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"Role of the MUTYH protein in the response to oxidative damage to DNA"

"Ruolo della proteina MUTYH nella risposta al danno ossidativo al DNA"

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ABBREVIATIONS

AP sites: Apurinic/Apyrimidinic sites

APE1: AP endonuclease

Aza: Azathioprine

BER: Base Excision Repair

DDR: DNA-damage response

dNTPs: deoxynucleoside triphosphates

DSBs: double strand breaks

FAP: Familial adenomatous polyposis

HR: homologous recombination

IR: ionizing radiation

LCLs: lymphoblastoid cell lines

LP-BER: Long-Patch BER

MAP: MUTYH-associated polyposis

MEFs: Mouse embryo fibroblasts

me6-TG: methyl-6-TG

MMR: Mismatch Repair

NER: Nucleotide Excision Repair

NHEJ: nonhomologous end-joining

Pol: polymerase

RNS: reactive nitrogen species

ROS: reactive oxygen species

SSBs: single-strand breaks

SP-BER: Short-Patch BER

ssDNA: single-strand DNA

UV: ultraviolet radiation

5'dRp: 5'-deoxyribose-5'-phosphate

6-TG: 6-thioguanine

8-oxo-dG: 7,8-dihydro-8-hydroxyguanine

8-oxo-dGTP: 8-oxo-2'-deoxyguanosine triphosphate

8-oxo-dGMP: 8-oxo-2'-deoxyguanosine monophosphate

9-1-1 complex: Rad1, Rad9, and Hus1 complex

RIASSUNTO

Da un numero sempre più crescente di evidenze sperimentali emerge che il danno al DNA è uno dei principali fattori causali nell'insorgenza del cancro e di molte patologie legate all'invecchiamento. Il DNA, al pari di tutte le molecole biologiche, rappresenta un target preferenziale di potenziali agenti di danno, sia esogeni che endogeni, che ne mettono a repentaglio l'integrità.
Le radiazioni ultraviolette, i raggi X, gli agenti chimici genotossici come anche i prodotti del metabolismo endogeno o di reazioni chimiche spontanee possono indurre lesioni permanenti nel genoma con gravi conseguenze a livello cellulare.
Le rotture a singolo (SSBs) o doppio filamento (DSBs) come anche gli appaiamenti errati di basi azotate, i foto-prodotti indotti dai raggi ultravioletti e le modificazioni chimiche a livello delle basi sono solo alcune delle potenziali modificazioni indotte da tali agenti di danno (Hoeijmakers, 2009), (figura 1, pagina 18). L'importanza del rischio associato a queste lesioni si riflette nell'esistenza di numerosi sistemi di riparazione del DNA (tabella 1, pagina 21), veri e propri meccanismi di salvaguardia del genoma dai quali dipende il destino della cellula in termini di sopravvivenza, senescenza o morte, in caso di danno. Nei mammiferi, alcuni fra questi sono rappresentati dal sistema di riparazione per escissione di basi (BER), dal sistema di riparazione per escissione di nucleotidi (NER), la riparazione degli appaiamenti errati (MMR) e la ricombinazione omologa (HR) e non omologa (NHEJ), (Maynard et al., 2009). Il processo di riparazione del danno, così come la replicazione del DNA, è coordinato in maniera fine e strettamente regolata con la progressione del ciclo cellulare attraverso dei complessi meccanismi di sorveglianza, noti come checkpoint del ciclo cellulare. Questi sono composti da una rete di sensori, trasduttori ed effettori (Bartek et al., 2004; Sancar et al., 2004), e vengono considerati essenziali per la sopravvivenza cellulare e dell'intero organismo (Brown et al., 2000; de Klein et al., 2000), (figura 2, pagina 23).
Alcuni fra i principali fattori endogeni di danno al DNA includono le specie reattive dell'ossigeno (ROS) generate dal normale metabolismo cellulare che, pur esercitando importanti funzioni in numerosi processi fisiologici, svolgono un ruolo causale in fenomeni quali la mutagenesi, la carcinogenesi e l'invecchiamento (Valko et al., 2006).
Le basi azotate sono fra i bersagli più sensibili all'ossidazione da parte dei ROS, e tra di esse la guanina risulta essere particolarmente suscettibile a causa del suo basso potenziale redox. Non sorprende dunque che la 8-oxo-7,8-diidroguanina (8-oxo-dG) sia una delle lesioni più abbondanti e meglio caratterizzate, spesso usata come bio-marcatore cellulare di stress ossidativo a carico del DNA. A causa della sua capacità di mimare funzionalmente la timina nella sua conformazione *syn,* essa si appaia stabilmente con l'adenina, introducendo nel genoma trasversioni di tipo G>T (David et al., 2007), identificate come mutazioni somatiche predominanti in diverse tipologie di cancro. Alla rimozione di tale lesione sono preposti molteplici sistemi di riparazione del DNA e tra

questi il BER è in prima linea. Il processo di eliminazione della 8-oxo-dG catalizzato dalle proteine del BER consiste in una sequenza di reazioni finemente coordinate in cui l'evento chiave è l'idrolisi del legame *N*-glicosidico tra la base modificata e il deossiribosio, catalizzata da DNA glicosilasi aventi ciascuna una diversa specificità di substrato (Sharma and Dianov 2007), (figura 3, pagina 29). Tra queste, la proteina MUTYH (mutY homolog (E.Coli)) rimuove l'adenina erroneamente appaiata con la 8-oxo-dG e contribuisce in tal modo, in cooperazione con la DNA glicosilasi OGG1 (8-Oxoguanine glycosylase), ad eliminare tale lesione dal genoma (figura 4, pagina 32).
Diversi domini funzionali sono stati identificati nella struttura proteica di MUTYH, deputati a processi quali il legame al DNA, la escissione della 8-oxo-dG e l'interazione con fattori della replicazione (PCNA, RPA), della segnalazione del danno al DNA (il complesso 9-1-1), o proteine di altri sistemi di riparazione come il MMR (MSH6) (Oka and Nakabeppu, 2011).
La capacità di MUTYH di interagire con proteine appartenenti a sistemi diversi dalla semplice riparazione del DNA suggerisce il suo possibile coinvolgimento in un *network* complesso di intercomunicazione che lascia supporre un suo ruolo più ampio e rilevante nel mantenimento dell'integrità del genoma.
L'importanza funzionale di questa proteina è inoltre sottolineata dall'associazione fra la presenza di mutazioni germinali nel gene *MUTYH* ed una malattia autosomica recessiva, la poliposi associata a MUTYH (MAP), caratterizzata da poliposi colorettale e da predisposizione al cancro. In tale sindrome ereditaria le mutazioni bialleliche di MUTYH sono tipicamente associate alla presenza di trasversioni G>T nel gene oncosoppressore *APC* (Adenomatous Polyposis Coli), oltre che, in un'alta percentuale di tumori derivati da pazienti MAP, nell'oncogene *K-RAS*.
Partendo da tali osservazioni, nel presente studio sono stati analizzati gli effetti prodotti dalla mutazione o dalla inattivazione del gene che codifica per questa proteina nella risposta biologica al danno ossidativo al DNA. In particolare, nella prima parte della tesi si è esaminato l'impatto prodotto a livello cellulare dalla presenza di mutazioni diverse di *MUTYH* in linee linfoblastoidi di pazienti affetti da MAP, in termini di ossidazione basale del DNA, riparazione del danno ossidativo, mutagenesi spontanea ed indotta.
Diverse varianti geniche di MUTYH sono state finora caratterizzate mediante l'uso di proteine purificate (Bai et al., 2005; Bai et al., 2007; Ali et al., 2008; Yanaru-Fujisawa et al., 2008; D'Agostino et al., 2010) sebbene gli studi biochimici siano stati limitati ad un numero relativamente ridotto di esse. Inoltre, mancano informazioni riguardanti il fenotipo mutatore associato all'inattivazione di *MUTYH* nell'uomo. Da uno studio condotto in precedenza nel nostro laboratorio era emerso che tutte le varianti di MUTYH associate a MAP erano accomunate dall'accumulo di 8-oxo-dG nel DNA e dall'ipersensibilità agli effetti citotossici del bromato di potassio ($KBrO_3$), un noto agente ossidante. Tale studio era stato condotto utilizzando un saggio in cui le singole proteine mutanti venivano espresse in fibroblasti embrionali di topo

(MEFs) derivate da topi *Mutyh* difettivi (Molatore et al., 2010). Tale tipo di approccio, tuttavia, non può essere usato per analizzare mutazioni eterozigoti composte di *MUTYH*, che si riscontrano piuttosto comunemente tra i pazienti italiani affetti da MAP. Allo scopo di studiare tali varianti complesse abbiamo utilizzato linee linfoblastoidi derivate da pazienti MAP con mutazioni missenso e troncanti in condizioni di omozigosi o di eterozigosi composta (Tabella 2, pagina 35 e figura 5, pagina 36).
La quantificazione dei livelli di espressione di MUTYH mediante RT-PCR e Western blotting ha rivelato che, mentre in alcune linee cellulari esiste una buona correlazione fra i livelli di trascritto e quelli di proteina, in altri casi invece, per lo più in linee caratterizzate da mutazioni di tipo frameshift, la presenza del trascritto non corrisponde a quella della proteina (Figura 6, pagina 37), lasciando ipotizzare che alcune mutazioni siano associate ad instabilità dei relativi trascritti.
In seguito alla messa a punto di un saggio in cui viene valutata l'attività glicosilasica sia di MUTYH che di OGG1 (figura 7, pagina 38) è stato possibile dimostrare che tutte le linee mutanti sono difettive nella rimozione dell'adenina da un substrato 8-oxoG:A, pur presentando una normale attività della glicosilasi OGG1 (Figura 8, pagina 39). In accordo con quanto dimostrato da altri autori (Wooden et al., 2004; Parker et al., 2005; Ali et al., 2008; Kundu et al., 2009) si è identificata una residua benché minima attività glicosilasica nelle due varianti p.Tyr179Cys/Tyr179Cys e p.Tyr179Cys/Gly396Asp caratterizzate dalla presenza di livelli di proteina simili alle linee wild-type di donatori sani.
E'stato inoltre interessante osservare che mentre la quasi totalità delle linee esaminate mostra livelli basali di 8-oxo-dG maggiori rispetto alle linee wild-type, le linee caratterizzate dalle mutazioni frameshift p.Gly264TrpfsX7 e p.Ala385ProfsX23, che non esprimono livelli rilevabili di proteina, fanno eccezione. Tale osservazione potrebbe suggerire la presenza di un effetto dominante negativo associato alla proteina mutante, probabilmente connesso all'interferenza con altri meccanismi di riparazione della 8-oxo-dG (Figura 9A, pagina 40).
Con l'obiettivo di verificare se l'incremento nei livelli basali della base ossidata fosse dovuto ad una sua difettiva rimozione nelle linee mutanti, si è eseguito un saggio di cinetica di riparazione dopo trattamento con $KBrO_3$, un agente ossidante che introduce un alto livello di 8-oxo-dG nel DNA. Tutte le linee con mutazioni di *MUTYH* hanno mostrato in effetti una rimozione più lenta della lesione quando confrontate con una linea di donatore sano (Figura 9B, pagina 40). La presenza di specifiche trasversioni G>T nei geni *APC* o *K-RAS* nei tumori di pazienti MAP può essere plausibilmente ritenuta un'evidenza indiretta della esistenza di un fenotipo mutatore associato alla presenza di una proteina MUTYH non funzionale (Al-Tassan et al., 2002; Jones et al., 2002, Lipton et al., 2003; van Pujienbroek et al., 2008). Partendo da tale osservazione, si è misurata, per la prima volta in linee di pazienti MAP, la frequenza di mutazioni spontanea, utilizzando una metodologia innovativa messa a punto dal gruppo di L. Luzzatto

(Araten et al., 2005) basata sulla determinazione per via citofluorimetrica della frequenza di mutazione di un gene sentinella, *PIG-A*, già applicata allo studio del fenotipo mutatore associato ad altri difetti della riparazione del DNA. Dai risultati ottenuti si è identificato un aumento medio di quattro volte nel valore della frequenza di mutazione di *PIG-A* nelle linee di pazienti rispetto al valore medio di tre linee wild-type (Figura 10, pagina 42). Inoltre, la ricomparsa di cellule mutanti dopo previa eliminazione tramite "sorting" dalla popolazione cellulare iniziale, in una delle linee con più alta frequenza di mutazione, ha dimostrato che il fenotipo mutatore identificato è una caratteristica intrinseca della linea cellulare. Nella stessa linea mutante trattata con $KBrO_3$, si è per di più osservata sia ipersensibilità agli effetti citotossici dell'agente ossidante che un fenotipo di iper-mutabilità rispetto ad una linea di donatore sano (Figura 11, pagina 43), confermando la presenza di un forte impatto biologico associato alla presenza di mutazioni di *MUTYH* nella risposta al danno ossidativo.

Nella seconda parte della tesi sono invece riportati e discussi i risultati ottenuti in un progetto condotto in parallelo in cui si sono studiati gli effetti dovuti all'assenza di MUTYH nella risposta ad un interessante modello di danno ossidativo prodotto dalla interazione del farmaco immunosoppressore Azatioprina (Aza) con i raggi ultravioletti di tipo A (UVA).

Come suggerito da diversi studi, il danno al DNA indotto dalla combinazione di tali agenti è stato riconosciuto come uno dei possibili fattori responsabili dell'aumentata insorgenza di cancro della pelle in pazienti immunosoppressi con Aza dopo trapianto d'organo (Brem et al., 2009). Il trattamento sistemico con Aza causa l'incorporazione nel DNA di 6-tioguanina (6-TG) (Relling and Dervieux, 2001) che, a differenza delle basi azotate canoniche assorbe le radiazioni UVA, agendo da cromoforo e generando ROS.

La stessa 6-TG è fortemente esposta al processo di ossidazione. In particolare, la sua interazione con i raggi UVA genera una lesione nota come guanina 6-sulfonato che costituisce un blocco alla replicazione del DNA ed è potenzialmente mutagena.

Studi realizzati su cellule umane hanno dimostrato che la 6-TG e i raggi UVA sono sinergicamente citotossici e mutageni inducendo pericolose lesioni a livello del DNA e delle proteine (O' Donovan et al., 2005).

Sulla base di tali osservazioni, e considerando l'importante funzione di MUTYH nella risposta al danno ossidativo, abbiamo esaminato il ruolo di questa proteina nella risposta al danno cellulare indotto dalla combinazione di 6-TG ed UVA. Dato il suo coinvolgimento nella regolazione della risposta cellulare alla 6-TG come anche nella rimozione dell'8-oxo-dG abbiamo esteso parte degli studi anche alla proteina MSH2 facente parte del sistema MMR.

Dai nostri saggi di sopravvivenza *in vitro* sulle MEFs è risultato che la combinazione di 6-TG e raggi UVA produce effetti tossici in cellule wild-type (WT), mentre il trattamento singolo con 6-TG o con UVA non ha effetti rilevanti sulla sopravvivenza cellulare. Sorprendentemente, sia l'assenza di *Mutyh* che di *Msh2* conferisce resistenza all'effetto tossico prodotto dal

trattamento combinato, (Figura 12, pagina 44) sebbene tutte le linee, indipendentemente dal genotipo, mostrano aumenti simili dei livelli di 8-oxo-dG nel DNA (Figura 13, pagina 45).
Poichè numerosi dati sperimentali indicano che, in cellule trattate con 6-TG, tale analogo di base risulta essere presente nel "pool" cellulare di deossinucleosidi trifosfato (dNTPs) dove può agire da fonte di ROS per esposizione ai raggi UVA (Cooke et al., 2008), si è pensato di determinare il potenziale contributo di questa "riserva" di 6-TG agli effetti biologici osservati.
Esperimenti svolti a tale scopo (Figura 14A, pagina 46) hanno rivelato che anche nel sistema da noi analizzato la 6-TG del "pool" contribuisce notevolmente sia agli effetti di citotossicità (Figura 14B, pagina 47) che di ossidazione (Figura 15 pagina 47) osservati dopo il trattamento combinato.
Con l'obiettivo di studiare il meccanismo alla base della tossicità riportata nelle cellule WT ed il ruolo esercitato da MUTYH in questo processo abbiamo analizzato la progressione del ciclo cellulare mediante citometria a flusso. Dai profili ottenuti è emerso che l'assenza MUTYH è associata al mancato arresto delle cellule in fase S dopo esposizione a 6-TG ed UVA, un arresto che si osserva invece in maniera prominente nelle cellule WT (Figura 16, pagina 48). Tale fenomeno si riflette in una diversa cinetica di attivazione del checkpoint: l'attivazione per fosforilazione della proteina Chk1, coinvolta nell'attivazione del checkpoint di fase S e G2/M, si osserva a tempi brevi dal trattamento combinato nelle cellule WT, mentre nelle cellule *Mutyh* difettive il segnale della proteina fosforilata compare in tempi molto più lunghi e con un'intensità notevolmente ridotta (Figura 17, pagina 49). Tali osservazioni sembrano supportare il coinvolgimento di MUTYH nell'attivazione precoce come anche nella regolazione del checkpoint di fase S in risposta a tale tipo di danno. E' noto che la fosforilazione di Chk1 da parte della chinasi ATR è coinvolta nel controllo della riparazione dei DSBs mediante il sistema di riparazione HR (Sørensen et al.; 2005). La quantificazione dei DSBs misurati come numero di foci dell'istone fosforilato, γH2AX, ha rivelato che, anche nel nostro modello murino, come nelle cellule umane (Brem et al.; 2010), il trattamento combinato con 6-TG e raggi UVA produce questo tipo di danno al DNA, e che i livelli sono paragonabili fra i due genotipi. Sorprende tuttavia che le due linee si distinguano nella modalità di processamento di tale danno. La determinazione numerica dei foci della proteina RAD51, facente parte del sistema HR, ha infatti rivelato che se da un lato nelle cellule WT vi è un'induzione più elevata di tale proteina nonché una sua persistenza a tempi lunghi dopo il trattamento con 6-TG ed UVA, dall'altro invece l'assenza di MUTYH è associata a livelli decisamente più bassi di foci e ad un graduale decremento degli stessi nel tempo (Figura 18, pagina 50). Se si considera che RAD51 esercita una funzione chiave nella sopravvivenza cellulare contro i potenziali effetti letali associati ai DSBs, l'ipersensibilità delle cellule WT alle conseguenze del trattamento combinato è in apparente contraddizione con l'induzione di una risposta chiaramente protettiva. Una possibile spiegazione di questo fenomeno consiste nell'ipotizzare

che il tentativo di riparazione del danno prodotto dall'interazione della 6-TG con i raggi UVA mediante attivazione del sistema HR induce la formazione di una quantità considerevole di intermedi riparativi estremamente tossici incompatibili con la sopravvivenza cellulare. In quest'ottica, l'assenza MUTYH e quindi di una modalità efficiente e/o canonica di riparazione del danno comporterebbe un vantaggio selettivo per la cellula stessa in termini di vitalità, associato tuttavia ad un possibile aumento dell'instabilità genomica.

