Identification of Factors Interacting with hMSH2 and hMLH1 in the Fetal Liver and Investigations of how Mitochondrial Dysfunction Creates a Mutator Phenotype

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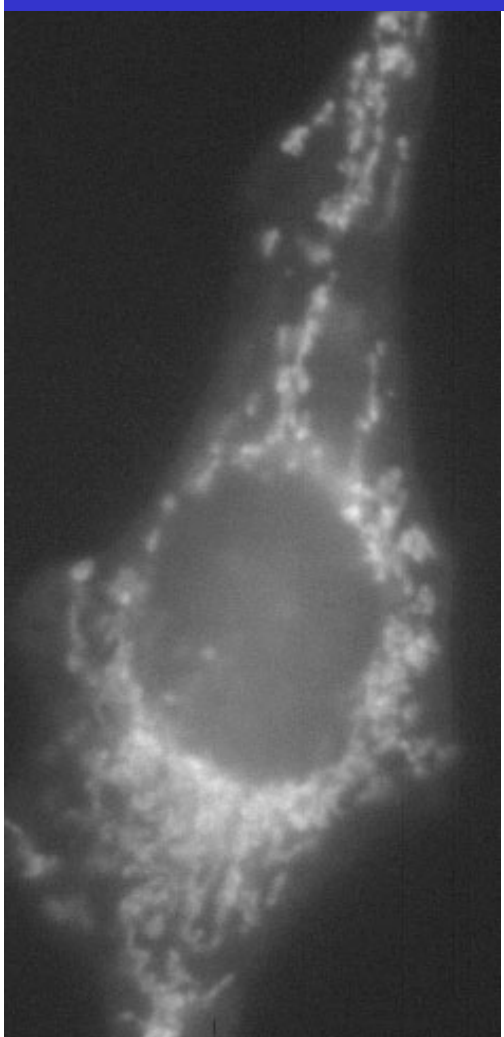

Anne Karin Rasmussen

Ph.D. Thesis

December 2001

The Johns Hopkins Oncology Center
NCI CCC
A Comprehensive Cancer Center Designated by the National Cancer Institute

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Preface

This Ph.D. thesis is based on work carried out at Department of Life Sciences and Chemistry, Roskilde University, Denmark in Dr. Lene Juel Rasmussen's laboratory and at Johns Hopkins Oncology Center, Baltimore, MD, USA in Dr. Keshav K. Singh's laboratory from 1998 to 2001.

On the beginning, my Ph.D. research focus area was primarily the DNA mismatch repair pathway. However, after a stay at Johns Hopkins the scope of my thesis ended up being more comprehensive. In the Ph.D. thesis I am also addressing the following issues: molecular mechanisms associated with DNA damage and DNA repair, and mutagenesis in mitochondria.

Front cover: HeLa cells stained with MitoTracker Red

Acknowledgements

First, I wish to express my gratitude to Dr. Lene Juel Rasmussen for critical reading of my manuscripts and thesis and particularly for being an extraordinarily positive, dedicated and stimulating supervisor.

Secondly, I wish to give my thanks to Dr. Keshav Singh for letting me work in his laboratory at Johns Hopkins and for widening my intellectual horizon in the field of mutagenesis in mitochondria. Furthermore, I want to thank him for the critical reading of my manuscripts and for being an inspiring supervisor.

Thirdly, I want to express my thanks to Lene Markussen and Gerda Olesen for excellent technical assistance.

In addition, I want to give my thanks to all my colleagues and friends at Department of Life Sciences and Chemistry at Roskilde University and at Johns Hopkins Oncology Center. A special thanks to Rob Delsite and Barbara Sigala for technical support and to Anne Lützen and Jonas Andreasen for critical reading of my thesis and for creating a cheerful atmosphere.

Finally, I want to express my gratitude to my dearest family and boyfriend for all the love and support they gave me while completing my Ph.D.

Anne Karin Rasmussen
Copenhagen 2001

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Publications/Manuscripts

The present thesis is based on one original publication (I), one submitted manuscript (III), and two manuscripts in preparation (II, IV). The manuscripts referred to in the thesis are identified by the following roman numerals:

I. Lene Juel Rasmussen, Merete Rasmussen, Byung-In Lee, **Anne Karin Rasmussen**, David M. Wilson III, Finn Cilius Nielsen, and Hanne Cathrine Bisgaard. Identification of factors interacting with hMSH2 in the fetal liver utilizing the yeast two-hybrid system. In vivo interaction through the C-terminal domains of hEXO1 and hMSH2 and comparative expression analysis. **Mutation Research** (2000) 460, 41-52.

II. **Anne Karin Rasmussen**, Keshav K. Singh, and Lene Juel Rasmussen. Characterization of O^6-MeG DNA Methyltransferase (MGMT) protein in repairing human mitochondrial DNA. **Manuscript in preparation.**

III. **Anne Karin Rasmussen**, Aditi Chatterjee, Lene Juel Rasmussen and Keshav K. Singh. Mitochondria as determinant of genetic stability in *Saccharomyces cerevisiae*. **Nature Genetics** (submitted).

IV. **Anne Karin Rasmussen,** Lene Juel Rasmussen, and Keshav K. Singh. Mitochondrial dysfunction suppresses the mutator phenotype of *Saccharomyces cerevisiae* superoxide dismutase deficient cells. **Manuscript in preparation.**

Structure

The thesis is divided into two sections:

1. Section one is a general introduction where important results from my research are included. The section is divided into three parts: The first part is an introduction to the publication (I). The second part is an introduction to manuscript II. The third part is an introduction to manuscripts III and IV.
2. Section two consists of one published paper and three manuscripts.

