels of apoptosis in wild type cells after UVC-treatment, since the differences in apoptosis were marginal. Furthermore, at a UVC-dose of 0.5 J/m^2, a dose that hardly induces apoptosis, MutSα-deficient cells were still hypermutable in comparison to wild type cells. The mutational spectrum of the *Hprt* gene in both wild type and MutSα-deficient cells was subsequently analysed after UVC-treatment and it was found that although

the absolute number of mutations in MMR-deficient cells was markedly higher, the types (transitions/transversions) and sites (transcribed versus non-transcribed strand; location in the *Hprt* gene) of mutations were generally similar in both genotypes. An exception was found at position 599 in the *Hprt* gene, which appeared to be a spectral hotspot for C to T transitions in wild type cells at which the mutation frequency was not affected by MMR status.

From the data presented in **chapter 2** we discuss two models to explain the anti-mutagenic activity of MMR after UVC irradiation. In the first model a MMR-induced S-phase delay allows the repair of deaminated cytosine dimers prior to mutagenic translesion synthesis that would otherwise result in C to T transitions. In the second model, that we favour (see below), MMR directly recognizes and removes mismatches in UVC-induced compound photolesions. In the case of UVC irradiation, a compound lesion consists of a mismatch opposite an UVC-induced photolesion.

Chapter 2 furthermore describes that exclusion from the wild type mutational spectrum of the UVC-induced spectral hotspot at position 599 in *Hprt* resulted in a marginally higher C to T transition frequency in *Msh2*-deficient cells compared to wild type cells (31% in wild type cells versus 47% in MutSα-deficient cells), an observation that according to our favoured model indicates that C:A mismatches in UVC-induced compound lesions were particular permissive to MMR.

In **chapter 3** it is shown that UVC induces a cell cycle delay during S/G2-phase that was partially dependent on the presence of the MMR recognition dimer MutSα. The UVC-induced cell cycle delay coincided with phosphorylation of checkpoint kinase Chk1. It is subsequently demonstrated that the UVC-induced MutSα-dependent cell cycle delay is not the main cause for the six times higher mutation induction in MutSα-deficient cells after UVC-treatment (as descibed in chapter 2) since abolition of the cell cycle delay in both genotypes with UCN-01 still resulted in a markedly higher mutation induction in MMR-deficient cells. This observation is in conflict with the model described above proposing that the MMR-

induced cell cycle delay plays a major role in counteracting the induction of C tot T transitions by enhancing repair of deaminated cytosine dimers.

Chapter 4 is an account of experiments showing that UVC and also the cross-linking agent MMC and alkylating agent ENU efficiently induce loss of the wild type *Msh2* allele in *Msh2*$^{+/-}$ mouse ES cells. The mechanism by which this occurred depended on the property of the genotoxic agent: MMC exclusively induced loss of heterozygozity (LOH) whereas UVC and ENU frequently induced mutations in the wild type *Msh2* allele. In addition, we observed a high frequency of spontaneous LOH of the *Msh2* allele in the *Msh2*$^{+/-}$ population. These observations are of significant importance for HNPCC patients who are heterozygous for one of the MMR genes (frequently *MSH2*) and thus possibly at risk to lose the wild type allele either spontaneously or by exposure to a genotoxic agent. As described above, the thus originated MMR-deficient cells will display a mutator phenotype that can have deleterious consequences such as the development of cancer.

In **chapter 5** the data of the preceding chapters are discussed and compared to the prevailing 'futile cycling model' and the 'direct signalling model' used to explain the MMR dependent toxicity/mutagenicity of S_N1-methylating agents. The suitability of either model to explain the observations described in chapter 2 and 3 is discussed. Both models are based on the findings of experiments with S_N1-methylating agents in the presence of the methyltransferase inhibitor O6-benzylguanine. The 'futile cycling model' explains the MMR dependent toxicity of methylating agents by stating that the methylating agent-induced O6-meG:C and O6-meG:T basepairs induce futile MMR ('futile' because the O6-methyl group is not removed due to the inhibition of the methyltransferase and a MMR repair event will result again in a O6-meG:C or O6-meG:T base pair). Continuous futile repair results in permanently present single strand DNA stretches that will create cytotoxic double strand breaks upon replication. The induction of apoptosis prevents the accumulation of cells with mutations, illustrated by the hypermutability of MMR-deficient cells upon treatment with S_N1-methylating agents. According to the 'direct signalling model' the MMR dependent toxicity/mutagenicity of S_N1-methylating agents is caused by signalling of MutSα