Lo dimostrano infatti i dati derivati da uno studio parallelo condotto *in vivo* su animali wild-type e *Mutyh*-difettivi, sottoposti a trattamenti singoli o combinati (Aza somministrata per via intraperitoneale seguita o meno da esposizione ad UVA) per 12 mesi consecutivi. Come nel modello *in vitro*, il trattamento con entrambi gli agenti ha determinato una notevole differenza nella tossicità: l'80% dei topi difettivi è sopravvissuto al trattamento contro il 20% dei topi wild-type. Il trattamento singolo con i raggi UVA non ha invece prodotto effetti sulla sopravvivenza in nessuno dei due genotipi, mentre l'immunosoppressione prodotta dall'Aza ha determinato livelli di mortalità simile nei due genotipi.

E' stato inoltre osservato un incremento notevole nei livelli di 8-oxo-dG nella pelle di entrambi i gruppi di animali, sebbene non siano state riscontrate differenze significative nei livelli di ossidazione del DNA tra i due genotipi (Figura 19A, pagina 52). E' degno di nota, invece, che l'analisi istologica della pelle ha identificato la presenza di due carcinomi a cellule squamose in due topi *Mutyh*$^{-/-}$ (Figura 19B, pagina 52) dopo trattamento combinato suggerendo che l'assenza di una proteina fondamentale nella salvaguardia del genoma è associata ad una possibile predisposizione all'insorgenza di tumori della pelle.

Considerati nel loro complesso i dati ottenuti da tali studi mettono in luce l'importanza dell'effetto protettivo esercitato dalla proteina MUTYH nella risposta al danno ossidativo al DNA. Le osservazioni condotte sulle linee di pazienti affetti da MAP, oltre a sottolineare l'aspetto di patogenicità associato alla presenza della proteina mutante, consentono anche di individuare nell'accumulo di danno ossidativo nel DNA e nella presenza di un fenotipo mutatore sia spontaneo che indotto da stress ossidativo, fattori di estrema rilevanza per una migliore valutazione clinica della patogenesi associata alle varianti di MUTYH. Infine, i risultati ottenuti dallo studio condotto nel modello murino di inattivazione di MUTYH confermano la rilevanza biologica di tale proteina nella salvaguarda della stabilità genomica, indicando tuttavia un suo ruolo più complesso nella risposta al danno al DNA che va oltre il semplice meccanismo di riparazione del danno ossidativo.

SUMMARY

The oxidized base 7,8-dihydro-8-hydroxyguanine (8-oxo-dG) is one of the most deleterious injuries induced by oxidative stress. Multiple DNA repair proteins have evolved to protect the genome against the detrimental effects of this promutagenic lesion. One of the major ones is the Base Excision Repair (BER) MUTYH DNA glycosylase that removes adenine from 8:oxoG-containing mispairs originated by DNA polymerases and *via* the OGG1 DNA glycosylase contributes to 8-oxo-dG repair. Germline mutations in the *MUTYH* gene lead to MUTYH-associated polyposis (MAP), an autosomal recessive syndrome characterized by colorectal polyposis and cancer predisposition. Although several reports characterized MUTYH variants using purified proteins, relatively few mutations have been investigated from the biochemical point of view. In addition no information is available on the mutator phenotype associated with *MUTYH* inactivation in humans. In our previous study accumulation of 8-oxo-dG and hypersensitivity to killing by the oxidizing agent $KBrO_3$ were identified as a common phenotype among the investigated MAP-associated variants. These results were based on an assay in which single mutant MUTYH proteins were expressed in mouse embryonic fibroblasts (MEFs) derived from *Mutyh*-null mice (Molatore et al., 2010). This approach however cannot be exploited to analyze compound heterozygous *MUTYH* mutations, a very common situation among Italian MAP patients. With the aim of studying these particularly complex variants, we derived human lymphoblastoid cell lines (LCLs) from MAP patients harbouring missense and truncating mutations. RT-PCR and Western blotting analyses revealed that while in some cell lines there is a good correlation between transcripts and protein levels, in other instances no MUTYH protein is detectable.

When basal levels of 8-oxo-dG were measured in these cell lines, increases were detected in DNA of six LCLs expressing MUTYH variants when compared to two wild-type cell lines. Interestingly the only two exceptions were cells in which no detectable expression of the MUTYH protein could be identified.

To determine whether this increase in steady-state levels was due to a defective repair of 8-oxo-dG in these mutants, repair kinetics of this oxidized base were determined following exposure to $KBrO_3$. All the LCLs harbouring MUTYH mutations showed a defective repair of 8-oxo-dG when compared to a cell line from a healthy donor.

Results of a novel assay where both MUTYH and OGG1 activity could be evaluated indicate that all these variants were defective in removing adenine from an 8-oxoG:A DNA substrate, but retained wild-type OGG1 activity. Mutation frequency measurements at the *PIG-A* gene identified a four-fold increase in spontaneous mutagenesis in six LCLs from MAP patients when compared to three LCLs from healthy donors. Finally $KBrO_3$ hypersensitivity was accompanied by a hyper-mutable phenotype in a MUTYH mutant cell line. These observations support the pathogenic role of these *MUTYH* mutations and

identify accumulation of 8-oxo-dG and a mutator phenotype as relevant factors for a better clinical assessment of MUTYH variant pathogenesis.
In the second part of this thesis the results of a parallel study on the effects of MUTYH loss in response to a different type of oxidative damage are reported. This study addresses the toxicity and carcinogenicity of the anti-cancer immunosuppressant drug Azathioprine (Aza) combined with UVA radiation. Systemic treatment with Aza causes the incorporation of 6-thioguanine (6-TG) into DNA. 6-TG is a chromophore which generates reactive oxygen species **(**ROS) on exposure to UVA and is itself highly susceptible to oxidation. DNA damage induced by the Aza/UVA combination is thought to contribute to the huge incidence of skin cancer in immunosuppressed organ transplant patients. Several studies have shown indeed that Aza combined with low doses of UVA may cause mutagenic damage in human cells. In particular, 6-TG/UVA generates a novel DNA lesion, the guanine-6-sulfonate that blocks DNA replication and is potentially mutagenic. Here we examined the role of the MUTYH DNA glycosylase and the mismatch repair (MMR) MSH2 protein in the cellular response to 6-TG/UVA-induced DNA damage.
6-TG and UVA were synergistically toxic to wild-type mouse embryo fibroblasts (MEFs) while neither 6-TG or UVA alone detectably affected survival. *Mutyh*- or *Msh2*-defective cells were more resistant than wild-type MEFs to killing by 6-TG/UVA. Nevertheless, the combined treatment significantly increased the levels of DNA 8-oxo-dG irrespectively of the genotype. Interestingly, we also found in wild-type cells that the deoxynucleoside triphosphates (dNTP) pool contributed to both the increased levels of DNA 8-oxo-dG and the enhanced toxicity of a combined 6-TG/UVA treatment.
To better understand the mechanism of 6-TG/UVA toxicity and the relative role of the MUTYH protein we also analysed cell cycle progression by flow cytometry. The data suggest that the MUTYH protein is involved in the S phase arrest induced by the combined 6-TG/UVA treatment
We also reported a difference between the two cell lines in the timing of the checkpoint activation: phosphorylation of the Chk1 protein occurred immediately after 6-TG/UVA treatment in wild-type cells, while a later and weaker appearance of phosphorylated Chk1 occurred in *Mutyh*$^{-/-}$ cells. These data suggest that MUTYH might be involved in the activation of an S phase checkpoint following this type of oxidative damage. We also confirmed that in this mouse model, as in human cells, treatment with 6-TG/UVA produces an increase in double strand breaks (DSBs). These DSBs, as measured by γH2AX foci, were formed immediately and maintained up to 48 hr from the end of the treatment. The comparable increase in DSBs observed in *Mutyh*-defective cells indicates that the initial level of DNA damage is similar in the two genotypes. However analysis of the homologous recombination (HR) protein RAD51 foci following the combined treatment indicates that the DSBs resolution differs between the two genotypes. *Mutyh*$^{-/-}$ MEFs showed a significantly lower

induction of RAD51 foci in comparison to wild-type cells with a gradual decrease in the levels of foci at late post-treatment times. This contrasts with the persistence in wild-type cells of high levels of these foci indicating the presence of unresolved DSBs.

Finally we report the results of *in vivo* experiments in which the long-term toxicity of single or combined Aza/UVA exposures was analysed in wild-type and *Mutyh*-defective animals. Mice were treated with Aza given by intraperitoneal injection and/or UVA for 12 months. A differential toxicity between WT and *Mutyh*-defective animals was observed as consequence of the Aza plus UVA exposure. In fact, a high level of toxicity was identified in the group of wild-type mice (only 20% of the animals survived this exposure), while the 80% of *Mutyh*$^{-/-}$ animals survived this treatment. Survival was 100% in both UVA treated groups, while immunosuppression in Aza-treated groups was associated with some mortality, which was unaffected by the genotype.

A significant increase of DNA oxidation in the skin of animals exposed to the combined treatment was also observed, irrespectively of the genotype. Intriguingly when histopathological examination of the skin was performed two squamous cell carcinomas were identified only in *Mutyh*$^{-/-}$ mice exposed to Aza plus UVA, revealing a possible skin cancer proneness conferred by loss of this protein.

I. INTRODUCTION

1. Damage to DNA and human diseases

The genome integrity is pivotal in the maintenance of life. A variety of both exogenous and endogenous factors may compromise the architecture of the DNA double helix. Differently from proteins, lipids and RNA that, if damaged, are usually subjected to degradation, DNA lesions should be repaired. If unrepaired they may alter normal cell physiology and cell viability or even result in genomic instability (Radak and Boldogh, 2010).
If we bear in mind that the genome is a critical target for time-dependent deterioration, it is not surprising that DNA damage has been considered a critical factor for many diseases associated with aging.
There is a multitude of potential damage sources that may challenge DNA integrity and compromise its function. Hydrolysis, the principal spontaneous reaction occurring in a cellular environment, is intrinsic to the chemical nature of DNA in aqueous solution and it is responsible for creation of abasic sites and deamination events.
DNA is also continuously exposed to metabolism products, such as reactive oxygen and nitrogen species (ROS and RNS), endogenous alkylating agents, estrogen and cholesterol metabolites, reactive carbonyl species and lipid peroxidation products. Finally, some sources of carcinogenic DNA lesions originate in the environment: various exogenous physical and chemical agents, could represent a risk for DNA stability. Such chemicals can attack DNA, leading to adducts that impair base-pairing and/or block DNA replication and transcription, causing base loss and DNA single-strand breaks (SSBs). Ultraviolet light (UV) and ionizing radiation (IR) also generate various forms of DNA damage. The most toxic lesions caused by IR are double-strand breaks (DSBs). The biological consequences of both endogenous and exogenous injury factors generally depend on the location and number of lesions, the cell type, as well as the stage in the cell cycle and during differentiation (Figure 1) (Hoeijmakers, 2009).
If not properly removed, DNA damage can lead to DNA mutations and/or cell death, especially in the case of cytotoxic lesions that block the progression of DNA/RNA polymerases (Maynard et al., 2009). If not repaired, DNA mutations and large genomic alterations such as deletions, translocations, loss of heterozygosity, and amplifications may lead to cancer onset, while the occurrence of cell death or senescence may cause the acceleration of the aging process. Cell senescence and apoptosis are in fact suspected causes of aging under biological conditions associated to stem-cell exhaustion. Interestingly, whereas p53-induced cell death protects against tumorigenesis, pro-apoptotic p53 activity is harmful in settings such as stroke or heart attack. Induction of p53

by different sources of DNA damage can also affect the development of atherosclerosis (Jackson and Bartek, 2009).

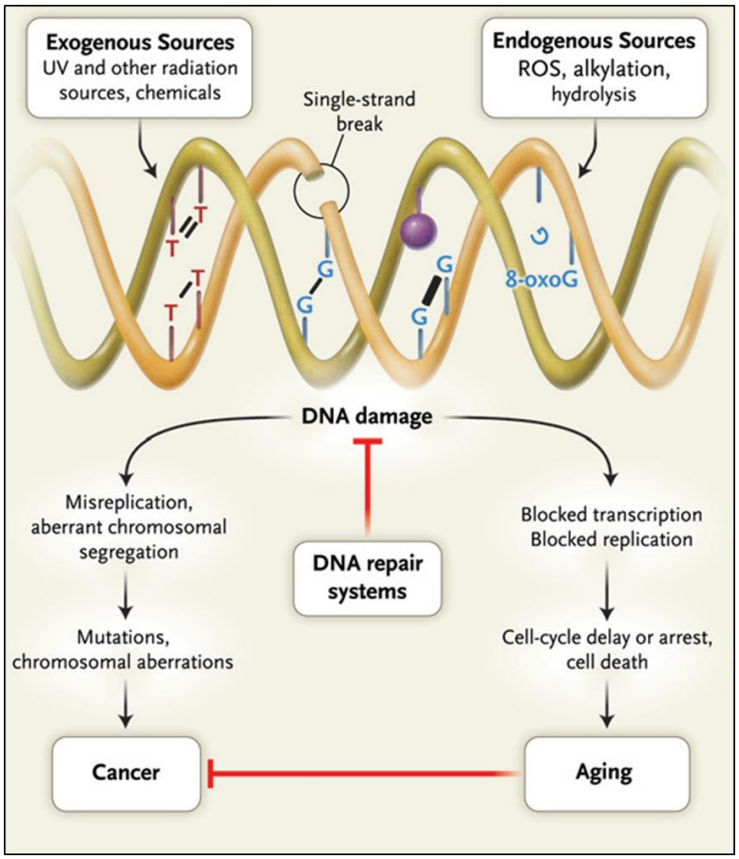


Figure 1. Sources and consequences of DNA Damage (Hoeijmakers, 2009)

1.1. The response to DNA damage: the genome-maintenance network

"We totally missed the possible role of.. [DNA] repair although I later came to realize that DNA is so precious that probably many distinct repair mechanisms would exist" Francis Crick, Nature, 26 April 1976

Genome instability caused by the continual attack to DNA from endogenous and environmental agents, would be an overwhelming problem for cells and organisms in the absence of DNA repair. In consideration of the great variety of DNA-damaging factors, it is not surprising that cells have evolved mechanisms – collectively termed the DNA-damage response (DDR) to detect DNA lesions, signal their presence and promote their repair (Wood et al., 2001).
Highly conserved pathways are tailored to deal with different classes of lesions, although some share many components and they usually occur by a common general program (Table 1). For example, three types of excision repair have been described: mismatch repair (MMR), nucleotide excision repair (NER) and base excision repair (BER) (Risinger and Groden, 2004). The principal aspects of the first two systems have been comprehensively summarized below, while the BER system will be analysed in full detail later on.
MMR is a central system in the repair of DNA replication errors and in the inhibition of recombination between non-identical DNA sequences. The key proteins in MMR are highly conserved from bacteria to mammals: in eukaryotes there are multiple homologs of the key bacterial MutS and MutL MMR proteins. (Harfe and Robertson, 2000). Six human mismatch repair genes (*MSH2*, *MSH3*, *MSH6*, *MLH1*, *PMS1* and *PMS2*) have been identified as components of this repair system that efficiently corrects single base mismatches and loops of one to three extrahelical nucleotides. The MSH2 and MSH6 proteins compose the heterodimer hMutSα that recognizes single base-base mismatches and small insertion/deletion mispairs. The *MSH2* and *MSH3* gene products form a heterodimer recognizing larger insertion/deletion mispairs, while the MLH1 protein together with PMS2 forms the human homolog of MutL, the hMutLα complex (Gu L et al., 1998).
The binding to DNA by one of the two mismatch recognition complexes (MutSα, MutSβ) is the first step in the error correction process. Complete excision and replacement of the mismatched section of DNA involves the MutLα complex (or another heterodimeric complex formed by MLH1 and hMLH3) as well as PCNA, RPA, DNA polymerase δ (pol δ) and EXO1 (Kunkel and Erie, 2005; Jiricny, 2006). MMR is also one of the alternative pathways involved in minimizing the toxic and mutagenic effects of the DNA 7,8-dihydro-hydroxyguanine (8-oxo-dG), a harmful DNA oxidation product causing G>T transversions and implicated in frameshift formation. The microsatellite instability and the mutator phenotype of MMR-defective cells strongly support a crucial role of MMR in the DDR (Macpherson et al, 2005). Moreover, inherited

MMR defects are associated with the hereditary nonpolyposis colorectal cancer (HNPCC), an autosomal dominant disorder.