Abbreviations

A	Adenine
AD	Activation Domain
ANT	Adenine Nucleotide Translocator
AP	Apurinic/apyrimidinic
ATP	Adenosine triphosphate
BD	Binding Domain
BER	Base Excision DNA Repair
C	Cysteine
CAT	Catalase
Complex I	NADH dehydrogenase or NADH:ubiquinone oxidoreductase
Complex II	Succinate dehydrogenase
Complex III	Ubiquinol:cytochome c oxidoreductase
Complex IV	Cytochrome c oxidase
Complex V	ATP synthase
CoQ	Coenzyme Q, also called ubiquinone
DNA	Deoxyribonuceic acid
E. coli	*Escherichia coli*
FEN1	Flap endonuclease-1, *S. cerevisiae* rad27 homolog
G	Guanine
GGR	Global genome repair
GPx	Glutathione peroxidase
H_2O_2	Hydrogen peroxide
hAPE1	Human AP endonuclease
hEXO1	Human exonuclease 1
hMLH1-hMLH3	*E. coli* MutL homolog complex
hMLH1-hPMS1	*E. coli* MutL homolog complex
hMLH1-hPMS2	*E. coli* MutL homolog complex
hMSH2-hMSH3	*E. coli* MutS homolog complex, recognizes insertion/deletions
hMSH2-hMSH6	*E. coli* MutS homolog complex, recognizes base:base mispairs and insertion/deletions
hMYH	Glycosylase, Human MutY Homolog
HNPCC	Hereditary Non-Polyposis Colon Cancer
hNTH1	Glycosylase, Human Nth homolog
hOGG1	8-OxoGuanine DNA Glycosylase
$HO^{\bullet}$	Hydroxyl radical
hUNG1	Human mitochondrial UNG
hUNG2	Human nuclear UNG
3-MeA	3-methyladenine
3-MeG	3-methylguanine
MGMT	O^6-methylguanine-DNA methyltransferase
MMR	Mismatch repair
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
MNU	Methylnitrosourea
MPG	N-methylpurine-DNA glycosylase
Msh1	Mitochondrial homolog of the *E. coli* MutS
mtDNA	Mitochondrial DNA
nDNA	Nuclear DNA
NER	Nucleotide Excision Repair
NLS	Nuclear Localization Signal
NPC	Nuclear pore complexes
O_2^-	Superoxide radical
O^4-MeT	O^4-methylthymine
O^6-MeG	O^6-methylguanine
8-oxoG	7,8-dihydro-8-oxoguanine
PCNA	Proliferating Cell Nuclear Antigen
POL G	Polymerase γ
Pol ε	DNA polymerases (Pol II)
Pol δ	DNA polymerases (Pol III)
rho^-	Mutations in the mitochondrial genome
rho^0	Lack of mitochondrial genome
ROS	Reactive oxygen species
S. cerevisiae	*Saccharomyces cerevisiae*
SOD1	Nuclear superoxide dismutase
SOD2	Mitochondrial superoxide dismutase
SSB	Single-strand binding protein
T	Thymine
TCR	Transcription coupled repair
UNG	Uracil-DNA Glycosylase
XPG	*S. cerevisiae* Rad2 homolog

Summary

Increased spontaneous mutation frequency is associated with increased cancer risk. However, the relative contribution of spontaneous endogenous mutagenesis to carcinogenesis is not known today. Defects in the postreplication DNA mismatch repair (MMR) pathway are recognized to increase spontaneous mutations. Mutations in MMR genes cause hereditary non-polyposis colon cancer.
In an effort to identify unidentified genes involved in MMR and tissue-specific MMR-associated factors, we employed the yeast two-hybrid system, using the human hMSH2 as bait and a human fetal liver cDNA library as prey. We demonstrated that hMSH2 interacts with a human 5' $\rightarrow$ 3' exonuclease 1 (hEXO1).

Data presented in this thesis also support the conclusion that mitochondrial dysfunction leads to spontaneous nuclear DNA damage. We employed the yeast *Saccharomyces cerevisiae* as a model system to investigate a potential link between mitochondrial activity and genomic instability. Mitochondrial dysfunction and genetic instability are characteristic features of cancer cells. Furthermore, mitochondrial dysfunction is a key feature of aging due to accumulation of mutations in mtDNA. Our studies in a yeast model system suggest that mitochondria contain some intrinsic properties that control the generation of the mutator phenotype associated with cancer cells. We hypothesize that cancer cells by losing their mitochondrial function create a mutator phenotype.

Given the importance of maintaining the integrity of the mitochondrial genome we have found that it might be valuable to further investigate the molecular processes and components responsible for mtDNA repair. It has recently been recognised that base excision DNA repair (BER) is operating in the mitochondria, however, knowledge about other repair pathways is still very limited. We decided to investigate O^6-methylguanine-DNA methyltransferase (MGMT) because of the fact that its sub-cellular localization has not been determined. We determined that it was localized to nucleus but not to mitochondria in HeLa and breast epithelial cells.

Sammendrag (Summary in Danish)

En øget frekvens af spontane mutationer er forbundet med øget risiko for cancer. Hvor stor en del af cancinogenese som skyldes spontan endogen mutagenese, er endnu ikke kendt. Fejl i det postreplikative DNA mismatch repair (MMR) system har imidlertid vist sig at forøge den spontane mutationsfrekvens, da mutationer i MMR-generne kan forårsage hereditær non-polypos colon cancer.

For at finde uidentificerede gener, som er involveret i MMR, samt vævsspecifikke MMR-associerede faktorer, gjorde vi brug af gær two-hybrid systemet. Vi brugte hMSH2 som "bait" og et humant føtalt lever cDNA bibliotek som "prey". Vi viste, at hMSH2 interagerer med en human 5' $\rightarrow$ 3' exonuclease 1 (hEXO1).

Endvidere indeholder afhandlingen resultater, som understøtter konklusionen om, at mitokondriel dysfunktion fører til spontan nukleær DNA skade. Vi brugte *Saccharomyces cerevisiae* som modelsystem til at undersøge en mulig sammenhæng mellem mitokondriers aktivitet og genomisk stabilitet. Mitokondriel dysfunktion og reduceret genetisk stabilitet er karakteristiske egenskaber for cancer celler. Yderligere er mitokondriel dysfunktion forbundet med aldring på grund af akkumulering af mutationer i mtDNA. Vore studier i et gær modelsystem indikerer, at mitokondrier har egenskaber som kan styre udviklingen af den mutator fænotype der er forbundet med cancer celler. På baggrund af dette opstiller vi en hypotese om, at cancer celler ved at miste deres mitokondrie funktion kan frembringe en mutator fænotype.

Givet vigtigheden af at opretholdeholde/reparere det mitokondrielle genom for at forhindre mitokondriel dysfunktion, fandt vi det værdifuldt yderligere at undersøge de molekylære processer og komponenter ansvarlige for mtDNA reparation. Det er i dag anerkendt at base excision DNA repair (BER) virker i mitokondrierne. Viden om andre reparationssystemer i mitokondrierne er dog stadig meget begrænset. Vi undersøgte O^6-methylguanine- DNA methyltransferase (MGMT) subcellulære lokalisation, og fandt at MGMT i bryst epithelial-celler er lokaliseret til kernen, men ikke til mitokondrierne.