to the checkpoint machinery of the cell upon recognition of O6-meG:C and O6-meG:T basepairs resulting in the induction of apoptosis.

We believe that the 'direct signalling model' is less relevant for the explanation of the hypermutable phenotype of MMR-deficient cells after UVC treatment, amongst others because abolition of checkpoint activation did not alleviate the hypermutable phenotype of MMR-deficient cells as described above. Also, the model predicted that a decrease in the efficiency of CPD removal consequent to increased S-phase progression might result in increased mutations from photoproducts at the transcribed strand in these cells (CPDs are never prereplicatively repaired in the non-transcribed strand in rodent cells, except when they contain a deaminated cytosine). This difference in strand bias was not found, thus we believe that the reduced cell cycle delay in MutSα-deficient cells does not result in a mutational transcribed strand bias in these cells due to a decrease in the CPD removal efficiency from this strand. Thus we believe that the observed cell cycle delay is rather an indirect effect of mismatch removal from UVC-induced compound lesions by MMR than enabling enhanced excision repair. In the absence of a functional MutSα dimer the UVC-induced mutation frequency increases at sites in *Hprt* were MMR would normally occur. The hotspot of UVC-induced mutations at *Hprt* position 599 in wild type cells can be explained by the fact that mismatches at this position are refractory to MMR.

Samenvatting

Organismen groeien door middel van celdeling. Echter ook nadat de groei voltooid is, blijft celdeling noodzakelijk. Cellen hebben namelijk een beperkte levensduur en dienen vervangen te worden wanneer deze verstreken is. Verder verloopt het proces van wondheling via celdeling waarbij het beschadigde weefsel wordt vervangen door weefsel bestaande uit nieuw aangemaakte cellen. De functie die de cel vervolgens in een specifiek weefsel moet gaan vervullen wordt bepaald door het arsenaal aan eiwitten dat in de cel wordt aangemaakt. De informatie voor het maken van deze eiwitten ligt gecodeerd in het erfelijk materiaal, het DNA, van de cel. Het DNA dat de informatie bevat voor één eiwit wordt een gen genoemd. Het is belangrijk dat elke nieuwe cel het DNA coderend voor de benodigde eiwitten tot zijn beschikking krijgt. Daarom dupliceert de cel voorafgaand aan de celdeling zijn DNA waarna beide dochtercellen een copie van het genoom toebedeeld krijgen.

DNA bestaat uit twee strengen van opeengestapelde eenheden, de DNA basen. Er zijn vier typen basen in het DNA, te weten adenine, cytosine, guanine en thymine, afgekort door de letters A,C, G en T. Elke base van de ene streng heeft zijn specifieke bindingspartner in de tegenoverliggende streng. Op het bestaan van deze specifieke baseparing is het principe van DNA duplicatie gebaseerd. Een T paart altijd met een A en een G altijd met een C. De informatie die ligt opgeslagen in DNA leidt tot de productie van eiwitten middels de synthese van een intermediair, het RNA. Een van beide DNA strengen wordt in RNA vertaald, dit is de getranscribeerde streng. Het RNA wordt vervolgens in een eiwit vertaald. De DNA streng die niet codeert voor RNA wordt aangeduid als de niet-getranscribeerde streng.