NER is a highly versatile and sophisticated DNA damage removal pathway mainly defending mammalian cells against UV-induced DNA damage. Besides cyclobutane pyrimidine dimers and (6-4) photoproducts produced by UV rays, it also deals with bulky adducts resulting from exposure to various agents like cisplatin. The first step in the NER pathway is the lesion recognition process involving the *ERCC1, XPA* and *XPF* gene products followed by the interaction with the TFIIH transcription factor. A dual incision event is accomplished by the *ERCC1* and *XPG* gene products, and this is followed by exonuclease activity. DNA is then synthesized to fill the gap using the undamaged strand as a template, and the ends are ligated (Bohr , 1995). Defects in NER are reflected in the severe photosensitivity and predisposition to skin cancer associated to the prototype repair syndrome Xeroderma pigmentosum (de Laat et al., 1999) .

DSBs represent one of the most severe type of DNA damage and two additional types of DNA repair, homologous recombination (HR) and non-homologous end-joining (NHEJ), are tailored to deal with this type of lesion. Using a copy (usually the sister chromatid available during the S and G2 phases of the cell cycle) of the damaged segment as a template to mediate a faithful repair, HR is considered an error-free pathway. NHEJ, on the contrary, is an error-prone pathway, since free ends are joined without the use of a template *via* very small microhomologous repeats. As a result, repair may be associated to loss of nucleotides or translocations (Risinger and Groden, 2004). In NHEJ, DSBs are recognized by the Ku proteins that then bind and activate the protein kinase DNA-PKcs, leading to recruitment and activation of end-processing enzymes, polymerases and DNA ligase IV (Jackson and Bartek, 2009).

HR is always initiated by single-strand DNA (ssDNA) generation, which is promoted by various proteins including the MRE11-RAD50-NBS1 (MRN) complex. In events catalyzed by RAD51 and the breast-cancer susceptibility proteins BRCA1 and BRCA2, the ssDNA invades the undamaged template and, following the actions of polymerases, nucleases, and helicases, DNA ligation and substrate resolution occur. HR is also used to restart stalled replication forks and to repair interstrand DNA crosslinks.

Table 1. DDR mechanisms and components

DDR mechanism	Prime lesions acted upon	Key protein components
Direct DNA-lesion reversal	UV photo-products O^6 alkylguanine	Photolyase O^6-methylguanine methyltransferase (MGMT)
Mismatch repair (MMR)	DNA mismatches and insertion/deletion loops arising from DNA replication	Sensors MSH2-MSH6 and MSH2-MSH3 plus MLH1 PMS2, MLH1-PMS1, MLH1-MLH3, EXO1, polymerases δ and ε, PCNA, RFC, RPA, ligase I
Base excision repair (BER) and single-strand break repair (SSBR)	Abnormal DNA bases, simple base- adducts, SSBs generated as BER intermediates by oxidative damage or by abortive topoisomerase I activity	DNA glycosylases (sensors), APE1 endonuclease, DNA polymerases (β, δ, ε) and associated factors, flap endonuclease FEN1, ligase I or ligase III. SSBR can also involve polymerase β lyase activity, XRCC1, PARP-1, PARP-2, polynucleotide kinase (PNK) and aprataxin (APTX)
Nucleotide excision repair (NER)	Lesions that disrupt the DNA double-helix, such as bulky base adducts and UV photo products	Sensors elongating RNA polymerase, XPC-HR23B and DDB1/2, plus XPA, XPE, XPF/ERCC1, XPG, CSA, CSB, TFIIH (containing helicases XPB and XPD), DNA polymerases and associated factors, RPA, ligase I
Trans-lesion bypass mechanisms	Base damage blocking replication-fork progression	"Error-prone" DNA polymerases, including polymerases eta, iota, kappa, REV3 and REV1; plus associated factors
Non-homologous end-joining (NHEJ)	Radiation- or chemically-induced DSBs plus V(D)J and CSR intermediates	Sensors Ku and DNA-PKcs plus XRCC4, XLF/Cernunnos and ligase IV. Can also employ the MRE11-RAD50-NBS1 (MRN) complex, Artemis nuclease, PNK, Aprataxin and polymerases μ and λ
Homologous recombination (HR)	DSBs, stalled replication forks, inter-strand DNA cross-links and sites of meiotic recombination and abortive Topoisomerase II action	RAD51, RAD51-related proteins (XRCC2, XRCC3, RAD51B, RAD51C, RAD51D, DMC1), RAD52, RAD54, BRCA2, RPA, FEN1, DNA polymerase and associated factors. Promoted by MRN, CtIP, BRCA1, and the ATM signalling pathway
Fanconi anaemia (FANC) pathway	Inter-strand DNA cross-links	FA-A, C, D1/BRCA2, D2, E, F, G, I, J, L, M, N plus factors including PALB2 and HR factors
ATM-mediated DDR signalling	DSBs	ATM, MRN and CHK2. Promoted by mediator proteins such as MDC1, 53BP1 MCPH1/BRIT1, and by ubiquitin ligases RNF8, RNF168/RIDDLIN and BRCA1
ATR-mediated DDR signalling	ssDNA, resected DSBs	Sensors ATR ATRIP and RPA plus the RAD9-RAD1-HUS1 (9-1-1) complex, RAD17 (RFC1-like) and CHK1. Promoted by MRN, CtIP and mediator proteins such as TOPBP1, Claspin, MCPH1/BRIT1 and BRCA1

(Jackson and Bartek, 2009)

1.2. DNA-damage repair and checkpoint pathways

DNA repair, as well as DNA replication, is strictly coordinated with cell cycle progression. DNA damage and replication blocks activate signals that arrest cell cycle progression providing time for repair or trigger cell apoptosis when repair cannot be completed. These complex surveillance mechanisms, known as cell-cycle checkpoints, are controlled by a network of DNA damage sensors, transducers and effectors (Bartek et al., 2004; Sancar et al., 2004)(Figure 2).
Highly conserved DNA repair and cell cycle checkpoint pathways allow cells to deal with both endogenous and exogenous sources of DNA damage (Kastan and Bartek, 2004). Originally defined as dispensable regulatory pathways, DNA damage checkpoints are now considered as integrated components of the larger DDR. Recent lines of evidence demonstrated, in fact, the existence of an intimate connection between checkpoint components and DNA repair proteins (Zhou and Elledge, 2000).
Several checkpoint genes are also essential for cell and organism survival (Brown et al., 2000; de Klein et al., 2000) suggesting that their function is not restricted to DNA surveillance but is essential for cellular physiology.
Partly overlapping or redundant checkpoint pathways operate in various cell-cycle phases. Cells in G1 or G2 phases can counteract genotoxic stress by promoting checkpoints that provoke an arrest in G1 or G2 before re-entry into S phase or M phase of cell cycle, respectively. During DNA replication, on the contrary, the cellular response to potential DNA damage factors leads to a delay of cells progression through the S phase in a transient manner. If damage is not repaired, cells exit S phase and arrest at the G2 checkpoint (Bartek J et al., 2004). Despite the recent explosion of information regarding the molecular components of cell cycle checkpoints in eukaryotic cells, there is not a wide understanding of the identity of the DNA damage sensors or the mechanisms through which they initiate and terminate the activation of checkpoints. However, members of the Rad group of checkpoint proteins, which include Rad17, Rad1, Rad9, Rad26, and Hus1 are widely expressed in all eukaryotic cells where act as damage sensors (Green et al. 2000; O'Connell et al. 2000). Three of these Rad proteins, Rad1, Rad9, and Hus1 form a heterotrimeric DNA damage responsive complex (the 9-1-1 complex) that exhibits structural similarity with the homotrimeric clamp proliferating cell nuclear antigen, PCNA, that has the ability to slide across double-strand DNA (Majka et al., 2003). The 9-1-1 complex physically and functionally interacts with several DNA repair proteins, including factors involved in the BER pathway suggesting the presence of a close connection between checkpoint activation by 9-1-1 complex and recruitment of repair machinery (Meister et al., 2003). The 9-1-1 complex is loaded onto DNA by the Rad17/RFC2-5 complex and subsequently serves as a recruitment platform for several proteins, such as the Chk1 and Chk2 serine/threonine kinases. These effectors kinases promote cell cycle arrest,

transcriptional activation and apoptosis by phosphorylating critical targets (Guan et al., 2007). Chk1 and Chk2 are targets of regulation by two signal transducers proteins, ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3-related protein) kinases, respectively. ATM and ATR are PIKK (phosphoinositide three-kinase-related kinases) that share a number of phosphorylation substrates, even if they respond to different type of DNA damage. ATR plays a central role in the response to certain types of genotoxic agents, including hydroxyurea and UV and seems to have a central function in the S and G2 checkpoints. On the contrary, ATM has a major function for management of the G1 checkpoint and in contrast to ATR, provides a rapid protective response to an extremely lethal form of DNA damage, the DSBs. (Abraham, 2001). The main relevance for cell and organism life of this "network of genome surveillance" is reflected in the evidence that a major mechanism whereby tumor cells acquire genetic instability is through the acquisition of mutations that modify checkpoints. This feature renders them more dependent on the remaining intact pathways to promote repair and arrest the cell cycle, representing an important approach for the development of therapeutic strategies in the personalized cancer treatment (Medema and Macurek, 2011).

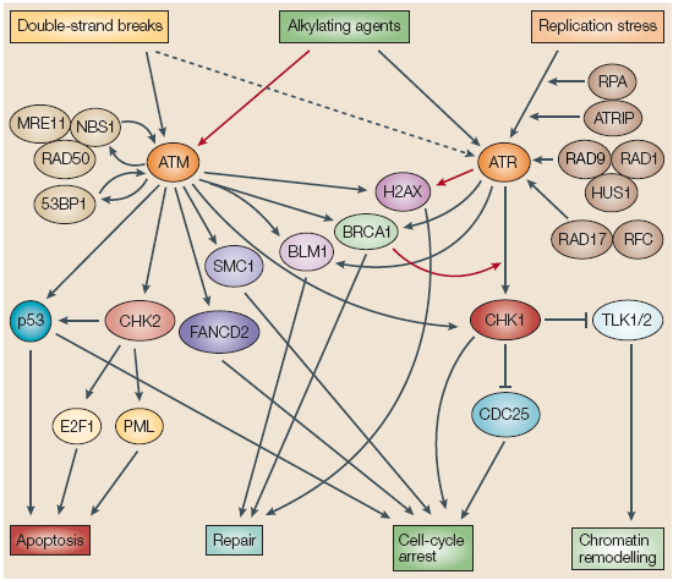


Figure 2. DNA-damage-response signal-transduction network (Zhou and Bartek, 2004)

2. The oxidative damage to DNA

The variety of reactions in which oxygen is implicated in the cellular environment leads to the formation of chemical intermediates known as ROS, that account for the background levels of oxidative DNA damage detected in normal tissue (Cooke et al., 2003).

During oxidative metabolism in mitochondria, oxygen is mainly converted to water and only a small percentage (0.2–2%) leads to ROS, because of the leakage of electrons directly to oxygen leading to formation of superoxide anions ($\bullet O2^-$). Either spontaneously or through catalysis by superoxide dismutases, superoxide anions can be further converted to hydrogen peroxide (H_2O_2), that can be next reduced to H_2O or partially reduced to the hydroxyl radical ($\bullet OH$), a very powerful oxidant (van Loon et al., 2010).

ROS may also be generated by IR or UV radiation, chemotherapeutic drugs and environmental exposures to transition metals and chemical oxidants (Maynard et al., 2009). Although ROS have a physiological role in numerous signaling pathways, in inflammatory processes and in preventing infections, they can also be genotoxic and oxidize various cellular components such as lipids, proteins and nucleic acids (Mitra et al., 2001). These "oxidative damage" events are thought to be involved in mutagenesis, carcinogenesis and aging. Thus, oxidative stress is accepted as a critical pathophysiological mechanism in different frequent pathologies, including cardiovascular diseases, cancer, diabetes, rheumatoid arthritis, or neurological disorders such as Alzheimer or Parkinson disease (Mena et al., 2009).

ROS-induced DNA damage involves SSBs and DSBs, purine, pyrimidine, or deoxyribose modifications, and DNA cross-links, that can lead to either arrest or induction of transcription, activation of signal transduction pathways, replication errors and genomic instability (Valko et al., 2006).

More than 100 products have been identified as generated by the oxidation of DNA, with bases in DNA being particularly sensitive to ROS oxidation. The low redox potential of guanine makes this base particularly susceptible to oxidation resulting in the formation of a great variety of potential oxidized products. Thus, not surprisingly, 8-oxo-dG is one of the most abundant and well-characterized DNA lesions generated by ROS, often used as a biomarker in cells to indicate the extent of DNA oxidative stress. It arises by the introduction of an oxo group on the carbon at position 8 (C8) and a hydrogen atom to the nitrogen at the position 7 (N7). 8-oxo-dG in *syn* conformation is particularly mutagenic because of its strong ability to functionally mimic T and to form a stable base pair with adenine. In contrast to many other types of DNA damage, these structural features provide for efficient, though inaccurate, bypass of the lesion by replicative DNA polymerases representing a direct source of C:G to A:T transversion mutations (David et al., 2007). The estimated steady-state level of

8-oxo-dG lesions is about 10^3 per cell/per day in normal tissues and up to 10^5 lesions per cell/per day in cancer tissues.
Recent extensive studies of the patterns of somatic mutations in genomes of different cancer types shed light on the abundance of C:G to A:T transversion mutations and identified them among the most predominant somatic mutations in lung, breast, ovarian, gastric and colorectal cancers (van Loon et al., 2010).

2.1. Response to oxidative damage: the special problem of 8-oxo-dG

The removal of oxidative DNA lesions has a crucial role in the limitation of mutagenesis, cytostasis, and cytotoxicity. The existence of multiple overlapping repair processes of oxidative DNA damage introduces a fail-safe element to DNA repair, such that attenuation or elimination of one repair process does not preclude removal of a particular lesion (Cooke et al., 2003).
The deleterious effect of 8-oxo-dG, in particular, is emphasized by the evolution, from bacteria to humans, of several mechanisms to neutralize it. The main protective device that many organisms have developed is a three-component enzyme error-preventing system (termed the "GO system" after 8-oxo-dG). In humans this system consists of three repair proteins: the 8-oxo-dGTPase (MTH1), the 8-oxo-dG DNA glycosylase (OGG1) and the MutY glycosylase homologue (MUTYH), that will be described in details below. The MTH1 protein, the homolog of *E.Coli* MutT, acts by hydrolyzing 8-oxo-dGTP to 8-oxo-dGMP, thereby eliminating it from the pool of DNA synthesis precursors so that it cannot be incorporated into DNA by DNA polymerases (Tsuzuki et al., 2007).
OGG1 targets the C:8-oxo-dG mispair, removing the lesion. Upon DNA binding, the C:8-oxo-dG base pair is disrupted and the 8-oxo-dG flipped out of the double helix. Several studies have indicated that OGG1 initiated repair follows the SP-BER pathway (Dianov et al., 1998), in which DNA pol β seems to be responsible for the re-synthesis step (Fortini et al., 1999).
MUTYH acts instead by removing the misincorporated adenine opposite an 8-oxo-dG localized in the template strand. The direct association of MUTYH with both PCNA and RPA is fundamental to direct the MUTYH activity to the newly synthesized strand. This is of great importance in order to prevent a possible fixation of an A:T to C:G mutation. Interestingly, MUTYH was also found to excise another type of oxidative lesion, the 2- hydroxyadenine from DNA, (Ohtsubo T et al., 2000), suggesting that its function may be broader than the removal of A from 8- oxoG:A mispairs.
There are several lines of the evidence indicating that even though the "GO system" is the most important repair mechanism of 8-oxo-dG from genomic DNA, cells possess alternative repair pathways that can handle 8-oxo-dG damage (Klungland et al., 1999). Besides OGG1 and MUTYH, the two bifunctional DNA glycosylases NEIL1 and NEIL2 might be involved in BER of

8-oxo-dG. NEIL1 can in fact excise an 8-oxo-dG from a duplex DNA containing C:8-oxo-dG base pair (Hazra et al.,2002). NEIL2 can also excise 8-oxo-dG, but only when it is present inside a bubble structure suggesting that it may function during BER linked to transcription or replication (Dou et al., 2003). The MMR proteins also contribute to 8-oxo-dG repair. The human MutSα complex efficiently recognizes DNA 8-oxo-dG as well as another oxidation product, 2-hydroxyadenine, in some contexts that resemble frameshift intermediates (Macpherson et al., 2005; Barone et al., 2007). It has been shown that MMR also provides supplementary protection by excising incorporated 8-oxo-dGMP (Colussi et al., 2002) and this 8-oxo-dG derived from an oxidized pool of deoxynucleoside triphosphates (dNTPs) is an important cofactor in the genetic instability that characterizes MMR-deficient cells. Indeed hMTH1 overexpression in MMR-defective *Msh2*$^{-/-}$ mouse embryonic fibroblasts (MEFs) drastically attenuates their mutator phenotype (Russo et al., 2004).
There is also evidence that NER might act as a backup for the repair of this lesion (Reardon et al., 1997). Several data suggest a role of both the Cockayne syndrome B (CSB) and A (CSA) proteins, in this process. The similar impairment of 8-oxo-dG repair observed in the absence of the CSB (Licht et al., 2003) or CSA (D'Errico et al., 2007) proteins suggests a role in the same pathway of response to oxidative stress.
Furthermore, CSB interacts with BER proteins such as APE1 (Wong HK et al., 2007) and there are some evidence of a possible interplay between CSB and OGG1 (Khobta et al., 2009). Another member of the NER pathway, the Xeroderma Pigmentosum complementation group C (XPC) protein, might also be involved in 8-oxo-dG repair. Mice deficient in XPC display an elevated sensitivity to oxidative damage, susceptibility to lung carcinogenesis (Melis et al., 2008) and increased levels of C:G to A:T transversion mutations in lympohocytes (Wijnhoven et al., 2000). Some data indicate also a role for XPC as a cofactor of BER by stimulating the activity of the DNA glycosylase OGG1 in the removal of 8-oxo-dG from DNA (D'Errico et al., 2006).