1. Analysis of Human DNA Mismatch Repair

1.1 Introduction

The postreplication DNA mismatch repair (MMR) pathway is responsible for the maintenance of DNA fidelity upon replication (Buermeyer *et al*., 1999; Kolodner & Marsischky, 1999; Harfe & Jinks-Robertson, 2000; Jiricny *et al*., 2000). MMR captures errors in the newly synthesized DNA strand that are missed by the polymerase proofreading and lowers the mutation frequency by a factor of 100-1000-fold as compared to MMR deficient cells (Bhattacharyya *et al*., 1994; Eshleman *et al*., 1995). In humans, accumulation of mutations is a critical step in carcinogenesis. Loss of a single allele of one of the mismatch repair proteins causes **H**ereditary **N**on-**P**olyposis **C**olon **C**ancer (HNPCC), a form of cancer that accounts for 1-5% of all cases of colon cancer (Lynch & de la Chapelle, 1999; Peltomäki, 2001). HNPCC is caused by inherited mutations in MMR genes. Most HNPCC families have germline mutations in the *hMSH2* (2p22-p21) and *hMLH1* (3p21) genes. Mutations in the other known MMR genes *hMSH6* (2p16), *hMSH3* (5q11-q12), *hPMSH2* (7p22), *hPMSH1* (2q31), and *hMLH3* (14q24) are either rare or non-existent in HNPCC families (Peltomäki & Vasen, 1997; Kolodner & Marsischky, 1999; Wood *et al*., 2001).

Some HNPCC families fail to display mutations in known MMR genes. Therefore, we argue that these HNPCC families must harbor mutations in yet unidentified genes that are involved in MMR. In an attempt to identify such genes, we employed the yeast two-hybrid system, using the human MMR proteins hMSH2 (Rasmussen *et al*., 2000) or hMLH1 as bait and a fetal liver matchmaker cDNA library as prey.

1.2 DNA Mismatch Repair

MMR in Escherichia coli

Mechanisms and functions of mismatch correction are best understood in *Escherichia coli*, therefore a short introduction to MMR in *E. coli* is given. MMR is directed by the state of adenine methylation of GATC sequences. Since DNA adenine methylation (Dam) occurs after replication, an unmethylated newly synthesized strand is temporarily paired with a fully methylated parental strand, which provides a strand discrimination signal for MMR in

E. coli (Lahue & Modrich, 1989). Initiation of MMR occurs via mismatch recognition and binding of a MutS homodimer followed by binding of a MutL homodimer. In *E. coli* MutL serves to couple mismatch recognition with downstream MMR events. Interactions between MutL, MutS, and ATP are believed to result in translocation of the MutS-MutL complex away from the mispair, leading to the activation of the MutH endonuclease (Allen *et al*., 1997; Hall & Matson, 1999). After activation, MutH introduces a nick in the nascent strand of the nearest hemi-methylated GATC sequences. MutL helps to load DNA helicase II (UvrD) at the nicked GATC site and UvrD unwinds DNA from the nick toward and past the mismatch (Hall *et al*., 1998). Removal of the error-containing DNA strand is facilitated by one of four single-stranded, DNA-specific exonucleases (RecJ, ExoI, ExoVII, ExoX) depending on the polarity of the reaction (Viswanathan & Lovett, 1998; Burdett *et al*., 2001). The resulting single-stranded gap, is stabilized by single-strand binding protein (SSB) and filled by DNA polymerase III holoenzyme. The remaining nick is closed by DNA ligase and Dam methyltransferase finishes the MMR pathway in *E. coli* by methylating the newly synthesized strand (Rasmussen *et al*., 1998).

Mismatch recognition - in eukaryotes

In eukaryotes, mispaired bases in DNA are recognized by the heterodimeric complexes, MSH2-MSH6 and MSH2-MSH3 (**M**ut**S h**omologs) (Acharya *et al.*, 1996; Guerrette *et al.*, 1998, Genschel *et al*., 1998). Analysis of mismatch binding specificities of the human hMSH2-hMSH6 and hMSH2-hMSH3 complexes showed that they were overlapping but not identical. The hMSH2-hMSH6 complex recognizes base:base mispairs and insertion/deletion mispairs of up to 8 unpaired bases. In comparison, hMSH2-hMSH3 has a high affinity for insertion/deletions of 2-8 unpaired bases, weak affinity for single-nucleotid insertion/deletion mispairs, and do not bind base:base mispairs (Drummond *et al.*, 1997; Genschel *et al*., 1998). The predominant DNA-binding protein in the hMSH2-hMSH6 complex appears to be hMSH6 when binding to a mismatched oligonucleotide (Matton *et al*., 2000). Hence, the hMSH2-hMSH6 complex appears to provide the predominant mismatch-binding activity in human cells (Genschel *et al*., 1998; Marra *et al*., 1998). The hMSH3 protein is believed to compete with hMSH6 for the available hMSH2, as the interacting regions of hMSH2 with hMSH3 and hMSH6 are identical (Guerrette *et al.*, 1998). This prediction is supported by two independent findings: extracts prepared from HCT15 cells, which lack hMSH6, contain approximately three-fold higher levels of hMSH2-hMSH3 complex compaired to MMR proficient cells (Genschel *et al*., 1998); and

cells overexpressing hMSH3 preferentially form hMSH2-hMSH3 complexes (Marra *et al*., 1998). Thus, hMSH3 interacts with all hMSH2 to form the hMSH2-hMSH3 complex, making cells functionally deficient in hMSH2-hMSH6 complex. Consequently, these cells lack base:base mispair correction (Drummond *et al*., 1997; Marra *et al*., 1998).