Om de informatie die opgeslagen ligt in het DNA foutloos van de ene cel op de andere over te dragen, is de cel uitgerust met eiwitten die gespecialiseerd zijn in het nagenoeg foutloos dupliceren van DNA. Deze eiwitten heten polymerases. Tijdens de DNA duplicatie worden de DNA strengen uit elkaar gehaald en maakt het polymerase een nieuwe DNA streng tegenover elk van de uit elkaar gehaalde DNA strengen. Hierbij zal het dus een C tegenover een G plaatsen en een T tegenover een A. Wanneer het polymerase hiermee klaar is bevat de cel

een dubbele hoeveelheid DNA die tijdens de celdeling over beide dochtercellen wordt verdeeld.

Als er een fout optreedt tijdens het proces van DNA duplicatie –bijvoorbeeld doordat het polymerase een C tegenover een A plaatst in plaats van een T, daarbij een 'mismatch' introducerend- wordt een gespecialiseerde set van eiwitten geactiveerd die tezamen het mismatch herstel mechanisme vormen (in het Engels wordt dit mechanisme afgekort tot 'MMR'). Een ander veelvoorkomende DNA duplicatiefout die door MMR wordt hersteld, is de insertie-of deletielus (IDL). Deze kan ontstaan wanneer het polymerase een keten van dezelfde opeenvolgende basen (microsatellieten genoemd) dupliceert. Hierbij kan het gebeuren dat het polymerase één of enkele basen te veel of te weinig invoegt, waarbij de tegenoverliggende base of de zojuist geïntroduceerde base partnerloos blijft en dus wordt uitgestoten. Hierbij ontstaan de bovengenoemde IDLs. Wanneer deze duplicatiefouten niet worden gerepareerd door MMR, zullen de ketens van repeterende basen kleiner of groter worden, een fenomeen dat wordt aangeduid met microsatelliet instabiliteit (MSI). Het niet verwijderen van mismatches leidt tot permanente basepaarveranderingen in het DNA, ook wel 'mutaties' genoemd.

De MMR eiwitten zijn evolutionair gezien heel oud en aanwezig in zeer uitlopende organismen, van bacteriën tot mensen. Dit geeft aan hoe belangrijk deze eiwitten zijn voor het goed functioneren van een organisme. In **hoofdstuk 1** worden de verschillende stappen van MMR bediscussiëerd en worden de betrokken eiwitten in prokaryoten (organismen waarbij het genetisch materiaal vrij ligt in het cytoplasma) en eukaryoten (organismen waarbij het erfelijk materiaal wordt omsloten door een membraan) beschreven. De experimenten die de kern vormen van dit proefschrift zijn uitgevoerd met eukaryote cellen, in het bijzonder embryonale stamcellen van de muis (ES cellen). In deze cellen worden DNA duplicatiefouten herkend door één van twee eiwitparen (die respectievelijk worden aangeduid met MutSα en MutSβ): een eiwitpaar van Msh2 en Msh6 herkent mismatches en kleine insertie/deletielussen en het Msh2-Msh3 eiwitpaar herkent zowel kleine als grotere IDLs. Na de herkenning van een DNA duplicatiefout wordt een deel van de streng bevattende de duplicatiefout verwijderd, waarna een nieuw stuk DNA wordt gesynthetiseerd. Tijdens de laatste

stap van MMR worden de nieuwe en de oude streng aan elkaar geplakt en is het DNA in zijn oude staat hersteld. Het belang van MMR wordt geïllustreerd in **hoofdstuk 1** door de beschrijving van de erfelijke ziekte 'hereditary non-polyposis colorectal cancer (HNPCC)'. Dit is een erfelijk kankersyndroom veroorzaakt door een aangeboren defect in één van de genen die coderen voor MMR eiwitten. HNPCC patiënten ontwikkelen kanker aanmerkelijk vroeger dan in de gemiddelde populatie gebruikelijk is, dit kan zijn rond hun 30e, 40e levensjaar. Veelal betreft dit kanker van de dikke darm, maar patiënten kunnen ook kanker ontwikkelen in andere organen, zoals de baarmoeder, dunne darm of maag.