2.2. Sources of cellular 8-oxo-dG: potassium bromate and combination of 6-thioguanine and UVA

Potassium bromate ($KBrO_3$) is an oxidising agent that has been used as a food additive and can also be produced from bromide during the disinfection of water by ozonation (Parsons and Chipman, 2000). Although its activity is only very weak in some microbial assays, there is no doubt that $KBrO_3$ can act as a mutagen as indicated by chromosome aberration and micronucleus tests, (Ishidate et al.,1984). Furthermore, $KBrO_3$ acts as a tumor promoter in renal cell tumours, mesotheliomas of the peritoneum and follicular cell tumours of the thyroid in F344 rats (Kurokawa et al., 1990).

Several observations, in particular the detection of increased levels of 8-oxo-dG in the kidney DNA of treated rats and the inhibition of this DNA oxidation by various antioxidants (Sai et al., 1992) led to a correlation between $KBrO_3$ induced-carcinogenicity in rodents with its ability to oxidize DNA.
The mechanism whereby $KBrO_3$ can oxidize DNA is however not clear. It is well known that glutathione, which is protective against most oxidants and alkylating agents, mediates $KBrO_3$ metabolic activation, while bromate itself does not react directly with DNA, despite its high oxidation potential. Even though the ultimate DNA damaging species has not yet been established, experiments under cell-free conditions suggest that ROS such as superoxide, hydrogen peroxide or singlet oxygen are not involved. Rather bromine radicals or oxides might be responsible (Ballmaier and Epe, 2006).
$KBrO_3$-induced DNA damage was found to consist mostly of base modifications sensitive to the Fpg protein, a glycosylase recognizing particularly 8-oxo-dG, while lesions such as SSBs and sites of base loss (apurinic/apyrimidinic sites, AP sites) were formed only in low amounts. A similar "damage profile" was also found in mammalian cells (Ballmaier and Epe, 1995).
As well as being produced as a result of simple oxidizing agents such as $KBrO_3$, DNA 8-oxo-dG might also be generated by more complex interactions among different chemical and/or physical agents. One of these is the combination between the thiopurine Azathioprine (Aza) and UVA radiation.
Aza is an anti-cancer immunosuppressant drug, often used in the treatment of inflammatory conditions. Thiopurines are all prodrugs and their metabolism results in the formation of 6-thioguanine (6-TG) nucleotides and, finally, in 6-TG incorporation into DNA (Relling and Dervieux, 2001). Long-term treatment with Aza results in detectable DNA 6-TG in patients' lymphocytes and in cells of the skin (Warren et al., 1995). In addition continuous immunosuppression in organ transplant patients is associated with an incidence of skin cancer that is up to 200-fold higher than that of non-immunosuppressed individuals (Brem et al., 2009). Although several factors may be involved in this phenomenon, some epidemiological evidences suggest that sunlight exposure is an important cofactor (Euvrard et al., 2003). Differently from the canonical DNA bases, thiopurines do absorb UVA (wavelengths 320–400 nm) and incorporated 6-TG therefore introduces into DNA a strong UVA photosensitizer. This suggests a possible mechanism by which sunlight and Aza might interact to promote the development of skin cancer (Brem et al., 2009). The interaction between DNA 6-TG and UVA is an important source of oxidative stress, generating ROS. The oxidative damage caused to DNA by ROS might therefore contribute to the development of transplant-related skin cancer (Zhang et al., 2007). ROS can inflict collateral modifications on surrounding normal DNA bases, sugars and DNA-associated proteins. As well as being a source of ROS, 6-TG itself is also a target for oxidation and one oxidized form of the thiobase is guanine-6-sulfonate (GSO_3), a bulky DNA adduct, incapable of stably pairing with any of the canonical DNA bases. This adduct can also block primer extension by DNA

polymerases and can be bypassed, in a potentially mutagenic strategy, only by DNA polymerases with a relatively low fidelity (O' Donovan et al., 2005).
A further outcome of the enhanced reactivity of the thiol group is the major susceptibility of 6-TG to other chemical modifications such as methylation. Similarly to 6-TG, methyl-6-TG (me6-TG) codes ambiguosly during replication and directs C and T insertion approximately equally. DNA me6-TG base pairs are recognized by MMR in a futile processing effort that causes cell death. One important consequence of this phenomenon is the possible selective proliferation of rare MMR-defective cells following long exposures to this base analog, and since MMR defects are associated to a marked mutator phenotype, this might favour the development of malignancy (Karran and Attard 2008).

2.3. The Base-excision repair: structural and functional aspects

The BER pathway is the primary mechanism for repair of oxidized base lesions. Besides 8-oxo-dG, other base modifications repaired by BER include formamidopyrimidines (4,6-diamino-5-formamidopyrimidine, FapyG) uracil and 3-methyladenine, resulting from cytosine deamination and alkylating agents, respectively (Krokan et al., 2002, Sedgwick et al., 2007). An overlap of the other DNA repair systems with BER has also been described. MMR and recombinational repair, in fact, provide protection against base pair mismatches and strand breaks (Russo et al., 2007), while NER can overlap with BER in repairing a variety of single-base DNA modifications (Memisoglu and Samson L, 2000). Although the understanding of the mammalian BER system is not yet complete, the fundamental enzymatic steps operating in this pathway have been extensively studied. The sequence of reactions consists of base removal, strand cleavage at the AP site, processing of the incised strand, gap filling DNA synthesis and DNA ligation (Wilson et al., 2010). Since unattended BER intermediates are cytotoxic and highly mutagenic (Loeb, 1985), a sequential step-to-step coordination is probably operating to sequester these dangerous repair intermediates, as described in the suggestive “passing the baton” model elaborated by Wilson and Kunkel (Wilson et al., 2000).
DNA glycosylases initiate the BER process by catalyzing hydrolysis of the N-glycosylic bond between the sugar and the base, thus releasing the damaged base to form an AP site. Eleven DNA glycosylases, with various specificities have been described so far in mammalian BER and classified in two distinct mechanistic classes: mono-functional such as UDG, and bi-functional such as OGG1. The first type DNA glycosylases create AP sites through cleavage of the N glycosylic bond using an activated water molecule as an active site nucleophile (Sharma and Dianov, 2007). The major 5’-AP endonuclease, APE1, utilizes the AP site and generates a DNA repair intermediate that contains a SSB with 3’-hydroxyl and 5’-deoxyribose-5’ phosphate (5’dRp) termini (Demple et al., 1991). The 5’dRp terminus is next excised by the dRp lyase activity of pol β

and a one-nucleotide gap is created. Bi-functional glycosylases have an associated AP lyase activity which can further process the AP site by incising the DNA backbone 3' to the AP site. The next step in the BER repair process consists of two distinct sub-pathways (Figure 3): short-patch BER (SP-BER) and long-patch BER (LP-BER), depending on the damage and the responsive enzymes. They are differentiated by the size of the repair patch synthesized by the repair DNA polymerases: one nucleotide in the case of SP-BER (Dianov et al., 1992) and two to 7-8 nucleotides in the case of LP-BER (Frosina et al., 1996**)**. DNA pol β is the major repair DNA polymerase involved in SP-BER. In the LP-BER DNA pol β has been described to most likely incorporate the first nucleotide (Podlutsky et al., 2001), while the subsequent elongation step is carried out by the replicative DNA pols ε or δ. LP-BER also involves flap endonuclease 1 (FEN1), proliferating cell nuclear antigen PCNA, replication factor C (RFC), DNA ligase I in addition to DNA glycosylase and AP endonuclease (Klungland and Lindahl, 1997). The final ligation step in SP-BER is coordinated by DNA ligase III/X-ray repair cross complementing 1 protein (XRCC1) complex and in LP-BER by DNA ligase I. (Tomkinson et al., 2001).

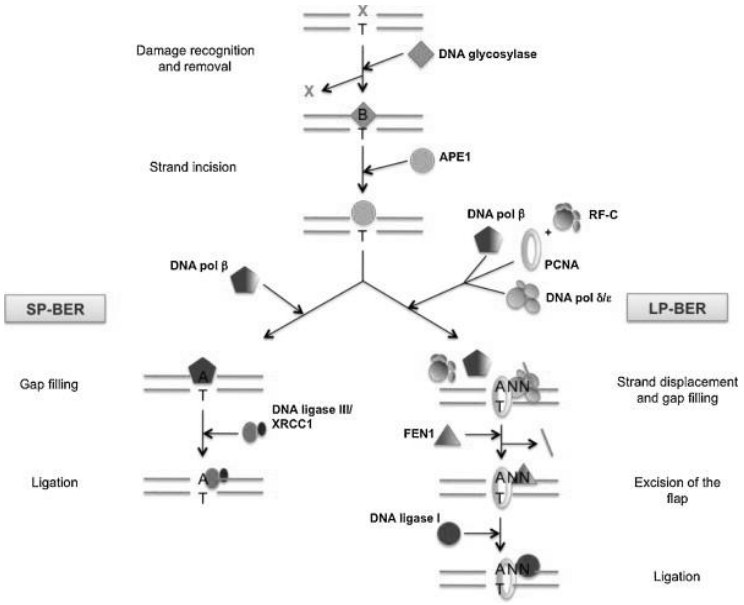


Figure 3. Short-patch (SP) and long-patch (LP) base excision repair (BER) pathways (van Loon et al., 2010)

The importance of BER as a critical process for genomic maintenance, is highlighted by the severe phenotypes observed in animals deficient in BER function, including cancer, premature aging and metabolic defects. Mouse knockouts of genes coding for core BER proteins, including XRCC1, POLβ, APE, FEN1 and DNA ligase I are embryonic lethal. On the contrary, *Mutyh* and *Ogg1* knockout mice show a much more moderate phenotype (Maynard et al., 2009): they are generally characterized by increases in DNA 8-oxo-dG levels in an age- and tissue-specific fashion accompanied by moderate increases in mutation rates (Russo et al, 2004 and 2009). Nevertheless recent reports showed an increased cancer susceptibility of *Ogg1*$^{-/-}$ and *Mutyh*$^{-/-}$ mice affecting respectively the lung and the gastrointestinal tract and occurring late in life (Sakumi et al, 2003; Sakamoto et al, 2007).
Genetic diseases caused by mutations in the BER pathway genes are rare. Up to now the only known example is the MUTYH associated polyposis (MAP) caused by inherited biallelic mutations in the *MUTYH* gene.

2.4. The problem of 8-oxo-dG: the special function of MUTYH

MUTYH, the human *E. coli* mutY homolog, is a 59-kDa protein encoded by a gene located on the short arm of chromosome 1 (1p32.1-p34.3) that spans 11.2 kb and contains 16 exons (Out et al., 2010). Transcription of *MUTYH* is initiated from three distinct exon 1 sequences and results in the production of three different primary transcripts: α, β and γ with different 5'-untranslated regions. These transcripts are furthermore subjected to alternative splicing in exon 1β and exon 3 with the production of 15 additional transcripts encoding at least nine different isoforms of the MUTYH protein (Oka and Nakabeppu, 2011). The functional significance of these isoforms is not entirely clear: they might have different glycosylase activity levels and/or different expression levels in various tissues (Ma et al., 2004).
The most abundantly expressed is the isoform 2, which has been found localized in mitochondria, while the isoform 4 is the most abundant nuclear isoform (Ohtsubo et al., 2000; Takao et al.,1999). For what concerns the structure, MUTYH is characterized by the presence of 15 functional domains involved in DNA binding, base flipping, catalysis, excision, 8-oxo-dG and adenine detection/recognition, and interaction with other DNA replication and repair proteins (Out et al., 2010). In particular, MUTYH consists of a catalytic core domain with an [4-Fe–4S] iron sulfur cluster at the N-terminus (Guan et al., 1998) and a C-terminal "MutT-like" domain (Shibutani et al., 1991). The N-terminal domain also contains a mitochondrial localization signal and the interacting motif with RPA, while the C-terminal domain is involved in the nuclear localization sequence and in the interaction with PCNA (Takao et al., 1998; Parker et al., 2001).

As previously mentioned, the MUTYH protein protects the cells from the mutagenic effects of 8-oxo-dG. In particular its function, as part of the BER pathway, consists in the removal of adenines misincorporated opposite 8-oxo-dG. The structural analysis of the bacterial protein MutY revealed the biochemical basis for recognizing both bases in the A:8oxoG pair and for catalysing the removal of adenine. Through the catalytic core and the MutT-like domains, MutY encircles the DNA making close contacts to the appropriate DNA strand. In a second step, MutY completely extrudes the substrate adenine nucleoside from the DNA helix and inserts it into a deep extrahelical active site pocket on its N-terminal domain. The oxo-dG lesion is, on the other hand, fully intrahelical and establishes extensive contacts with the MutT-like domain (Lee and Verdine, 2009). After the lesion recognition, the following step is the glycosidic bond cleavage through acid catalyzed protonation of the nucleobase (Figure 4).

The catalytic activity of MUTYH is probably subjected to an accurate modulation through the interaction with other proteins. Several MUTYH interactors have been hitherto identified, such as APE1, PCNA, RPA, Hus 1 (9-1-1 complex), MSH6 as well as other proteins involved in BER (Oka and Nakabeppu, 2011).

The interaction with APE1, PCNA, and RPA suggests that MUTYH catalyzes the base excision repair via a PCNA-dependent LP-BER route. Moreover, the docking of MUTYH onto PCNA and RPA couples BER to DNA replication: in this way the MUTYH activity can be directed to repair of the misincorporated adenines on the newly synthesized strand, but not on the parental strand (Parker et al., 2001). On the other side, the physical and functional interaction of MUTYH with the 9-1-1 complex, mainly via Hus1, might promote its catalytic activity in a stress-inducible way and supports a model in which MUTHY might act as an adaptor for sensor checkpoint proteins (Shi et al., 2006). MutSα through a direct interaction of MUTYH with MSH6 has been proposed to promote MUTYH activity, enhancing the binding affinity of the enzyme for A:8oxo-G containing DNA substrates. Thus MMR enzymes might efficiently assist the repair activity of a BER component. Taken together, these evidences suggest that the MUTYH protein is engaged in a network of molecular interactions, which is suggestive of a complex role of this protein outside the repair of oxidative DNA damage. Thus the role of this protein might be more complex than previously thought, reflecting its fundamental role in genome integrity maintenance (Parker and Eshleman, 2003).

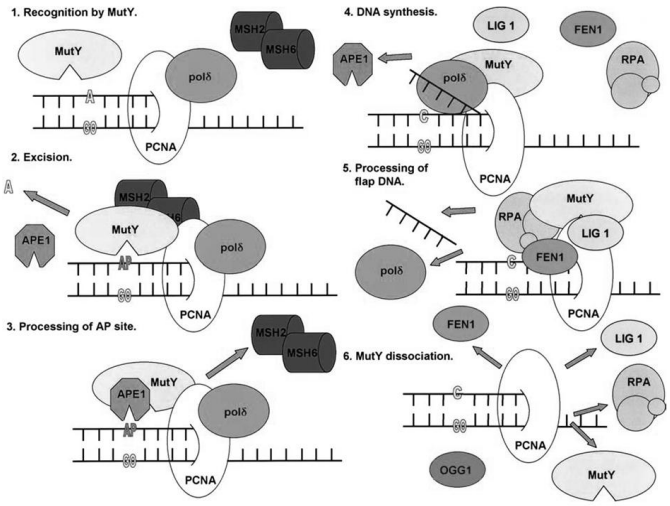


Figure 4. Proposed mechanism of repair by MUTYH : step 1, recognition; step 2, Excision; step 3, processing of the AP site; step 4, DNA synthesis; step 5, processing of flap structure; step 6, MUTYH dissociation (Parker and Eshleman, 2003)

2.5. Defective *MUTYH*: MUTYH-associated polyposis

The significance of preventing mutations caused by 8-oxo-dG is emphasized by the functional consequences of germline biallelic mutations in the *MUTYH* gene. MAP is a recessively heritable sindrome, characterized by the development of multiple colorectal adenomas, resulting in an increased risk of colorectal cancer (Al-Tassan et al., 2002; Jones et al., 2002; Sieber et al., 2003; Poulsen and Bisgaard, 2008; Jasperson et al., 2010).