Table 1.1 • DNA mismatch repair proteins
(Kolodner & Marsischky, 1999; Burdett *et al*., 2001; Wood *et al*., 2001)

E. coli	***S. cerevisia***	***H. sapiens***	**Function**
MutS	Msh2-Msh6	hMSH2-hMSH6	Recognizes single-nucleotide and insertion/deletion mispairs.
	Msh2-Msh3	hMSH2-hMSH3	Recognizes insertion/deletion mispairs.
MutL	Mlh1-Pms1	hMLH1-hPMS2	Couples mismatch recognition with downstream MMR events in *E. coli*. The function in eukaryotes is unclear.
	Mlh1-Mlh2	hMLH1-hPMS1	
	Mlh1-Mlh3	hMLH1-hMLH3	
MutH	*not identified*	*not identified*	Endonuclease; nicks hemimethylated GATC sequences.
UvrD	*not identified*	*not identified*	Helicase; facilitates DNA unwinding
RecJ & ExoVII	*not identified*	*not identified*	5'→ 3'single-stranded DNA exonucleases
ExoI & ExoX	*not identified*	*not identified*	3'→ 5' single-stranded DNA exonucleases
not identified	Exo1	hEXO1	5'→ 3' DNA exonucleases that have a preference for degrading double-stranded DNA

The step after mismatch recognition - in eukaryotes

Except for the initial mismatch recognition step, relatively little is known about the MMR mechanism in humans. However, human MutL homologs have been identified (table 1.1 & figure 1.1). The human MutL complexes consist of three different heterodimers: hMLH1-hPMS2, hMLH1-hPMS1 and hMLH1-hMLH3.

A glutathione-S-transferase (GST) fusion protein assay has shown that the interactions between hMLH1 and hPMS2 (Guerrette *et al*., 1999), hMLH1 and hPMS1 (Kondo *et al*., 2001), and hMLH1 and hMSH3 (Lipkin *et al*., 2000) all are mediated through the same C-terminal region of hMLH1 (Kondo *et al*., 2001). This fact could imply that hPMS2, hPMS1 and hMSH3 are competing for hMLH1.

To date, the exact biochemical roles of hMLH1-hPMS1 and hMLH1-hMSH3 complexes have not been determined. Only hMLH1-hPMS2 has been shown to be involved in MMR (Li & Modrich, 1995; Nicolaides *et al*., 1995), although, hMLH1 seems to have greater affinity for hPMS1 than for hPMS2, when measured in the yeast two-hybrid assay (Räschle *et al*., 1999) and hPMS1 has been shown to be mutated in one HNPCC family (Nicolaides *et al*., 1994).

Overproduction of an hMLH3 N-terminal deletion protein is associated with a microsatellite instability phenotype (Lipkin *et al*., 2000). Furthermore, results obtained in *S. cerevisiae* imply that the Mlh1-Mlh3 heterodimer repairs insertion/deletion mispairs, most likely in cooperation with the Msh2-Msh3 heterodimer (Flores-Rozas & Kolodner, 1998). These results suggest that the hMLH1-hMLH3 complex can substitute for the hMLH1-hPMS2 complex in the repair of insertion/deletion loops recognized by the hMSH2-hMSH3 complex (figure 1.1).

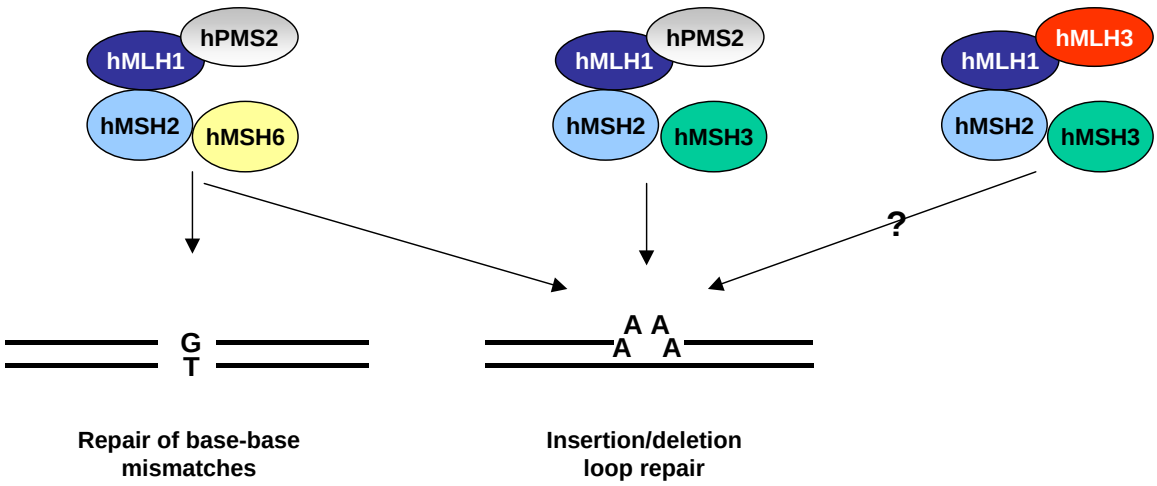


Figure 1.1: Protein complexes involved in MMR and their diverse functions. Base:base mispairs are only recognized by hMSH2-hMSH6/hMLH1-hPMS2 complexes. Whereas insertion/deletion loops are recognized by hMSH2-hMSH6/hMLH1-hPMS2 and hMSH2-hMSH3/hMLH1-hPMS2. Studies in yeast suggest that hMSH2-hMSH3/ hMLH1-hMLH3 recognize insertion/deletion loops but this has not yet been proven in human, therefore the question mark.

As in *E. coli*, the human MutL homologs have been shown to interact with the mismatch recognition complex; hMSH2, hMSH6, hMLH1, and either hPMS2 or hPMS1 (but not both together) have been co-precipitated from HeLa nuclear extracts in the absence of added ATP

(Matton *et al*., 2000). The exact function of the MLH heterodimers as well as the signal for strand discrimination is not clearly defined in eukaryotes. However, it is known that strand-specific mismatch repair in human cells can be directed by a single strand nick in the DNA helix (Holmes *et al*., 1990; Thomas *et al*., 1991).