In het tweede gedeelte van **hoofdstuk 1** wordt een meer recent ontdekte functie van MMR eiwitten bediscussiëerd. Dit betreft een functie in de respons van cellen op de behandeling met stoffen die het DNA beschadigen (genotoxische stoffen). Het is gebleken dat een intact mismatch herstel mechanisme van groot belang is om het kankerverwekkende effect van verschillende genotoxische stoffen tegen te gaan. Bewijs voor deze rol is geleverd door proeven die aantonen dat behandeling met bovengenoemde stoffen van cellen met een MMR defect leidt tot een groter aantal mutaties dan het geval is in cellen met intact MMR. Zoals eerder beschreven bevat het DNA de informatie die voor het vervaardigen van eiwitten noodzakelijk is. Mutaties kunnen leiden tot de productie van niet goed functionerende eiwitten, hetgeen de integriteit van de cel in gevaar brengt. Wanneer de mutaties worden veroorzaakt in genen die coderen voor eiwitten die een belangrijke rol spelen in het reguleren van de celdeling, kan het zijn dat de cel overgaat tot ongecontroleerde celdeling, een eigenschap die wordt aangetroffen in kankercellen. Mutatie-inducerende stoffen, ook wel mutagene stoffen genoemd, behoren dan ook vaak tot de categorie van kankerverwekkende stoffen.

Wanneer cellen behandeld worden met genotoxische stoffen reageren zij vaak door het proces van DNA duplicatie tijdelijk tijdelijk stil te leggen, dit wordt een tijdelijk celcyclusarrest genoemd. Het arrest wordt vervolgens benut om het beschadigde DNA te herstellen. Dit is een zeer belangrijk proces aangezien beschadigd DNA de foutloze duplicatie van het DNA bemoeilijkt. Het is gebleken dat de eiwitten van het mismatch herstel mechanisme een belangrijke rol spelen in het stil leggen van de DNA duplicatie na behandeling met genotoxische stoffen.

Over de anti-mutagene invloed van MMR eiwitten na behandeling met DNA beschadigende stoffen is nog niet zo heel veel bekend. In **hoofdstuk 2** worden de mutagene eigenschappen van kortgolvig UV licht (UVC) in cellen met een intact en een defect mismatch herstel systeem behandeld. Uit de in dit hoofdstuk beschreven proeven blijkt dat UVC zes keer zoveel mutaties induceert in cellen met een defect mismatch herstel mechanisme in vergelijking met cellen met intact MMR. Dit verschil kan niet verklaard worden door een verhoogde toxiciteit van UVC in cellen met intact MMR, want de verschillen in toxiciteit tussen beide cel typen waren marginaal. Bovendien bleken de verschillen ook aanwezig na het bestralen van cellen met een hele lage dosis UVC, een dosis waarbij bijna geen sprake was van toxiciteit.

Vervolgens wordt in **hoofdstuk 2** beschreven welke mutaties UVC induceert in cellen met een intact en een defect mismatch herstel systeem. Om dit te kunnen bepalen is gekeken naar de UVC-geïnduceerde mutaties in het *Hprt* gen. De soort mutaties geïnduceerd door UVC verschilden weinig tussen beide celtypen. In beide gevallen troffen we veel mutaties aan waarbij de C was vervangen door een T. Ook observeerden we nagenoeg geen verschillen in welke van de twee DNA strengen was gemuteerd, de getranscribeerde of de niet-getranscribeerde streng. Tot slot zagen we dat ook de locaties van de plaatsen met een verhoogde mutatie-inductie (zogenaamde 'hotspots') in *Hprt* bijna niet verschilden tussen de cellen met een intact mismatch herstel mechanisme en cellen met een defect mismatch herstel mechanisme. Het bleek dat UVC mutaties induceerde op plaatsen verspreid over het gehele *Hprt* gen, waarbij over het algemeen de frequentie van de gevonden mutaties hoger was in de cellen met een defect mismatch herstel mechanisme. Een uitzondering werd gevormd door positie 599 in het *Hprt* gen. Dit bleek een hotspot te zijn voor mutaties waarbij de C was vervangen door een T in cellen met een intact mismatch herstel systeem. Op deze plaats werd de mutatiefrequentie dus niet bepaald door het wel of niet aanwezig zijn van intact MMR.