The colorectal phenotype of MAP closely resembles Familial adenomatous polyposis (FAP), an autosomal dominantly inherited syndrome, caused by mutations in the adenomatous polyposis coli (*APC*) gene. However, in contrast to FAP, defective MUTYH in MAP results in a typical pattern of somatic G:C to T:A transversions in the *APC* gene (Pope et al., 2005). This is a novel mechanism by which inherited defects in a gene for a BER enzyme leads to somatic mutations in another cancer predisposing gene (*APC*). In addition to *APC*, also the *K-RAS* oncogene harbors G to T transversions in the first G of codon 12 in a high proportion of tumors from patients with biallelic *MUTYH* mutations. This is consistent with the typical spectrum of somatic mutations in MAP tumors reflecting both selection and hypermutation to which certain guanine residues are particularly prone (Lipton et al., 2003). The same type of *K-RAS* mutation was also observed in mice deficient in both *Mutyh* and *Ogg1*

genes, exhibiting a high susceptibility to tumor formation, predominantly lung and ovarian tumors and lymphomas (Xie et al., 2004).
Other groups successively confirmed the description of MAP by Al-Tassan in 2002 as the first autosomal recessive inherited form of colorectal cancer. These findings led to the conclusion that biallelic mutations in the *MUTYH* gene drive genomic instability in colorectal epithelial cells and result in an increased risk of neoplastic transformation in the colon (Wang et al., 2004; Farrington et al., 2005; Cardoso et al., 2006; Cleary et al., 2009). Currently, testing for mutations in *MUTYH* is recommended for patients exhibiting clinical features of FAP that are negative for inherited mutations in *APC* or do not show a family history consistent with dominant transmission of FAP (Lipton and Tomlinson, 2004).
To date, more than 299 *MUTYH* unique variants among MAP patients and/or controls have been described in the LOVD database (Out et al., 2010), with the p.Tyr179Cys and p.Gly396Asp variants as the most common documented mutations in Caucasian populations (Al-Tassan et al., 2002; Gismondi et al., 2004; Isidro et al., 2004; Cheadle and Sampson, 2007).
Considering the very recent detection of MAP as a disease, the lack of knowledge on molecular and functional characteristics of MAP-associated MUTYH variants makes diagnosis and clinical treatment of affected patients and their family members particularly complex. Most MUTYH variants are indeed missense mutations and their effect on protein function is difficult to predict when present in homozygosity and even more in a genetic condition of compound heterozygosity. In addition there is a need to set up functional tests, which take into account the biological context in which the variants are present. These might represent the basis for establishing genotype-phenotype correlations, thus providing an important tool in clinical practice as well as new insight into how *MUTYH* mutations contribute more globally to cancer.

II. RESULTS

Part 1

LCLs derived from MAP patients show different MUTYH expression levels

LCLs were established from eight MAP patients carrying biallelic, either compound heterozygous or homozygous, *MUTYH* variants. The phenotypic features of these patients are shown in Table 2. The family history in all biallelic mutation carriers corresponded to an autosomal recessive mode of inheritance, although four out of eight patients occurred as sporadic cases (FAP117, FAP278, FAP349, FAP483). According to the average MAP features, all studied biallelic carriers were phenotypically similar to attenuated APC-polyposis patients and characterized by a limited number of colorectal polyps (range 30-150), with the first diagnosis in adult ages (range 37-51 years). The pathology records of four patients (FAP236, FAP349, FAP483 and FAP527) reported however co-existing adenomatous and hyperplastic polyps. With the exception of two patients (FAP12 and FAP117), in which adenomas had been detected in small intestine, no apparent extracolonic disease manifestations were observed in the other biallelic mutation carriers.

Table 2. Phenotypic features and germline mutations identified in MUTYH mutation carriers

Patient ID	1st mutation	2nd mutation	Sex	Age	Polyp No.	CRC	Extracolonic disease
FAP 12	c.536A>G p.(Tyr179Cys)	c.536A>G p.(Tyr179Cys)	F	42	<100	YES	ileal adenoma
FAP 117	c.933+3A>C p.(Gly264TrpfsX7)	c.1437del p.(Glu480del)	M	46	>100	NO	duodenal adenoma
FAP 182	c.733C>T p.(Arg245Cys)	c.1187G>A p.(Gly396Asp)	F	37	<50	YES	NO
FAP 236	c.1187G>A p.(Gly396Asp)	c.1227_1228dup p.(Glu410GlyfsX43)	F	42	>100	NO	NO
FAP 278	c.933+3A>C p.(Gly264TrpfsX7)	c.1147del p.(Ala385ProfsX23)	F	45	<100	YES	NO
FAP 349	c.933+3A>C p.(Gly264TrpfsX7)	c.C933+3A>C p.(Gly264TrpfsX7)	F	51	<50	NO	NO
FAP 483	c.734G>A p.(Arg245His)	c.734G>A p.(Arg245His)	M	50	<50	NO	NO
FAP 527	c.536A>G p.(Tyr179Cys)	c.1187G>A p.(Gly396Asp)	M	45	<50	YES	NO

The position of the variants in relation to the *MUTYH* coding region together with the estimated locations of the assumed functional domains of the MUTYH protein (Out et al., 2010) are shown in Figure 5.

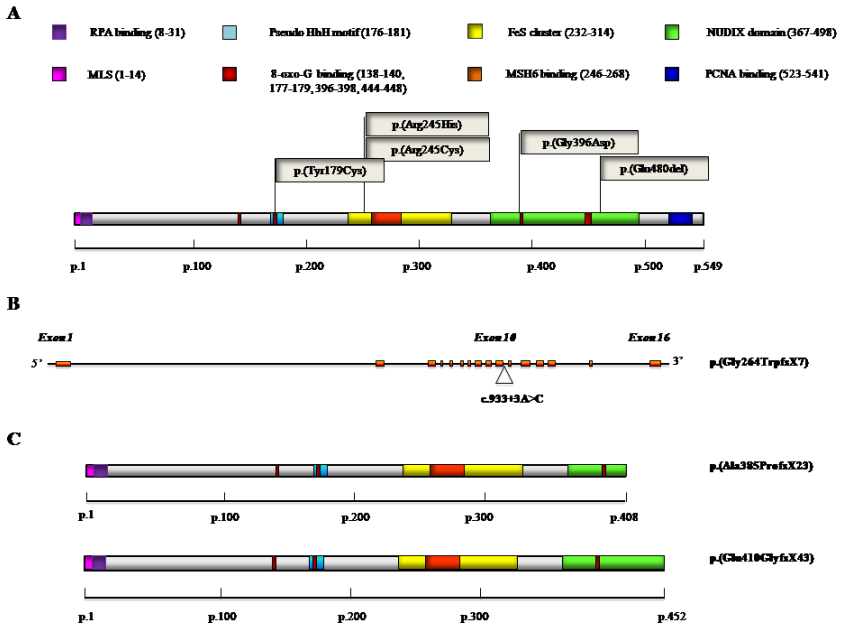
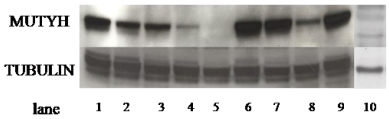


Figure 5: Location along *MUTYH* of the variants analyzed in this study.
A) Localization of missense (p.Tyr179Cys, p.Gly396Asp, p.Arg245Cys and p.Arg245His) and small in-frame deletion (p.Glu480del) mutations in the *MUTYH* gene in relation to the putative functional domains of the protein. **B)** Graphic representation of the c.933+3A>C splicing variant leading to skipping of exon 10 and the possible formation of a truncated protein (p.Gly264TrpfsX7).
C) Predicted truncated gene products of the p-Glu410GlyfsX43 and p.Ala385ProfsX23 mutations.

Expression of *MUTYH* in these variants was analysed by Real-time PCR using specific primers and a probe located at the junction between exons 5 and 6 of the gene. This allows detecting both mitochondrial and nuclear isoforms of *MUTYH*.
The comparison of *MUTYH* expression levels, normalized using the mean of two housekeeping genes (β-actin and β-tubulin), between eight LCLs from MAP patients and three LCLs from healthy donors indicate that only in two instances *MUTYH* levels were similar to wild-type cells (p.Tyr179Cys/Tyr179Cys and p.Tyr179Cys/Gly396Asp).

Thus a 20-35% decrease in *MUTYH* transcripts was observed in four LCLs harbouring the p.Arg245Cys/Gly396Asp, p.Gly264TrpfsX7/Glu480del, p.Arg245His/Arg245His and p.Gly264TrpfsX7/Gly264TrpfsX7 variants. These levels were further decreased to 50% of the wild-type protein in LCLs carrying the p.Gly264TrpfsX7/Ala385ProfsX23 and p.Gly396Asp/Glu410GlyfsX43 variants (data not shown).
Protein levels were then measured by Western blotting (Figure 6). Reproducible levels of the MUTYH protein were observed in three independent experiments and a comparison with two wild-type LCLs (lanes 6, 7) from healthy donors confirms that in some cell lines there is a good correlation between transcripts and protein levels (lanes 1, 2, 3 and 9). In other instances, often in LCLs harbouring frameshift mutations, no MUTYH protein is detectable (lanes 5 and 10), mainly because these truncated proteins might not be identifiable by our antibody that recognizes an epitope at the C-terminus of the protein (aa 435-535). We cannot exclude however that the transcripts undergo nonsense-mediated decay. Finally a low level of the MUTYH protein is also observed in LCLs with the homozygous Arg245His mutation suggesting that this specific mutation confers instability to the MUTYH protein (Figure 6).



MUTYH variants	Transcript (%)	Protein (%)
1. p.Tyr179Cys/Tyr179Cys	88	100
2. p.Gly396Asp/Glu410GlyfsX43	49	56
3. p.Arg245Cys/Gly396Asp	78	72
4. p.Gly264TrpfsX7/Glu480del	77	19
5. p.Gly264TrpfsX7/Ala385ProfsX23	45	<1
8. p.Arg245His/Arg245His	70	28
9. p.Tyr179Cys/Gly396Asp	100	89
10. p.Gly264TrpfsX7/Gly264TrpfsX7	64	5

Figure 6: **Quantitative analysis of MUTYH in wild-type and MUTYH variant LCLs.**
Relative levels of MUTYH transcript and protein in the eight MUTYH variant LCLs. Total amount of MUTYH mRNA (nuclear and mitochondrial forms) was obtained by real-time PCR. Reference values for transcripts are the mean of MUTYH expression in the wild-type BR806, BR805, BR77 LCLs. Relative expression of MUTYH protein was determined by Western blotting on eight MUTYH variants *versus* the wild-type BR806 cell line A representative Western blot of the MUTYH protein in whole cell extracts is also shown. Lanes 1 to 10 correspond to the LCLs indicated in the left panel, lanes 6 and 7 correspond to the wild-types cell lines BR806, BR805.

All LCLs derived from MAP patients have a defective MUTYH activity

In the majority of reports, with a few exceptions (Gu and Lu, 2001; Alhopuro et al., 2005; Parker et al., 2005), purified or partially purified MUTYH proteins were used to investigate the DNA glycosylase activity of MUTYH variants (Bai et al., 2005; Bai et al., 2007; Ali et al., 2008; Yanaru-Fujisawa et al., 2008; D'Agostino et al., 2010). Here we report a novel protocol to measure both MUTYH and OGG1 glycosylase activity in cell free extracts from human LCLs. Whole cell extracts prepared from wild-type BR77 cells line showed increasing DNA glycosylase activity as a function of protein concentration on a 30-mer containing a single 8-oxo-dG:A mispair (lanes 3-5, Figure 7A). A similar level of activity was observed in extracts from a second wild-type cell line, the BR806 LCL, with incision levels reaching 15-20% of the DNA substrate (Figure 7B). A dose response for the OGG1-dependent removal of 8-oxo-dG from a duplex with an 8-oxoG:C mispair was also identified in cell extracts prepared from wild-type BR77 cells line (Figure 7C).

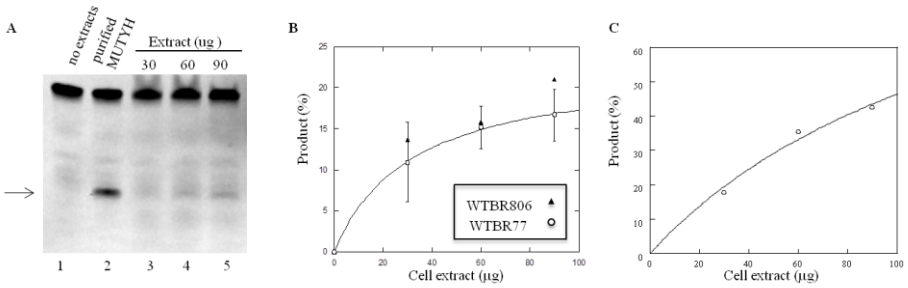


Figure 7: Dose response of MUTYH and OGG1 DNA-glycosylase activity in cell extracts from wild-type BR77 LCL.
A) A representative gel of the reaction products due to MUTYH activity on an 8-oxoG:A substrate is shown. A 3'-end fluorescent labelled DNA substrate was incubated with increasing amount of cell extracts. The product of the DNA-glycosylase activity in cell-free extracts is a fragment of 16 bases (indicated by an arrow) that co-migrates with the product of the purified MUTYH protein.
Control DNA (lane 1); reaction product of a purified MUTYH protein (lane 2), reaction products following incubation with 30, 60 and 90 µg of cell extracts from WTBR77 LCL (lanes 3-5).
B) Fluorescent band intensities from triplicate gels (as in A) were quantified using the public domain NIH *ImageJ* software and data analysis was performed with Kaleidagraph software. The percentage of reaction product following incubation with increasing amount of cell extracts from the wild-type BR77 cells (empty circle) are shown together with values obtained in a second wild-type cell line (BR806, full triangle). **C)** Plot of the percentage of reaction product due to OGG1 activity on an 8-oxoG:C substrate following incubation with increasing amount of cell-free extracts from WTBR77 LCL.

MUTYH DNA glycosylase activity was then investigated in cell-free extracts prepared from LCLs from MAP patients (Figure 8A, top panel). In six LCLs no MUTYH activity was identifiable, while a residual 5% enzymatic activity was still present in the p.Tyr179Cys, either in homozygousity or in combination with the p.Gly396Asp variant (lanes 5 and 6, Figure 8A and B). Parallel measurements of OGG1 activity indicate that these cell lines are similarly efficient in the removal of 8-oxodG from 8-oxoG:C mispairs (Figure 8A, bottom panel).
These data demonstrate that all the eight LCLs derived from MAP patients share a clearly defective MUTYH activity but still maintain a functional OGG1 glycosylase.

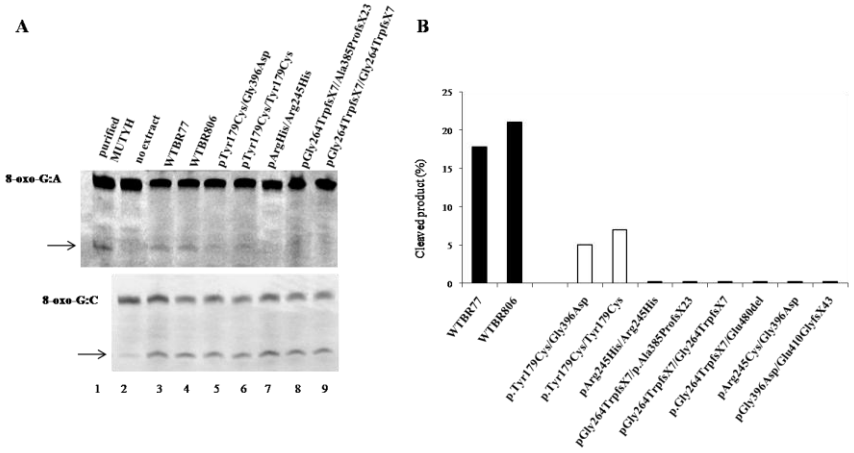


Figure 8: **MUTYH and OGG1 DNA-glycosylase activity in cell extracts from LCLs from different MAP patients.**
A) Representative gels of MUTYH and OGG1glycosylase activity in different LCLs. 6-FAM-labelled 8-oxoG:A (top) and 8-oxoG:C DNA substrate (bottom) were reacted with 90 ug of cell extracts at 37°C for 1h and treated with NaOH to visualize abasic site formation. Positive control with MUTYH purified protein (lane 1); control DNA (lane 2). **B)**The percentage of product obtained after reaction of the 8-oxoG:A DNA substrates with LCL cell extracts as described in Figure 8 A is shown.

MUTYH variants show a defective repair of DNA 8-oxo-dG

Inactivation of the *MUTYH* gene in rodents is associated with an accumulation of DNA 8-oxo-dG observed both *in vitro* and *in vivo* (Molatore et al., 2010; Russo et al., 2009). To verify whether this occurs also in human cells, steady-state levels of this oxidized purine were measured by HPLC/EC in LCLs from MAP patients and compared to control LCLs derived from two healthy donors.