It has been demonstrated that PCNA (Proliferating Cell Nuclear Antigen)[1] is required for initiation of repair as well as for resynthesis in MMR (Umar *et al.* 1996; Gu *et al*., 1998). Studies (two-hybrid screens) in yeast have shown that Mlh1 interacts with the replication accessory factor PCNA, suggesting that Mlh1 heterodimers serve as an interface between Msh heterodimers (mismatch recognition) and DNA replication components (Umar *et al*., 1996). The involvement of PCNA in MMR initiation has been supported by observations, which demonstrated that hMSH2, hMLH1, hPMS2, and PCNA can be co-immunoprecipitated from HeLa nuclear extracts in the presence of double-stranded circular DNA, Mg^{2+} and ATP. Furthermore, PCNA could not be precipitated from either *hMSH2*-defective LoVo or *hMLH1*-defective H6 cells or if ATP was eliminated from the immunoprecitation step suggesting that formation of the complex requires functional hMSH2 and hMLH1 proteins (Gu *et al*., 1998). Other research has suggested that PCNA plays an important role in steps proceeding mismatch recognition. Bowers *et al*. (2001) demonstrated that following mismatch recognition, PCNA could disrupt ternary complexes in yeast composed of Msh2-Msh6, Mlh1-Pms1 and linear mismatched substrate (Bowers *et al*., 2001).

Genetic analyses in yeast also indicate that mutations in PCNA can create mutator phenotypes (increase frameshifts in simple sequence repeats) consistent with disrupted MMR, possibly as the result of defects in strand discrimination (Johnson *et al.*, 1996; Kokoska *et al*., 1999; Chen *et al*., 1999). Recent work in yeast has suggested that PCNA-Msh2-Msh6 interactions play a key role in facilitating specific binding of Msh2-Msh6 to mispairs and/or that the specific activity of Msh2-Msh6 is increased by the interaction with PCNA (Flores-Rozas *et al*., 2000). The interaction between PCNA and Msh2-Msh6 seems to be mediated by a specific PCNA-binding site present in Msh6 (Flores-Rozas *et al*., 2000).

Therefore, PCNA seems to be implicated in steps in all phases of mismatch recognition. PCNA interacts with a number of DNA factors, including DNA polymerases (Pol δ, Pol ε),

[1] Proliferating cell nuclear antigen (PCNA) is a replication accessory factor encoded by the essential gene *POL30* in *Saccharomyces cerevisiae*. It is a homotrimeric ring-shaped protein that serves as an accessory factor for DNA polymerase δ (Pol δ) and DNA polymerase ε (Pol ε). DNA-bound PCNA forms a sliding clamp that tethers Pol δ and Pol ε to template DNA and thus promotes processive DNA synthesis (Chen *et al.*, 1999).

DNA endonucleases (FEN1, XPG), DNA ligases (Ligase 1), and methyltransferases (DNA-(cytosine-5) methyltransferase), indicating that it may have multiple roles in DNA repair (Gary *et al.*, 1999). However, it is still not clear how PCNA is involved in the MMR pathway.

Excision

One of the interacting proteins we found in the two-hybrid screening with hMSH2 as a bait was the human exonuclease 1 (hEXO1) (Rasmussen *et al*., 2000). Therefore, the excision step in MMR will be described in greater detail in the next section.

1.3 The Human Exonuclease 1

The human exonuclease 1 (hEXO1) belongs to a family of nucleases with structure-specific nuclease activity that is conserved from phage to human (Lieber, 1997; Ceska & Sayers, 1998). The strong sequence homologies are limited to two discrete regions, designated to the N (N-terminal) and I (internal) regions that comprise the catalytic domain responsible for exo- and endonuclease activities. Based on sequence comparisons, positioning of the N-terminal and internal regions, and their biochemical and biological functions, this nuclease family can be divided into three subfamilies (Lee & Wilson III, 1999).

One subfamily includes human XPG (xeroderma pigmentosum group G) and its *S. cerevisiae* (Rad2) and *S. pombe* (Rad13) homologs. Another consists of FEN1 (flap endonuclease-1), *S. cerevisiae* (Rad27) and *S. pombe* (Rad2) homologs. The third subfamily includes human exonuclease 1 (hEXO1), *S. cerevisiae* (Exo1) and *S. pombe* (Exo1).

The first subfamily – XPG

The XPG proteins possess both endonuclease and 5'→ 3' exonuclease activities and are known to operate in **N**ucleotide **E**xcision **R**epair (NER) (Habraken *et al*., 1994). Two NER subpathways exist; repair of damage that blocks elongating RNA polymerases, transcription coupled repair (TCR), and repair of lesions over the entire genome, referred to as global genome repair (GGR). In both the TCR and GGR pathways the XPG and ERCC1-XPF proteins are responsible for cleaving 3' and 5' respectively of the damaged strand which occurs after the damage is detected and a region around the damage site has been opened. Cleavages with XPG and ERCC1-XPF result in the removal of 24-32 nucleotides containing the lesion. The resulting gap is filled in by the combined action of DNA polymerase δ or ε,

PCNA, single-strand binding protein (RPA) and ligase. In mammalian cells, at least 25 polypeptides are required for the NER process (de Laat *et al.*, 1999; Hoeijmakers, 2001). Mutations in the *XPG* gene have been found to lead to the human disorder xeroderma pigmentosum, characterized by a hypersensitivity to sunlight and an increased likelihood of developing skin cancer (van Steeg & Kraemer, 1999).

The second subfamily – FEN1

The FEN1-like proteins exhibit a 5'→ 3' flap-specific exo/endonuclease that plays an important role in multiple DNA metabolic processes. The flap-specific endonuclease activity is required for branched DNA structures produced by DNA polymerase strand displacement during lagging strand DNA synthesis, or as intermediates during DNA recombination (Harrington & Lieber, 1994a, 1994b). The 5'nuclease function of FEN1 is responsible for the excision of Okazaki fragments, (FEN1 excises the final 5'-terminal ribonucleotide at the RNA-DNA junction) (Bambara *et al.*, 1997) and for long-patch base excision repair (Kim *et al.*, 1998).

In *S. cerevisiae*, a deletion of the *FEN1* homolog *Rad27* results in sensitivity to the alkylating agent methylmethane sulfonate, modest sensitivity to ultraviolet light, increased spontaneous chromosome instability, and temperature sensitivity (Johnson *et al.*, 1995; Reagan *et al.*, 1995; Vallen & Cross, 1995). These phenotypes are consistent with participation of FEN1 in both DNA replication and repair.

The third subfamily – EXO1

The human exonuclease 1 (*hEXO1*) gene consists of 14 exons, and is transcribed to yield a 3-kb mRNA. The *hEXO1* gene is located on chromosome 1 (1q42-43) (Schmutte *et al.*, 1998; Tishkoff *et al.*, 1998; Wilson III *et al.*, 1998).