Naar aanleiding van de data gepresenteerd in het eerste deel van hoofdstuk 2 behandelen we vervolgens twee modellen die de anti-mutagene werking van MMR na UVC behandeling zouden kunnen verklaren. Het eerste

model postuleert dat de inductie van een DNA duplicatie-arrest door MMR eiwitten herstel van UVC-geïnduceerde schade mogelijk maakt, waardoor de inductie van mutaties door foutieve replicatie van het beschadigde DNA voorkomen wordt. In het tweede model, het 'directe herstel model' hetgeen wij voorstaan (zie hieronder), herkent en verwijdert MMR de UVC-geïnduceerde samengestelde lesies. In het geval van UVC bestraling bestaat een samengestelde lesie uit een mismatch tegenover een door UVC geïnduceerde fotolesie.

Hoofdstuk 2 laat verder zien dat uitsluiting van de mutaties geïnduceerd op positie 599 in *Hprt* in cellen met intact MMR ervoor zorgt dat de hoeveelheid C naar T mutaties in de cellen met een defect mismatch herstel mechanisme enigszins hoger uitvalt (31% in mismatch herstel proficiënte cellen versus 47% in mismatch herstel deficiënte cellen). Deze observatie komt volgens het directe herstel model erop neer dat met name C:A mismatches in UVC-geïnduceerde samengestelde lesies toegankelijk zijn voor mismatch herstel.

In **hoofdstuk 3** wordt aangetoond dat UVC een celcyclusvertraging induceert dat voor een deel afhangt van de aanwezigheid van een intact mismatch herstel mechanisme. De celcyclusvertraging trad tegelijk op met de fosforylatie van het eiwit Chk1. Vervolgens laten we zien dat de MMR-afhankelijke celcyclusvertraging na UVC behandeling niet de voornaamste oorzaak is van de zes keer hoge mutatie-inductie in mismatch herstel deficiënte cellen (beschreven in hoofdstuk 2) aangezien het opheffen van de celcyclusvertraging in zowel MMR-proficiënte als -deficiënte cellen door behandeling met de stof UCN-01 nog steeds in een groot verschil in mutatie-inductie resulteerde na UVC behandeling.

Hoofdstuk 4 laat zien hoe in cellen die drager zijn van zowel een intacte als een defecte kopie van het gen *Msh2* de intacte kopie van dit gen verloren gaat na behandeling met verschillende DNA beschadigende stoffen zoals MMC en ENU of na bestraling met UVC. De manier waarop dit gebeurt hangt af van de soort DNA beschadiging die geïnduceerd wordt. MMC veroorzaakt voornamelijk deleties van het intacte gen, terwijl ENU en UVC ook mutaties in de intacte kopie van *Msh2* veroorzaken. Het verlies van *Msh2* door de inductie van een deletie

zagen we ook vaak optreden zonder dat de cellen behandeld waren met DNA beschadigende stoffen.

In **Hoofdstuk 5** worden de gegevens van de voorafgaande hoofdstukken bediscussiëerd en wordt er gekeken of de gegevens stroken met twee heersende modellen die gebruikt worden om de toxiciteit en de mutageniciteit van de zogenaamde S_N1-methylerende stoffen te verklaren. Deze stoffen methyleren het DNA, waarbij de gemethyleerde zuurstof (O) op de zesde positie in de base guanine (O^6-meG) de meest toxische lesie vormt. Het 'futile cycling model' gaat ervan uit dat de O^6-meG:C en O^6-meG:T baseparen die door S_N1-methylerende stoffen worden geïnduceerd continu MMR induceren doordat mismatch herstel van O^6meG:C en O^6meG:T baseparen wederom resulteren in O^6meG:C en O^6meG:T baseparen (de methylgroep blijft namelijk aanwezig, doordat het enzym dat deze groep kan verwijderen onschadelijk is gemaakt in deze experimenten). Het continu plaatsvinden van mismatch herstel zal leiden tot de permanente aanwezigheid van enkelstrengs DNA dat tijdens DNA duplicatie in een dubbelstrengs breuk resulteert hetgeen een zeer toxische lesie is. Een dubbelstrengs breuk induceert het proces van celcyclusarrest en/of geprogrammeerde celdood (apoptose). Beschadigde cellen die mogelijk G:T mismatches bevatten worden aldus geëlimineerd, hetgeen verklaart waarom S_N1-methylerende stoffen minder mutageen zijn voor cellen met een intact MMR systeem.