Basal levels of DNA 8-oxodG in the BR77 and BR806 wild-type cell lines were 0.44 and 0.49 residues $x10^{-6}$ dG, respectively (Figure 9A). Increases that ranged from 1.3 up to 1.7-fold were detected in DNA 8-oxodG levels of six LCLs expressing MUTYH variants ($p \leq 0.02$-0.001) (Figure 9A).

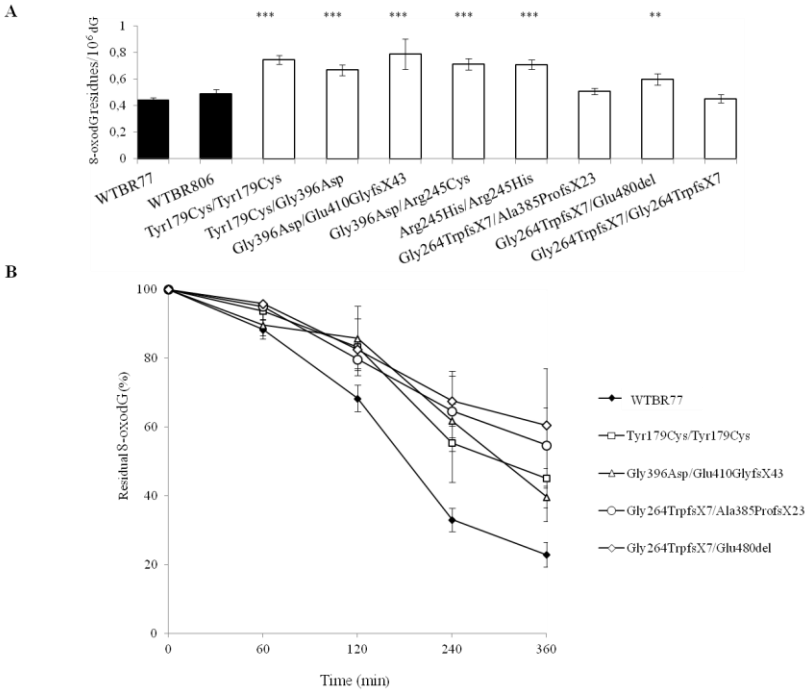


Figure 9: DNA 8-oxodG levels in wild-type and MUTYH variant LCLs.
A) Steady-state DNA 8-oxodG levels were measured in wild-type BR77 and BR806 LCLs by HPLC/EC. Data are mean±SE from 6-11 independent measurements. ** $p<0.01$, *** $p<0.001$ (Student's *t* test). **B)** Repair kinetics of DNA 8-oxodG following exposure to a 30-min treatment with 20mM $KBrO_3$ in wild-type and MUTYH variant LCLs. Rate of DNA 8-oxodG removal was measured at the indicated time points. Data are mean±SE from five experiments.

The two exceptions were cells harboring the p.Gly264TrpfsX7 variant either in homozygosity or in combination with the frameshift mutation p.Ala385ProfsX23. It is noticeable that these are the only cell lines in which no detectable expression of the MUTYH protein could be identified.
To confirm that the steady-state accumulation of 8-oxodG was due to a defective MUTYH activity, four LCLs were treated with the $KBrO_3$ oxidant, which introduces a high level of 8-oxo-dG in DNA, and levels of the oxidized purine were measured at different repair times (Figure 9B). All the LCLs with variant

MUTYH, including the p.Gly264Trpfs/Ala385ProfsX23 variant which does not express the protein, showed DNA 8-oxo-dG repair kinetics much slower than those observed in wild-type BR77 cells (Figure 9B), with almost a doubling in the half-life of the lesion (360 min vs. 180 min).
These data indicate that loss of MUTYH activity is always associated with an accumulation of DNA 8-oxo-dG in cells exposed to an oxidative stress. In the majority of cases this defect can also be identified at basal levels, with the expression of mutant MUTYH proteins leading to a more profound effect than the simple protein loss.

A defective MUTYH activity is associated with a spontaneous mutator phenotype

To investigate whether the defective MUTYH activity in LCLs from MAP patients is associated with increased mutagenesis, mutation frequency at the X-linked *PIG-A* gene were compared in wild-type and MUTYH variant LCLs. Mutations in this gene result in either complete or partial deficiency of glycosylphosphatidylinositol (GPI)-linked proteins from the cell membrane and GPI-negative cells can be identified by flow cytometry analysis as CD48, CD59 and CD55-negative cells (Araren et al., 2005; Peruzzi et al., 2010). The experimental procedure is shown in Figure 10A. When multiple measurements of mutation frequency at this gene were performed at weekly intervals in three wild-type cell lines (BR77, BR805 and BR806), a mean value of 21.7 x 10^{-6} GPI-negative events (10.9, 31.7 and 22.5 x 10^{-6}, respectively) was observed, with modest variations among different measurements.
This value is in the range of values previously reported for wild-type human cell lines (Araren et al., 2005; Peruzzi et al., 2010). LCLs from MAP patients, as a group, showed an increased mutation frequency when compared to values recorded in wild-type cells with a mean value of 87.6 x10^{-6} GPI-negative events, ranging from 42.2 (the homozygous pGly264TrpfsX7) to 157 x 10^{-6} GPI-negative events (pArg245His/Arg245His) (unpaired t-test, p=0.04) (Figure 10B).
In order to exclude that this high mutational load was due to a genetic drift of the cell population, GPI-negative cells were eliminated by flow sorting in the p.Gly396Asp/Glu410GlyfsX43 cell line. This sorted cell population was then expanded *in vitro* for several days and re-analyzed for mutation frequency at the *PIG-A* gene (Figure 10C). Once the initial GPI-negative events had been cleared, mutation frequency measured after several days in culture raised up to 149 mutation x 10^{-6} cells. Thus flow cytometry analysis of this cell population revealed the reappearance of the GPI-negative cells, demonstrating the tendency of this *MUTYH* defective cell line to accumulate mutations in the absence of any mutagenic stress. We can conclude that a spontaneous mutator phenotype is a characteristic feature of cells with a defective MUTYH activity.

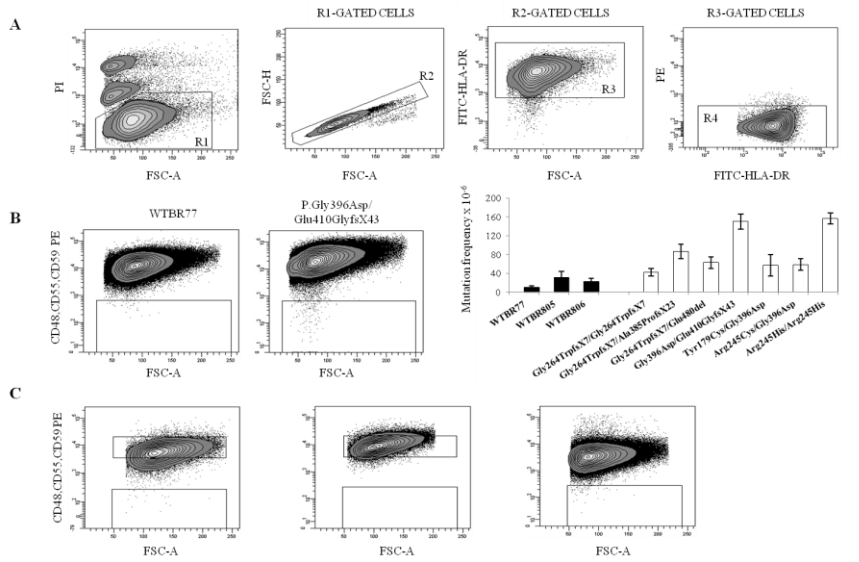


Figure 10: Mutation frequency at the *PIG-A* gene.
A)Flow cytometry methodology used to establish the population to analyze for the measurement of mutation frequency. Dead cells were excluded by propidium iodide staining (PI/FSC-A), while cells doublets were discriminated and excluded by comparing Area and Height signals of Forward scatter parameters (FSC-H/FSC-A) (first two panels). The FITC-HLA-DR positive population was gated (third panel) and evaluated in the PE channel (last panel), in order to obtain a correct compensation of FITC emission and to establish, at the same time, the gating necessary to determine the distribution of mutant cells in the PE channel. **B)** Representative flow cytometry dot plot analysis of WTBR77 (first panel) and p.Gly396Asp/Glu410GlyfsX43 (second panel) LCLs stained with antibodies against CD48, CD55, CD59 (all GPI-anchored proteins) and HLA-DR (a not GPI-anchored protein) antigens. Mutant cells, represented by GPI-negative events (lower quadrant of panels) do not express CD48, CD55, CD59 but do express HLA-DR. Spontaneous mutation frequencies, calculated as the fraction of GPI-negative events in HLA-DR positive cells (third panel) measured at one week intervals in wild-type (BR77, BR805 and BR806) and mutant MUTYH LCLs. Each bar represents the mean of 4-11 independent values. **C)** Cytometry dot plots of p.Gly396Asp/Glu410GlyfsX43 cells before (left) and after (center) sorting for GPI-positive population. Reanalysis of the same sorted population after several days in culture (right) shows the reappearance of GPI-negative cells.

MUTYH impairment causes a hypermutable phenotype in response to oxidative stress

We have previously shown that MUTYH-defective variants are hypersensitive to the toxic effects of oxidative damage introduced into DNA by $KBrO_3$ exposure (Molatore et al., 2010). To investigate whether this enhanced sensitivity to killing induced by an oxidant was also associated with a hypermutable phenotype, mutation frequencies were compared in wild-type BR77 and pGly396Asp/Glu410GlyfsX43 LCLs following treatment with increasing $KBrO_3$ concentrations (Figure 11).

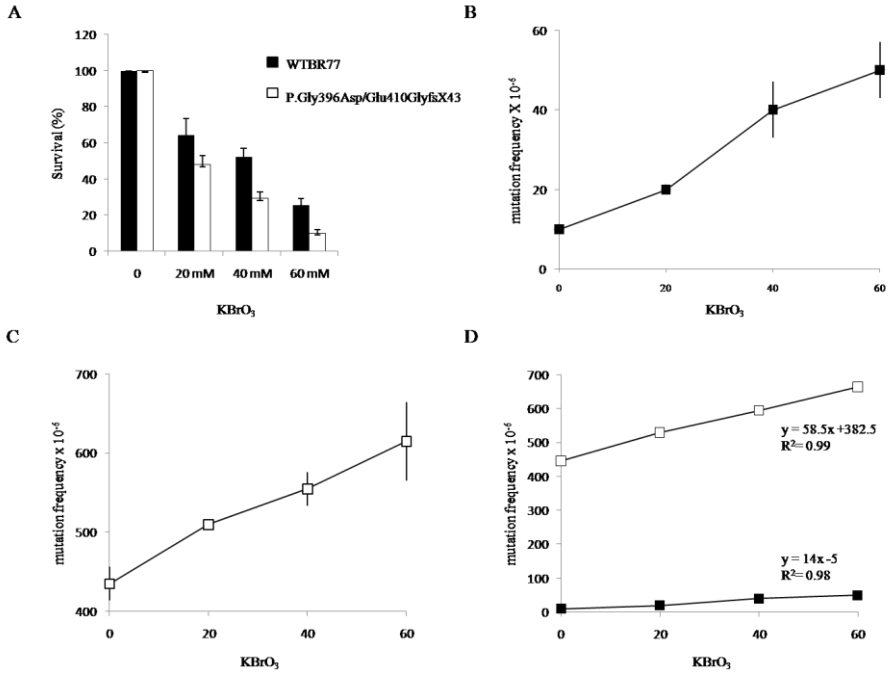


Figure 11:$KBrO_3$-induced mutations at the *PIG-A* gene in wild-type BR77 cells and MUTYH-defective p.Gly396Asp/Glu410GlyfsX43 cells.
A)Survival measured in wild-type BR77 cells (black) and p.Gly396Asp/Glu410GlyfsX43 cells (white) 7 days after a 30 min exposure to increasing concentrations of $KBrO_3$. **B)** and **C)** Mutation frequency measured 7 days after a 30 min treatment with increasing concentrations of $KBrO_3$ in wild-type BR77 (B) and p.Gly396Asp/Glu410GlyfsX43 (C) cells. Values are the mean and SD of two independent experiments. **D)** Comparison of the dose response curves for mutation induction by $KBrO_3$ exposure in WTBR77 and p.Gly396Asp/Glu410GlyfsX43 LCLs.

In comparison to wild-type cells the MUTYH-defective cell line was hypersensitive to killing by $KBrO_3$ (Figure 11A). Although a linear increase in mutation frequency was observed as a function of dose in both cell lines (Figure 11B and C), the number of mutations introduced into the genome by $KBrO_3$ exposure was much larger in pGly396Asp/Glu410GlyfsX43 than in BR77 cells (Figure 11D). This is evident by the comparison of the respective slopes (y=58.5x+382 *vs* y=14x-5, respectively) with an increment of 4.2 fold in the mutagenic response. These data indicate that the defective removal of 8-oxo-dG associated with a MUTYH defect leads to a hypermutable phenotype when cells are exposed to an oxidizing agent.

Part 2
***Mutyh* or *Msh2* deficiency confers resistance to the combined effects of 6-TG and UVA**

In order to investigate the role of the *MUTYH* gene in modulating the biological response to DNA damage induced by a combined 6-TG/UVA exposure, we set up an *in vitro* experimental model in which Mouse embryo fibroblasts (MEFs) derived from *Mutyh*- or *Msh2*- defective mice were compared to wild-type (WT) cells. As WT cells we used *Mutyh*$^{-/-}$ MEFs transfected with the pYMv200–MUTYH vector containing the human MUTYH cDNA (Molatore et al.; 2010).
Because of the established function of the MMR MSH2 protein in controlling 6-TG toxicity, as well as accumulation of 8-oxo-dG into DNA, we also aimed to examine the role of this protein in the 6-TG/UVA treatment-induced effects.
Viability, as determined by clonal assays, was compared in MEFs derived from *Mutyh*$^{-/-}$ and *Msh2*$^{-/-}$ mice as well as in WT cells. First of all the response to single treatments was analysed. As reported in human cells (O'Donovan et al.;2005), UVA exposure did not modify cell survival in any cell type. In contrast a single 6-TG exposure mainly affected, at a comparable level, survival of WT and *Mutyh*$^{-/-}$ cells, while *Msh2*$^{-/-}$ MEFs displayed the expected tolerance to this type of DNA damage (data not shown) (Bignami et al.; 2003). When cultures were exposed to a combined 6-TG/UVA treatment (growth in 60 nM 6-TG for 48 hours followed by UVA irradiation), WT MEFs showed a considerably reduced cell survival in comparison to *Mutyh*$^{-/-}$ and *Msh2*$^{-/-}$ MEFs (Figure 12).

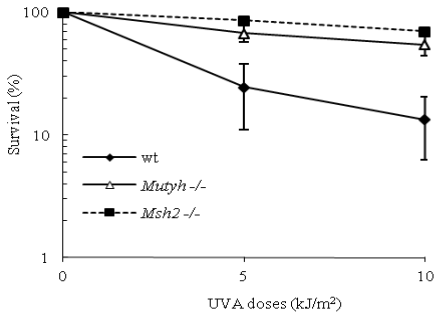


Figure 12: Survival following a combined 6-TG and UVA treatment.
WT, *Mutyh*-or *Msh2*-defective MEFs were exposed to a non-toxic concentration of 6-TG (60 nM) for 48 hours and irradiated with UVA (5 or 10 kJ/m^2). Survival was determined by clonal assays and expressed as percentage of the cloning efficiency in 6-TG. Data are mean±SE from at least 6 independent measurements.

The lethal effects were also found to be proportional to the UVA dose (5 and 10 kJ/m^2). We concluded that 6-TG and UVA are synergistically toxic to WT MEFs, while loss of either *Mutyh* or *Msh2* confers resistance to the detrimental effects of the combined treatment.

The combined 6-TG and UVA treatment significantly increases the levels of DNA 8-oxo-dG

To study the extent of oxidative stress associated with a combined 6-TG/UVA exposure, levels of DNA 8-oxo-dG were analysed in MEFs of different genotype (Figure 13).

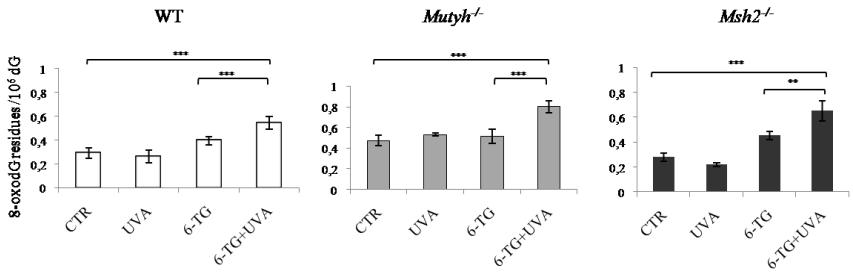


Figure 13: DNA 8-oxo-dG levels in WT, *Mutyh*- or *Msh2*-defective MEFs. The amount of 8-oxo-dG was measured by HPLC/EC in untreated MEFs as well as followingUVA exposure or a single or a combined exposure to 6-TG (0.6 mM for 48 h) and UVA (10 kJ/m^2).Data are mean±SD from at least 6 independent measurements. ** p<0.01, *** p<0.001 (Student's *t* test).