There exist two forms of exonuclease 1, hEXO1a/HEX1 and hEXO1b (Schmutte *et al.*, 1998; Tishkoff *et al.*, 1998; Wilson III *et al.*, 1998). The hEXO1a/HEX1 protein is 803 amino acids long, whereas the hEXO1b protein is 846 amino acids. This difference in length arises from alternatively spliced RNA transcripts involving only C-terminal content outside of the nuclease domain (Schmutte *et al.*, 1998; Tishkoff *et al.*, 1998).

The ratio between hEXO1a/HEX1 and hEXO1b ESTs, found during database searches, was 1:6, which suggests that hEXO1b is the more abundant species (Tishkoff *et al.*, 1998). The DNA sequences of the hEXO1 two-hybrid clones we isolated showed homology to the C-terminal region of hEXO1b (Rasmussen *et al.*, 2000).

It should be noted that hEXO1 will be used as nomenclature for human exonuclease 1 when hEXO1a/HEX1 and hEXO1b are not compared.

Interactions with hEXO1

The *S. cerevisiae* and human exonucleases 1 (Exo1 & hEXO1) interact with mismatch repair protein Msh2/hMSH2 as demonstrated by the two-hybrid system and immuno-coprecipitation, suggesting that hEXO1 may play a role in MMR (Tishkoff *et al.*, 1997; Schmutte *et al.*, 1998; Tishkoff *et al.*, 1998; Rasmussen *et al.*, 2000). The interaction is mediated through C-terminal domains for both the *S. cerevisiae* and human exonucleases 1 (Tishkoff *et al.*, 1997; Rasmussen *et al.*, 2000). We have shown that hMSH2 interacts with both forms of human exonuclease 1, suggesting that the interacting domain is located between exons 8 and 13 (~amino acids 384-870) (Rasmussen *et al.*, 2000). Recently, Schmutte *et al.* (2001) suggested that the carboxy-terminal amino acids 603-846 in hEXO1 are the specific interacting region with hMSH2 (Schmutte *et al.*, 2001).

Like hMSH2, the hMLH1 protein interacts with hEXO1 through the C-terminal domain of the hEXO1, as demonstrated by the two-hybrid system and via *in vitro* pull-down assay (Jäger *et al.*, 2001; Schmutte *et al.*, 2001). Two other MMR proteins, hMSH6 and hPMS2, have been shown not to interact with hEXO1 in the two-hybrid system (Rasmussen *et al.*, 2000; Jäger *et al.*, 2001). However, the N-terminal (amino acids 129-390) of hEXO1 seems to interact with the N-terminal of hMSH3 (Schmutte *et al.*, 2001). This result indicates that hEXO1 and hMSH3 proteins interact with same region of hMSH2. The interacting region of hMSH2 with hEXO1 are amino acids 261-671 of hMSH2 (261-669aa are essential for interaction and 261-600aa stabilize the interaction) and the interaction region between hMSH2 and hMSH3 are amino acids 378-625 of hMSH2. (Schmutte *et al.*, 2001).

Expression/localization of hEXO1

We have shown that *hMSH2* and *hEXO1* are co-expressed at high levels in fetal liver, adult testis and thymus. *hEXO1* transcripts are expressed in the fetal tissue of liver, spleen and kidney but not in adult liver, spleen, and kidney tissue, suggesting a role for hEXO1 in development of these tissues. Northern Blot analysis revealed that *hEXO1* is highly expressed in several liver cancer cell lines as well as in colon and pancreas adenocarcinomas but not in the corresponding non-neoplastic tissue (Rasmussen *et al.*, 2000).

Sub-cellular localization of hEXO1 was restricted to the nucleus of murine NIH3T3 cells transfected with YFP-hEXO1b plasmids (Jäger *et al.*, 2001), indicating that hEXO1 has no function in the mitochondria.

Mutator gene

Disruption of *EXO1* increased the mutation rate in *S. cerevisiae* cells, indicating a role for *EXO1* in DNA repair (Tishkoff *et al*., 1997; Tran *et al*., 1999). However, *S. cerevisiae exo1* mutants show a moderately lower mutator phenotype than that caused by mutations in *MSH2* (Tishkoff *et al*., 1997; Tran *et al*., 1999). This suggests that there are additional exonucleases involved in MMR in *S. cerevisiae*.

FEN1 may be another exonuclease involved in MMR. It has been observed that *rad27* and *exo1* mutations are lethal in combination with one another, unlike the corresponding single mutants (Tishkoff *et al*., 1997; Gary *et al*., 1999). Overexpressions of Exo1 or hEXO1 proteins suppress both the temperature sensitive and the spontaneous mutator phenotype of *rad27* mutants. (Tishkoff *et al*., 1997; Qiu *et al*., 1999).

hEXO1 remains unverified as a colon cancer predisposition gene. However, Wu *et al*. (2001) have detected germline *hEXO1* variants in HNPCC families. All *hEXO1* variants were identified in families in which no germline *hMSH2, hMLH1*, and *hMSH6* mutations had been found. One *hEXO1* variant found in a family with HNPCC resulted in a truncated protein that was 106 amino acids shorter than the wild-type gene product detected (Wu *et al*., 2001). Because hEXO1 interacts with hMSH2 through its C-terminal, the shorter gene product could have functional consequences for MMR.

DNA exonuclease activity

All of the Exo1 homologous (*S. cerevisiae, S. pombe,* and human) posses a 5'→ 3' double-stranded DNA exonuclease activity (Szankasi & Smith, 1995; Tishkoff *et al*., 1997; Qiu *et al*., 1999). The hEXO1 and Exo1 proteins act both on single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) substrate but have prefererence for dsDNA. The human hEXO1 does not discriminate between RNA and DNA substrates *in vitro* (Qiu *et al*., 1999). This suggests that the lethality of the *exo1rad27* double mutants are caused by inability to remove primer RNAs during lagging strand DNA synthesis. As mentioned earlier, the function of removing RNA primers of the lagging DNA strand is known to be performed by the FEN1/Rad27 nuclease in eukaryotes (Bambara *et al*., 1997). However, the efficiency of

hEXO1 to remove RNAs indicates that it may also be involved in removal of RNA primers during lagging strand DNA synthesis.
A detailed analysis of the nuclease domain (HEX1-N2) of hEXO1 showed that HEX1-N2 has 5'flap and pseudo flap-like structure-specific endonuclease activities analogous to FEN1 (Lee & Wilson, 1999). However, neither HEX1-N2 nor FEN1 were active on 3'-flap structures (Lee & Wilson, 1999). An analysis revealed that HEX1-N2 degrades blunt duplex substrate at a rate roughly 7-fold faster than ssDNA, but the analysis did not show specific endonuclease activity at 10-base pair bubble-like structures, G:T mismatches, or U:G mismatches (Lee & Wilson, 1999).