Het 'direct signalling model' wijt de mismatch herstel afhankelijke toxiciteit en mutageniciteit van S_N1-methylerende stoffen aan het feit dat mismatch herkennende eiwitten na het herkennen van de O^6-meG:C en O^6-meG:T baseparen signaleren naar eiwitten die het proces van celcyclus arrest en/of apoptose induceren.

Wij geloven dat het 'direct signalling model' minder relevant is voor de verklaring van de verhoogde mutatie-inductie in mismatch herstel deficiënte cellen na UVC behandeling. Zoals hierboven beschreven is één van de redenen hiervoor dat na het opheffen van de celcyclusvertraging na UVC behandeling de verhoogde mutatie-inductie in mismatch herstel deficiënte cellen niet verdwijnt. Het 'direct signalling model' voorspelt verder dat een afname van het herstel van UVC-

geïnduceerde fotolesies door het uitblijven van het induceren van een celcyclus arrest in MMR-deficiënte cellen leidt tot een verhoging van het aantal mutaties in de getranscribeerde streng (fotolesies in de niet-getranscribeerde streng worden op enkele uitzonderingen na niet hersteld in muizencellen). Wij vonden echter geen verschil in de hoeveelheid mutaties per streng tussen de mismatch-herstel-proficiënte en –deficiënte cellen. Om deze reden geloven wij dat de UVC-geïnduceerde celcyclusvertraging eerder een indirect effect is van het herstel van de mismatch in de UVC-geïnduceerde fotolesie. In de afwezigheid van MMR zal de mutatiefrequentie toenemen op die plaatsen waar normaal gesproken MMR zou plaatsvinden. De hotspot op positie 599 in het *Hprt* gen van cellen met intact MMR kan verklaard worden door het feit dat mismatches op deze positie niet door MMR worden hersteld.

Curriculum vitae

Viola Borgdorff werd op 2 maart 1975 geboren in 's-Hertogenbosch. Na het behalen van haar VWO diploma aan de Scholengemeenschap Assen (later Scholengemeenschap Vincent van Gogh geheten) in 1993 begon zij in september van dat jaar aan de studie biologie aan de Rijksuniversiteit Groningen (RuG). Na twee stages in het veld van de kanker-immunotherapie (academisch ziekenhuis Groningen, afdeling Fysiologische Chemie o.l.v. Dr. F. Pries; academisch ziekenhuis Verona, Italië, afdeling Immunologie en Infectieziekten, o.l.v. Dr. M. Colombatti) en een stage met als onderwerp de intracellulaire signalering in *Ras* getransformeerde cellen (Beth Israel Deaconess Medical Center, Boston, afdeling hematologie/oncologie o.l.v. Dr. R. Janssen) studeerde zij in augustus 1998 af in de studierichting Medische Biologie. Na een korte periode gewerkt te hebben als analist binnen de Unité Papillomavirus van het Institut Pasteur in Parijs (o.l.v. Dr. F. Breitburd) begon zij in maart 1999 aan haar promotieonderzoek op de afdeling Stralengenetica en Chemische Mutagenese (later Toxicogenetica geheten) o.l.v co-promotor Dr. N. de Wind met als promotoren Prof. Dr. L.H.F. Mullenders en Prof. Dr. R. Fodde. Sinds juli 2005 is zij als Postdoctoral Research Assistant werkzaam op de afdeling Cutaneous Research van het Institute of Cell and Molecular Science binnen de Queen Mary University of London (QMUL) o.l.v. Prof. Dr. D.H. Beach.