Irradiation with UVA alone did not modify steady-state levels of DNA 8-oxo-dG in any of the cell lines. In contrast 6-TG treatment of wild-type MEFs induced a small increase in DNA 8-oxo-dG (1.4-fold) when compared to untreated controls. This modest accumulation could also be appreciated in *Msh2*$^{-/-}$ MEFs, whereas the higher basal levels of the oxidized purine associated with MUTYH inactivation (Russo et al., 2009) masked this effect in *Mutyh*$^{-/-}$ MEFs. Interestingly, at comparable levels of 6-TG incorporation (data not shown), the synergistic effect of 6-TG/UVA interaction resulted in a remarkable increase in 8-oxo-dG levels (~2-fold in WT or *Msh2*$^{-/-}$ MEFs and ~1.7-fold in *Mutyh*$^{-/-}$ MEFs). Thus the combined treatment introduces oxidized bases in DNA and these enhanced oxidation levels are unaffected by the genotype.

Oxidation derived from dNTPs pool contributes to the detrimental effects of the combined treatment

Experimental evidence indicate that in 6-TG-treated cells the base analog can be found in the dNTPs pool where it can be a source of ROS upon UVA irradiation (Cooke et al., 2008).
To investigate the potential contribution of 6-TG present in the dNTPs pool to the biological response to the combined treatment, we depleted the dNTPs pool from 6-TG nucleotides by introducing a "chase" in our experimental settings. Thus cells were grown in a 6-TG containing medium for 24 and irradiated after a further 24 h incubation in 6-TG-free medium. (Figure 14A).

A

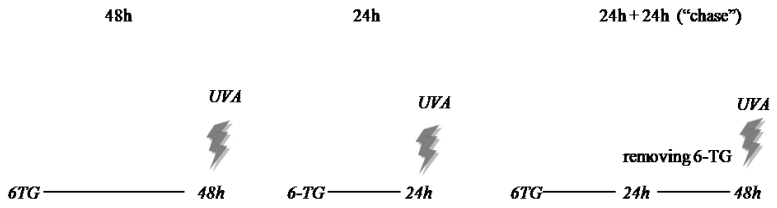


Figure 14: Effects of pool depletion on cellular survival
A) Experimental setting used to investigate the contribution of an oxidized dNTPs pool to 6-TG/UVA cytotoxicity. To deplete the cellular pool of 6-TG nucleotides, cultures were grown for 24 h in 0,6 μM 6-TG and UVA-irradiated (10 kJ/m^2) after a further 24 h growth in medium without 6-TG (24 + 24 h, "chase"); 48 h: classical experiment; 24 h: internal control of the "chase" experiment.

No difference in survival was observed between a 24 or 48 h treatment with 6-TG alone. A 24 h "chase" in 6-TG-free medium however drastically reduced killing induced by a combined 6-TG/UVA exposure. This protective effect on cell survival was particularly significant in WT cells, where the synergistic action of 6-TG and UVA on viability was much more dramatic than in the *Mutyh* or *Msh2*-defective cell lines (Figure 14B). In agreement with the capacity of 6-TG in the dNTPs pool to act upon UVA exposure as an oxidant towards DNA bases, a 24 h "chase" in WT cells reduced DNA 8-oxo-dG levels to steady state levels associated with the single 6-TG treatment (Figure 15A). A similar result was obtained in *Msh2*$^{-/-}$ MEFs and to a lower extent in *Mutyh*$^{-/-}$ cells.
Further confirmation that the oxidized pool contributes to oxidation of DNA bases comes from results obtained in a *Msh2*$^{-/-}$ clone overexpressing the hMTH1 hydrolase (Russo et al.; 2004). In this case high levels of hMTH1 activity completely abolished the increase of 8-oxodG levels associated with the combined treatment (Figure 15B).

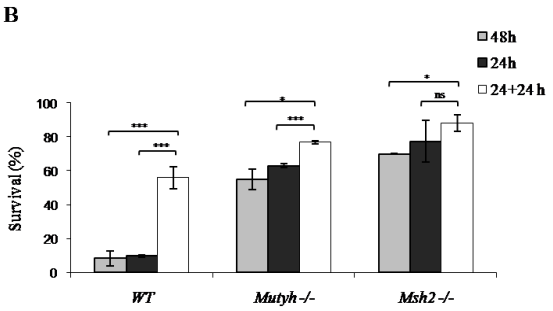


Figure 14: Effects of pool depletion on cellular survival
B) Sensitivity of cells to the combined 6-TG and UVA treatment after a "chase" experiment. WT, *Mutyh* -or *Msh2*-defective MEFs were exposed to 6-TG (60 nM) for 48 or 24 h and UVA-irradiated immediately afterwards (48 h e 24 h) or after a further 24 h growth in medium without 6-TG (24+24 h). Survival was determined by clonal assays and expressed as percentage of the cloning efficiency in 6-TG. Data are mean±SD from at least 6 independent measurements determinations. *p<0,05, *** p<0.001, ns: not statistically significant (Student's *t* test).

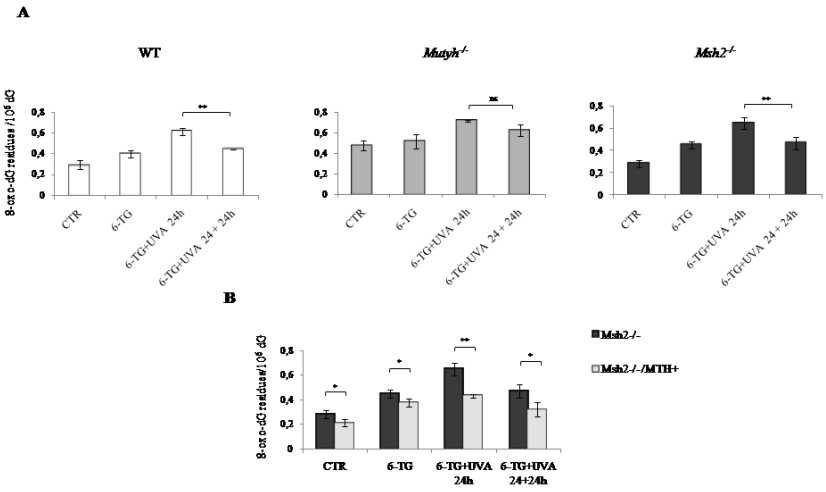


Figure 15: Effects of pool depletion on DNA 8-oxo-dG levels.
A) Measurement of DNA 8-oxo-dG in WT, *Mutyh* -or *Msh2*-defective MEFs irradiated with 10 kJ/m^2 UVA, following exposure to 6-TG (0.6μM) for 24 h or following a "chase" experiment. Data are mean±SD from at least 6 independent measurements. ** p<0.01, ns: not statistically significant (Student's *t* test). **B)** Measurement of DNA 8-oxo-dG levels in *Msh2*-defective MEFs and *Msh2*$^{-/-}$ clone overexpressing the hMTH1 hydrolase. Data are mean±SD from at least 6 independent measurements. *p<0,05, ** p<0.01 (Student's *t* test).

6-TG plus UVA treatment induce cell cycle perturbation

To better understand the role of MUTYH in 6-TG/UVA-induced toxicity, WT and *Mutyh*$^{-/-}$ MEFs were treated with 6-TG for 24 h, irradiated with UVA and analysed by flow cytometry after different recovery times (8, 24 and 48 h). As expected (Yamane et al., 2004) 6-TG treatment alone produced a progressive accumulation of WT cells in the G2-M phase (31% and 45% of the cell population at 24 and 48h, respectively) (Figure 16). In contrast the combined 6-TG/UVA treatment slows down the progression of the population through the S phase, with >than 75% of the cells blocked in the S phase at 24 h.
Following 6-TG incorporation *Mutyh*$^{-/-}$ cells showed a milder perturbation of cell cycle progression when compared to WT cells (a reduced percentage of cells are in G2-M phase, 20% and 25% of the cell population at 24 and 48h, respectively) and progressed into the G2-M phase after the combined 6-TG/UVA treatment (only 17% of cells were in the S phase at 24 h). These data suggest that MUTYH is needed for an efficient S phase arrest in the presence of lesions induced by the combined treatment. Finally irradiation with UVA alone did not produce changes in cell cycle progression in any cell type.

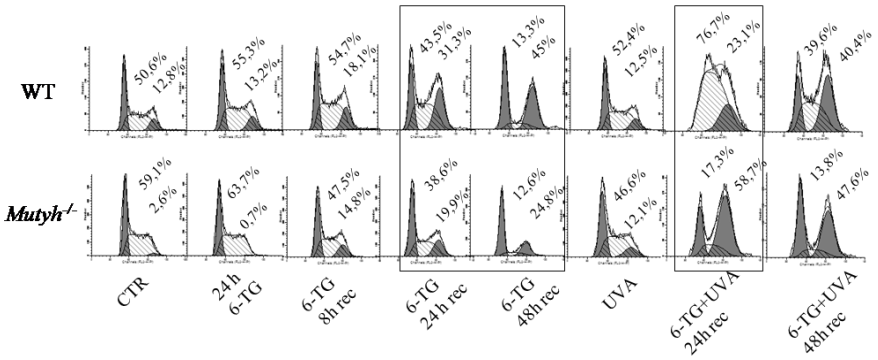


Figure 16:Cell cycle progression WT and *Mutyh*-defective MEFs were treated with 0.6 μM for 24 h, irradiated with 10 kJ/m^{2}UVA and analysed by flow cytometry at different recovery times (8, 24 and 48 h).Percentage of cells in S and G2/M phases are shown.

MUTYH sustains early checkpoint activation in response to the combined treatment

To investigate in more detail the mechanisms underlying the accumulation of WT cells in the S phase of cell cycle, after the combined treatment, the activated form of the Chk1 protein was examined by Western-blotting.

To this aim, cells were treated with 6-TG for 24 h and analysed after a recovery time of 1, 16, 20 and 24 h following UVA radiation.
In WT cells, phosphorylation of Chk1 on serine 345 occurred immediately after 6-TG/UVA treatment (1h), (Figure 17) and disappeared afterwards.

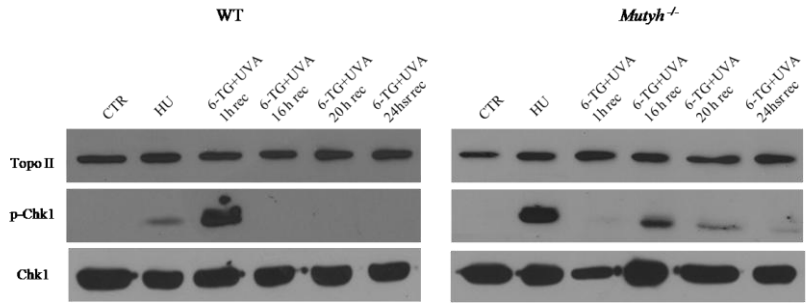


Figure 17:Analysis of Chk1 phosphorylation. WT and *Mutyh*-defective MEFs were grown in 0.6 μM 6-TG for 24 h and irradiated with 10 kJ/m^2 UVA. To detect the activated form of the Chk1 protein samples were collected 1, 16, 20 and 24 h after irradiation and analysed by Western blotting. Topoisomerase II (Topo II) and total Chk1 protein were used as loading controls. Cells treated were with 2 mM Hydroxyurea (HU) for 8 h and as a positive control.

In *Mutyh* $^{-/-}$ cells in contrast, phosphorylation of Chk1 was observed at 16 h after treatment and was maintained, thereafter, even though a gradual decrease was observed.
These data suggest that MUTYH activity might affect the early activation of the S phase checkpoint, as well as its regulation. No difference in Chk1 activation was detectable after exposure to 24 h 6-TG in the two genotypes (data not shown)

MUTYH affects the processing of damage by HR

Since loss of MUTYH leads to an easier progression through the cycle of presumably damaged cells, we investigated the initial levels of DSBs as well as their processing by HR in cells grown for 24 h in 6-TG and UVA irradiated immediately afterwards. We confirmed that, as in human cells (Brem R et al.; 2010) treatment of the cells with 6-TG/UVA produces an increase in DSBs as measured by the number of γH2AX foci, and these occurred immediately and were maintained up to 48 h from the end of the treatment (Figure 18A).

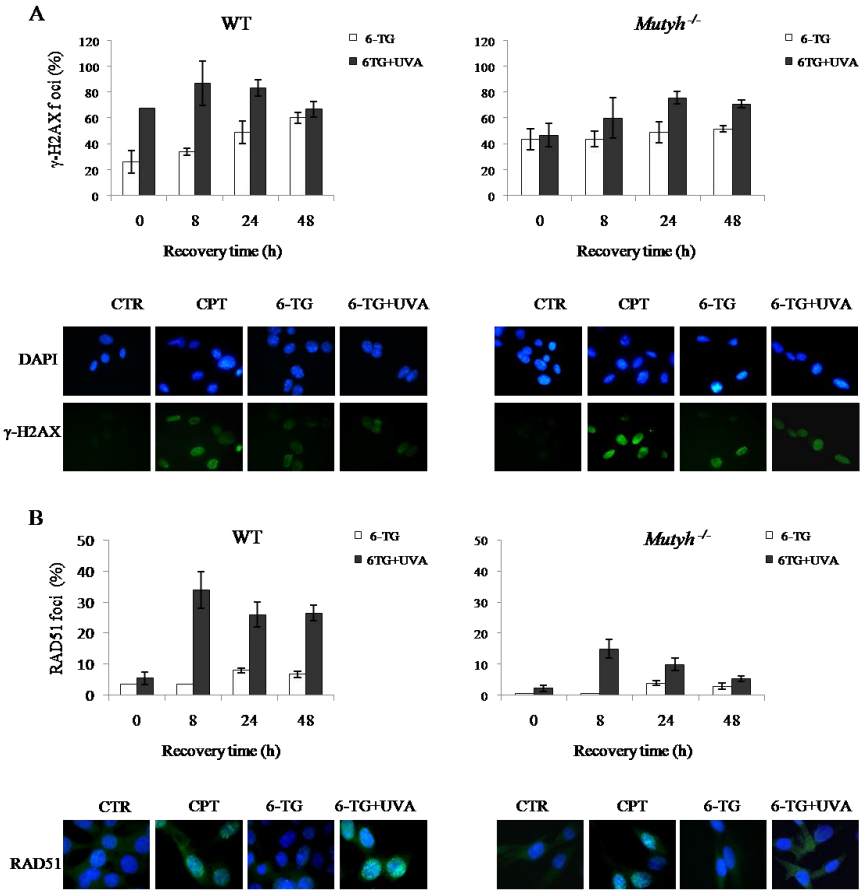


Figure 18: γ-H2AX and RAD51 foci formation. WT and *Mutyh*-defective MEFs were grown in the presence of 0.6 μM 6-TG for 24 h, irradiated with 10 kJ/m^2 UVA and collected for immunofluorescence analysis immediately afterwards, or after 8, 24 and 48 h. **A)** Graphs show the percentage of γ-H2AX-positive nuclei in each experimental condition (upper panel). **B)** Graphs show the percentage of RAD51-positive nuclei in each experimental condition (upper panel); cells treated with 30 μM camptothecin (CPT) for 4h were used as a positive controls. Representative images are shown in the lower panels.

This increase was of a comparable extent in *Mutyh*-defective cells, thus indicating that the initial level of DNA damage is similar in the two genotypes.
Analysis of RAD51 foci in WT MEFs indicated that DSBs are processed by HR, with RAD51 foci increasing 8 h after the end of 6-TG/UVA exposure and persisting up to 48 h, indicating the presence of unresolved DSBs. In contrast *Mutyh*$^{-/-}$ MEFs showed a lower level of RAD51 foci in comparison to WT cells with a gradual decrease at late post-treatment times (8-48 h) (Figure 18B).
We can conclude that exposure to 6-TG plus UVA introduces a similar level of

DNA damage in both genotypes. However the resolution of lesions induced by the combined treatment differs in WT and *Mutyh*$^{-/-}$ MEFS suggesting that the MUTYH protein plays a role in the processing of this type of DNA damage.

Mutyh$^{-/-}$ mice are resistant to the toxicity of the combined treatment but develop skin cancer

In order to analyse the effects of the single and combined 6TG /UVA treatment *in vivo*, WT and *Mutyh*-defective mice were treated with Aza, UVA alone or Aza plus UVA for 12 months. Following a series of short-term experiments in which toxicity associated with UVA exposure (by Minimal Erythemal Dose) and Aza-induced immunosuppression (by Mixed Lymphocyte Reaction) were evaluated in both WT and *Mutyh*$^{-/-}$ mice, the experimental conditions for a long-term experiment were identified.
WT and *Mutyh*$^{-/-}$ C57BL mice were exposed to 15mg/kg Aza given by intraperitoneal injection three times/week. A UVA dose of 150 kJ/m^2 was applied on the dorsal skin of shaved animals three times/week, one h following Aza injection. A differential toxicity between WT and *Mutyh*-defective animals was observed as consequence of the Aza plus UVA exposure.
In fact, while a high level of mortality was identified in the WT group (only 20% of the animals survived this exposure), 80% of *Mutyh*$^{-/-}$ treated animals survived the combined treatment. Survival was 100% in both UVA treated groups, while immunosuppression in Aza-treated groups was associated with some mortality, which was unaffected by the genotype (data not shown).
When DNA 8-oxo-dG levels in samples of spleen and skin were measured, a significant increase of DNA oxidation in the skin of animals exposed to the combined treatment was observed, irrespectively of the genotype (Figure 19A).
A trend of increased DNA oxidation, that did not reach statistical significance, was also observed in the spleen of both genotypes following the combined treatment. In addition *Mutyh*$^{-/-}$ mice showed a small but non-significant increase of the levels of 8-oxo-dG in the skin of UVA-treated group.
Interestingly, when histopathological examination of the skin was performed two squamous cell carcinomas, at a first stage of differentiation, were identified only in *Mutyh*$^{-/-}$ mice exposed to Aza plus UVA (Figure 19B).