Both hEXO1 and FEN1 appear to be directed by their C-terminal domains. As mentioned earlier hEXO1 associates with hMSH2 and hMLH1 through a C-terminal interaction, a physical association that may direct hEXO1 to sites of mispaired nucleotides. The C-terminus of FEN1 has been identified as the PCNA binding sequence (Gary *et al.*, 1997; Warbrick *et al.*, 1997). PCNA stimulates FEN1 nuclease activity and PCNA stabilizes FEN1 on a DNA substrate (Tom *et al*., 2000). These properties suggest that FEN1 and PCNA interact during the course of DNA replication, DNA repair, or both.
Gomes & Burgers (2000) have found that protein-protein contacts between FEN1 and PCNA differ depending on whether the proteins are in complex with DNA or not (Gomes & Burgers, 2000). In the absence of DNA, FEN1 interacts with PCNA mainly through the **I**nter**D**omain **C**onnector **L**oop (IDCL). However, when PCNA encircles the DNA, the C-terminal domain of PCNA rather than its IDCL is important for binding FEN1 (Gomes & Burgers, 2000). This interesting bimodal interaction between PCNA and FEN1 may represent a model for several other proteins with the PCNA-binding motif ($Q_1XX[ILM]_4XXF_7[FY]_8$). The PCNA-binding motif has been identified in a large number of proteins involved in DNA metabolic processes, such as DNA methylation (MCMT, cytosine-5-methyltransferase), NER (XPG endonuclease), base excision repair (hMYH glycosylase), MMR (hMSH3 and hMSH6) and cell cycle control (p21) (Warbrick, 1998; Tsurimoto, 1999; Boldogh *et al*., 2001; Kleczkowska *et al*., 2001).

3' → 5' exonuclease

Strand-specific mismatch repair in HeLa cell extracts can be directed by a single strand nick in a heteroduplex and the mismatch correction is independent of whether the nick occurs 3' or 5' to the mispair (Fang & Modrich, 1993). This suggests that the human MMR possesses a

bidirectional capability. Polymerase δ and ε are responsible for lagging and leading DNA strand replication. The 3' → 5' proofreading exonuclease activity of both polymerase δ and ε has been proposed to be functionally redundant with Exo1 in *S. cerevisiae* (Tran *et al.,* 1999). Yeast strains harboring inactivation of *exo1* and the 3' → 5' proofreading exonuclease function of polymerase ε led to an increase in the mutation rate of up to 55-fold over that found for either single mutant (Tran *et al*., 1999). Furthermore, yeast strains with mutation in the 3' → 5' proofreading exonuclease of DNA polymerase δ in combination with a deletion of *exo1* or *msh2* are lethal. These findings indicate that 5' → 3' activity of Exo1 and the 3' → 5' exonuclease activity of DNA polymerase δ participate in a bi-directional MMR (Tran *et al*., 1999).

1.4 Methods

We employed the yeast two-hybrid system in order to identify new MMR proteins. The yeast two-hybrid system is a genetic assay designed to detect protein-protein interactions *in vivo* and has been used with great success to identify new partners in multi-protein complexes (Fields and Song, 1989; Chien *et al*., 1991).

Principles of the two-hybrid system

The yeast two-hybrid system relies on the structure of particular transcription factors that have two physically separable domains. One domain (the **B**inding **D**omain) interacts with the DNA at an upstream activation site. The second domain (the **A**ctivation **D**omain) binds to the basal transcription apparatus and activates transcription. The MATCHMAKER GAL4 two-hybrid system (CLONTECH) utilizes the yeast GAL4 transcriptional activator which is required for expression of genes encoding proteins involved in galactose metabolism. In the two-hybrid system, the two GAL4 domains are separately fused to proteins, and the recombinant hybrid proteins are expressed in yeast. If the two hybrid proteins interact, the two GAL4 domains (BD and AD) will be in close proximity and will be able to activate transcription of reporter genes (e.i. *HIS* and *lacZ)* (Bai & Elledge, 1997).

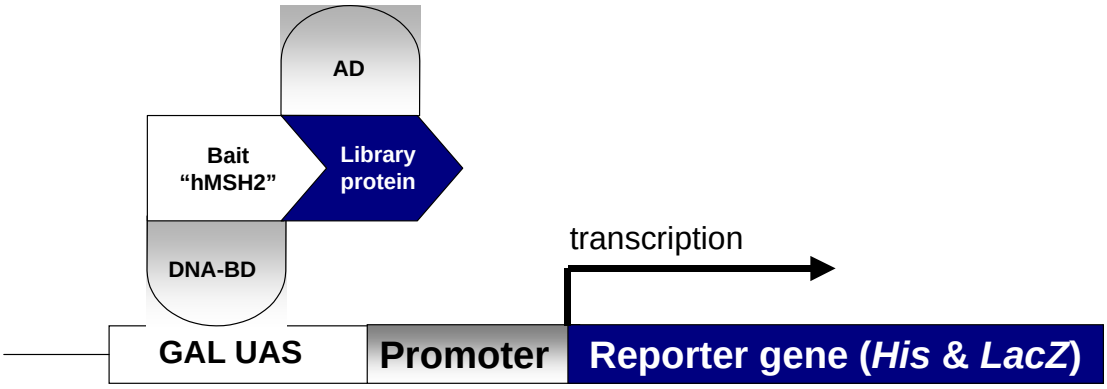


Figure 1.2. The principle of the two-hybrid system. The bait is cloned into the DNA-BD vector where it is expressed as a fusion to amino acids 1–147 of the yeast GAL4 protein. A second gene or cDNA library is cloned into the AD vector, where it is expressed as a fusion to amino acids 768–881 of the yeast GAL4 protein. When the fusion proteins interact, the DNA-BD and AD domains are brought into close proximity and can activate transcription of reporter genes. The DNA-BD target the transcription factor to a specific promotor sequence UAS (upstream activation sequence) whereas the DNA-AD domain facilitate assembly of the transcription complex allowing the initiation of transcription (Bai & Elledge, 1997).