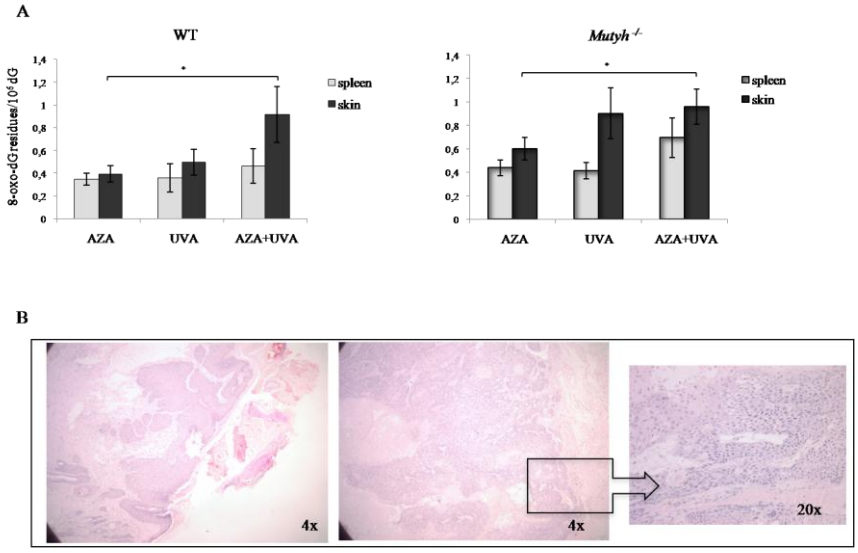


Figure 19: Effects of Aza/UVA exposures in WT and *Mutyh*-defective mice.
A) DNA 8-oxo-dG levels in spleen and skin samples of mice treated with 15mg/kg Aza and/or 150 kJ/ UVA m^2 three times/week for 12 months. Data are mean ± SE of at least 3 independent determinations *, $P< 0.05$ (Student's *t* test).**B)** Two examples of well-differentiated squamous cell carcinomas invading the dermis occurring in *Mutyh*-defective mice (first two panels). The third panel shows a microscopic magnification of a histological detail (*collaboration with MT Mancuso, ENEA*).

Taken together, the *in vitro* and *in vivo* experiments confirm the involvement of the MUTYH protein in the response to the damage induced by combined Aza and UVA treatment. Interestingly they also reveal that the absence of *Mutyh* is associated to an increased susceptibility to the carcinogenic effects of this type of DNA damage.

III. DISCUSSION

Extensive biochemical and genetic studies in model organisms demonstrate that the oxidative DNA lesion 8-oxo-dG is mutagenic and several DNA repair mechanisms operate to prevent its potentially detrimental effects. The importance of an efficient DDR in the protection against the carcinogenic outcome of oxidative lesions is indicated by the increased cancer susceptibility of individuals with germ-line mutations in genes involved in the repair of oxidative DNA damage. This includes biallelic germ-line mutations in the *MUTYH* gene, encoding a DNA glycosylase that is fundamental in the removal of 8-oxo-dG from DNA, which strongly predispose humans to MAP, a rare hereditary form of colorectal cancer.

MAP is an increasingly studied autosomal recessive disorder but functional analysis of *MUTYH* mutations is relatively limited and mainly related to the most recurrent missense variants. In the first part of this report we present a novel assay for analysis of MUTYH DNA glycosylase activity in LCLs from MAP patients. At variance with *in vitro* studies of purified MUTYH mutant proteins or when human variants are expressed in rodent cell lines (Molatore et al., 2010), this experimental approach allows studying the pathogenic consequences of mutant proteins in the biological context in which they are expressed.

Evaluation of MUTYH protein levels in LCLs from MAP patients indicates that analysis of transcript levels is not always exhaustive. Indeed in three out of four cells lines harbouring frameshift mutations the presence of transcripts results in a greatly reduced amount of the MUTYH protein. Although we cannot formally exclude that the predicted truncated proteins are undetectable by the antibody used in this study, it is more likely that these transcripts are subjected to nonsense-mediated decay. Interestingly, the substitution of Arg with His at the 245 amino acid position results in a reduced level of the MUTYH protein, suggesting that this mutation localized near the FeS cluster domain might affect both protein stability and function.

The results of the glycosylase assay indicate that all the LCLs from MAP patients are impaired in 8-oxodG:A cleavage when compared to WT cell lines. It's hardly surprising that cells characterized by an unstable MUTYH protein show no DNA glycosylase activity. In agreement with other reports (Wooden et al., 2004; Parker et al., 2005; Ali et al., 2008; Kundu et al., 2009), a residual but minimal enzymatic activity was retained in the p.Tyr179Cys/Tyr179Cys and p.Tyr179Cys/Gly396Asp variants, which are characterized by WT levels of the protein. Similarly the p.Arg245Cys/Gly396Asp variant, although expressed, was totally defective in its glycosylase activity, thus identifying the presence of a dysfunctional MUTYH protein. In comparison to DNA glycosylase assays based on immunoprecipitated MUTYH proteins (Parker et al., 2005; Alhopuro et al., 2005), the advantage of this assay resides in the relatively small number of cells

needed for the preparation of cell extracts and the possibility of analysing MUTYH function even when putative truncated proteins are present.
We previously demonstrated that MUTYH loss is always associated with increased steady-state levels of DNA 8-oxo-dG. In addition expression of some MUTYH variants leads to a more profound effect in the removal of the oxidized purine than the simple protein loss (Molatore et al., 2010). Indeed also in human cells defective MUTYH DNA glycosylase activity was reflected in an *in vivo* accumulation of DNA 8-oxo-dG following an oxidative stress induced by the $KBrO_3$ oxidant (Kawanishi and Murata 2006). Even in the absence of exogenous oxidation, when compared to wild-type cells, an increased steady-state level of DNA 8-oxodG was observed in the majority of LCLs harbouring MUTYH variants. Finally, LCLs harbouring the p.Gly264TrpfsX7 and p.Ala385ProfsX23 frameshift mutations, in which there is no detectable expression of the MUTYH protein, show a more moderate phenotype. Thus we suggest that also in humans the presence of a mutant MUTYH protein has a dominant negative effect, possibly interfering with others DNA repair pathways involved in the removal of 8-oxo-dG, for example MMR, other BER factors, nucleotide excision repair, or in signalling of DNA damage (the 9-1-1 complex) (Shi et al., 2006).
At variance with the mouse model where mutant MUTYH proteins are expressed in the same genetic background (*Mutyh*$^{-/-}$ MEFS), LCLs from different patients might have an ample range of cellular responses to oxidative stress. This is one of the reasons why both MUTYH and OGG1 activity were measured in parallel in cell-free extracts. The observation that all LCLs show wild-type OGG1 activity allows concluding that the accumulation of DNA 8-oxo-dG is a direct consequence of the MUTYH defect.
The information on the mutator phenotype associated with *MUTYH* inactivation is limited to MEFs and embryonic stem cells from *Mutyh*$^{-/-}$ mice, in which increases in mutation rates varied from 2- to 10-fold (Hirano et al., 2003; Russo et al., 2009). The presence of specific G>T transversions in *APC* or *K-RAS* genes in tumors occurring in MAP patients can also be considered an indirect evidence of a mutator phenotype associated with the presence of a non functional *MUTYH* (Al-Tassan et al., 2002; Jones et al., 2002, Lipton et al., 2003; van Pujienbroek et al., 2008). Here we investigated for the first time to which extent MUTYH impairment results in an increased mutation frequency in human cells. The gene we chose for this analysis is the X-linked *PIG-A* gene that has been successfully used to characterize the mutator phenotype associated with DNA repair defective human syndromes (Ataxia telangiectasia, Fanconi anemia) (Araten et al., 2005). When compared to the $21.7x10^{-6}$ mutational events at the *PIG-A* gene observed in wild-type cells, the group of LCLs from MAP patients showed a 4-fold increased mutation frequency.
The reappearance of *PIG-A* mutants in a cell line with one of the highest mutation frequency (p.Gly396Asp/Glu410GlyfsX43), following flow sorting to eliminate the pre-existing mutant cells, demonstrates that this phenotype is not due to a genetic drift of the cell population but it is an intrinsic feature of these

cells. The reported spontaneous mutation rate (μ) at this gene for normal cells ranges from 8.8 to 16.5 x 10^{-7} mutation per cell division (Peruzzi et al., 2010). A tentative μ value of 299 x 10^{-7} mutation per cell division was calculated on the p.Gly396Asp/Glu410GlyfsX43 LCLs following measurement of mutation frequency after six doublings. This value is at least 10-times higher that μ values for normal human cells (Araten et al., 2005). These data support the presence of a spontaneous mutator phenotype associated with defective MUTYH proteins and fit quite well with the proposed role of MUTYH variants in favouring the appearance of mutations in oncogenes relevant in the process of colorectal carcinogenesis (Al-Tassan et al., 2002; Jones et al., 2002).

Since oxidative damage in DNA can contribute to genome instability we also investigated whether MUTYH inactivation exposes cells to a further increase in their mutational burden in a condition of oxidative stress. Thus treatment of the biallelic p.Gly396Asp/Glu410GlyfsX43 variant LCLs with an oxidizing agent results indeed in a hypermutable phenotype. Oxidative stress might then play an independent and major role in causing genome instability in the presence of an inactivating MUTYH mutation. Although the mutator phenotype associated with MUTYH defects is considerably milder than that observed in MMR-defective cells, the hypermutability induced by specific genotoxic agents is a shared characteristic of cells with inactivation in either pathway (Glaab et al., 1998; Xu et al., 2001; Bignami et al., 2000; Sansom et al., 2003 and this study). Thus we suggest that the level of oxidative damage to DNA bases undergoing in specific districts of the body (and specifically in the gastrointestinal tract) might be a relevant issue to explain the tissue specificity of the increased cancer susceptibility of MUTYH- and MMR-defective patients.

By taking into consideration the prominent biological impact of pathogenic defects in the MUTYH protein when dealing with oxidative damage to DNA, we decided to investigate the effects of MUTYH impairment in response to another type of oxidative stress. In the second part of this thesis we report the results of this parallel study.

Combination of Aza, an inflammatory, anticancer and immunosuppressive drug, and UVA radiation is a major source of DNA 8-oxo-dG (Cooke et al., 2008). The interaction between 6-TG and UVA generates ROS and the resulting photochemical oxidation of DNA has been implicated as a possible contributor to the high skin cancer risk associated to long-term Aza treatment (Karran and Attard 2008). Experimental data obtained by Peter Karran's group suggest that the combination of 6-TG and UVA causes several types of damage, such as replication and transcription inhibition, DNA crosslinking, oxidation of DNA bases and proteins as well the covalent attachment of proteins to DNA. Many of these DNA lesions are difficult for cells to deal with, resulting in cytotoxic as well as mutagenic effects in human cells (O'Donovan et al.; 2005).

Here we examined the effects of 6-TG and UVA radiation in *in vitro* and *in vivo* mouse models, particularly regarding the impact of *Mutyh* loss on the biological response to this type of damage. As observed in human cells, the synergistic

action of 6-TG and UVA resulted detrimental to WT MEFs, with a clearcut killing effect. Interestingly, loss of the MUTYH or MSH2 proteins was associated with a considerable resistance to this type of damage.
In view of the extensive oxidation provoked by the combined treatment in human cells, we expected that this experimental setting associated with high ROS production might result in increased levels of DNA 8-oxo-dG in the mouse models.
Taken into account that both MUTYH and MSH2 control the amount of 8-oxo-dG in DNA (Russo et al., 2009), *Msh2*- and *Mutyh*-defective MEFs were expected to accumulate the oxidized lesion in their genome. Nevertheless, all the cell lines we examined, irrespectively of their genotype, showed similar levels of oxidative DNA damage following 6-TG and UVA treatment. Thus any discrepancy in the survival outcome cannot be ascribed simply to differences in the accumulation of oxidative lesions.
It's interesting that also in our mouse model 6-TG present in the dNTP represents a significant source of ROS upon UVA irradiation (Cooke et al., 2008; Brem et al., 2009). "Chase" experiments demonstrated that this non-DNA 6-TG makes indeed a considerable contribution to the 8-oxo-dG increase as well as to the killing effects induced the combined treatment in WT cells. The involvement of the oxidized nucleotide pool in DNA oxidation was confirmed by the great reduction in 8-oxo-dG levels observed in *Msh2*-defective MEFs overexpressing the hMTH1 hydrolase. This enzyme degrades 8-oxo-dGTP to 8-oxo-dGMP, thereby eliminating it from the pool of DNA synthesis precursors.
Attempts to investigate the mechanisms underlying the resistance against the type of DNA damage induced by 6-TG and UVA and associated to *Mutyh* loss included flow cytometry analysis of cell cycle progression and activation of checkpoints. The absence of MUTYH was indeed associated with a failure of S-phase halt, that in contrast is evident in WT cells, following the combined treatment. The apparent absence of a clear S phase arrest in *Mutyh*$^{-/-}$ cells was paralleled by a deficient Chk1 activation at early time points after treatment. ATR-mediated Chk1 phosphorylation, is known to play a critical role in regulating replication fork stability by arresting cells in S or G2/M phase (Sørensen et Syljuåsen, 2011). Thus our results revealed that MUTYH is involved in this step of the cellular response to DNA damage.
Recently it has been reported that MUTYH interacts with ATR and is needed for efficient Chk1 phosphorylation following UVB and HU treatments (Hahm et al.; 2009). Our data are consistent with an involvement of MUTYH in the activation of the S phase checkpoint following a different type of damage to DNA.
This observation might have consequences for cell killing. It is well known that ATR-dependent Chk1 phosphorylation controls the repair of DSBs by HR (Sørensen et al.; 2005). We confirmed that, as reported in human cells (Brem et al.; 2010), oxidative DNA damage produced by the combined 6-TG and UVA exposure results in DSBs formation also in MEFs. Even though no significant differences in the absolute levels of DSBs, as measured by γH2AX foci

formation, were observed between MUTYH-proficient and –deficient cells, a distinctive mode of DSBs repair was identified in the two genotypes. Quantification of RAD51 foci in WT and *Mutyh*-defective MEFs revealed persistence of unresolved lesions in WT cells, while loss of MUTYH protein resulted in a minor induction of RAD51 foci following the combined treatment. RAD51 is a well-known mediator of HR that plays a crucial role in protecting cells against the lethal effects of DSBs. If we consider HR as a pro-survival response to DNA damage, the hypersensitivity of WT cells in comparison to *Mutyh*-defective cells to killing by 6-TG/UVA is an apparent contradiction.
The attempt by HR of damage resolution might however introduce a heavy burden of dangerous repair intermediates that ultimately lead to a decrease in cell viability. Thus it is possible that loss of *Muthy* and the consequent inefficient or non-canonical DNA repair might represent a viable route of escaping from the lethal impact of 6-TG plus UVA exposure.
In this context, the apparent reduced activation of Chk1 in *Mutyh*$^{-/-}$ cells might be functionally correlated with the reduced RAD51 foci formation and with the enhanced survival, probably at the expense of genomy stability.
The striking observation that the 80% of *Mutyh*$^{-/-}$ mice *versus* the 20% of the wild-type counterpart survived the combined treatment confirms a pro-survival advantage associated with the absence of the *Mutyh* gene. However, the presence of two squamous cell carcinomas (the same tumor identified in Aza long-term treated patients) in *Mutyh*$^{-/-}$ mice exposed to Aza plus UVA is in agreement with a carcinogenic effect of the combined treatment. As in the *in vitro* model, measurement of 8-oxo-dG into DNA of skin and spleen cells of mice did not reveal significant differences in oxidative damage accumulation between the two genotypes (except for the UVA treatment alone). An excess of extracolonic tumors, including tumor of the skin, has been recently reported in MAP patients (Vogt et al., 2009). It is intriguing that the survival advantage in the response against the type of oxidative damage induced by Aza/UVA provided by loss of *Mutyh* and identified both in *vitro* and *in vivo*, comes at a price of increased cancer susceptibility in the skin. Whether the mechanism underlying this cancer proneness depends on the role of MUTYH in the cellular signaling and processing of oxidative damage induced by Aza/UVA remains to be ascertained.

IV. CONCLUSIONS

Our experimental investigation on the biochemical and biological consequences associated with the impairment or complete loss of the MUTYH protein strengthens the importance of this protein in protecting the genome against oxidative damage.
Moreover the novel functional assays for genotype-phenotype correlations of MUTYH variants we set up might provide a better understanding of MAP with possible implications for counseling, screening and clinical management of patients and their families. Additionally, the study on the effects of *MUTYH* loss in mice also provided new insights on the detrimental effects of Aza combined with UVA radiation and on possible novel functions of MUTYH more complex than previously thought.

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