The BD-plasmids GAL4-hMSH2 (pLJR105) or GAL4-hMLH1 (pACJ14) were transformed into *S. cerevisiae* strain Y190 as described in Rasmussen *et al*. (2000). The human fetal liver matchmaker cDNA library (CLONTECH, #HL4029AH) was sequentially transformed into Y190/pLJR105 or Y190/pACJ14.

Table 1.2 • Summary of Two-hybrid screening

Number of independent clones in human fetal liver cDNA library	$3.5x10^6$ *
Average insert size	1.5 kb
Amount of library plasmids used	40 μg
Cotransformation efficiency	$2.2x10^4$ - $8.2x10^4$ cfu/μg
Clones screened	$8.8x10^5$ - $3.2x10^6$
Positive colonies	20 - 40

*Libraries with at least $1x10^6$ independent clones are representative of the genomic DNA or mRNA population complexity (protocol #PT3061-1; www.clontech.com).

Interactors were selected on synthetic dextrose minimal medium (SD) lacking tryptophan (to maintain the GAL4 binding domain plasmids), leucine (to maintain the GAL4 activation domain plasmids), and histidine (to identify peptides capable of assembling a functional GAL4 transcription factor), and supplemented with 30 mM 3-amino-1, 2, 4-triazole (3-AT). The plates were incubated at 30^{o}C for 7-10 days and approximately 700 positive clones (minimum size ~ 1 mm) for each screen were picked from SD/-TRP/-LEU/-HIS + 3-AT plates and screened for β-galactosidase activity to verify positive interactions (for more method details see Rasmussen *et al*., 2000). The total number of positive interactions after β-galactosidase screening was therefore only ~ 30 positive clones per screen (table 1.2).

1.5 Results & Discussion

Library screenings with BD-hMSH2 and BD-hMLH1 were done twice for each MMR protein. The results presented in table 1.3A and 1.3B are a summary of four independent screens. A hMSH2-hMSH6 binding domain vector was also constructed by inserting the *hMSH2* coding sequence into the *Sal*I site of the pBridge binding domain vector (CLONTECH). The human *hMSH6* coding sequence was inserted into the *Not*I site. The *hMSH6* protein was in this way conditionally expressed from the P_{Met25} promoter. Because hMSH2 binds to a mismatch DNA sequence in complex with hMSH6, one could expect hMSH6 to work as a bridge protein that stabilizes a weak interaction between hMSH2 and another MMR protein, or as a modifier of hMSH2 or another MMR protein. However, a library screen using this construct gave no positive interactions.

Table 1.3A • Result of yeast two-hybrid screen

• hMSH2 as bait	Number of clones
Human exonuclease 1 (hEXO1)	7
Human G/T mismatch binding protein (hMSH6)	2
Human alpha1-antichymotrypsin	8
Human alpha-1-microglobin	1
Human alphaglobin	1
Human Alu RNA binding protein	1
Human colony stimulating factor 1 (CSF-1, FMS)	1
Human complement component 3 (C3)	1
Human cofilin (F-actin depolymerizing protein)	1
Human ectonucleoside triphosphate diphosphohydrolase 5 (ENTPD5)	1
Human fibrinogen	4
Human gammaglobin	5
Human glycine amidotransferase	1
Human haptoglobin	1
Human hemoglobin alpha (HBA1)	2
Human hemoglobin gamma G (HBG2)	5
Human homeobox protein HOX-B6 (Hox-2.2)	1
Human HU-K4	1
Human importin alpha homolog	1
Human mitochondrial COX1 (cytochrome C oxidase subunit I)	1
Human mitochondrial COX3 (cytochrome C oxidase subunit III)	1
Human mRNA for long-chain acyl-CoA synthetase	1
Human non-muscle myosin alkali light chain isoform	1
Human selenoprotein P (SEPP1)	1
Human serum albumin (ALB)	3
Human sodium phosphate transporter (NPT3)	1
Human translation elongation factor 1 alpha1 (EEF1A1)	1
ribosomal protein L13a	2
rRNA from: Homo sapiens mitochondrion"16S ribosomal RNA"	2
Unknown human cDNAs	18

Table 1.3B • Result of yeast two-hybrid screen

• hMLH1 as bait	Number of clones
Human homolog of mutL (hPMS1)	3
Human 14-3-3 gamma (YWHAG)	1
Human alpha-1-antichymotrypsin	10
Human alpha-2-HS-glycoprotein (AHSG)	1
Human EIF4A1 (translation factor 4AI)	1
Human fibrinogen beta (FGB)	1
Human fibrinogen gamma	2
Human H19	1
Human haptoglobin (HP)	1
Human hemoglobin gamma G (HBG2)	5
Human homeobox protein HOX-B6 (Hox-2.2)	3
Human H-protein (glycine cleavage system)	1
Human karyopherin alpha2 (importin alpha, Rch1, hSRP1)	1
Human ornithine decarboxylase antizyme	1
Human peroxiredoxin 2 NKEFB (natural killer cell enhacing factor)	1
Human poly(A)-binding protein-like 1 (PABPL1)	1
Human RAB22A (Ras oncogene family)	1
Human ribosomal protein L27a (RPL27A)	1
Human ribosomal protein S11 (RPS11)	1
Human serum albumin	1
KIAA0860	1
KIAA1018	2
Unknown human cDNAs	8

Two-hybrid screening with hMSH2 as a bait

Our screens identified 77 clones as interactors. Sequence analysis showed that the majority of the clones contained unidentified human cDNAs and extracellular proteins such as gammaglobin, fibrinogen and α-antichymotrypsin. Furthermore, we isolated two clones containing hMSH6, which is known to form a complex with hMSH2 and seven clones containing hEXO1 (Rasmussen *et al*., 2000). When full-length hEXO1 was fused to GAL4 activation domain in pACT2, we were unable to detect protein-protein interaction with any of our MMR (BD) constructs in the two-hybrid system. However, when full-length hEXO1 was

fused to the GAL4 binding domain in pAS2, we could detect interactions with MMR (AD) proteins in the two-hybrid assay. This could explain why we failed to isolate any full-length cDNAs of *hEXO1* in our two-hybrid screen (Rasmussen *et al*., 2000).

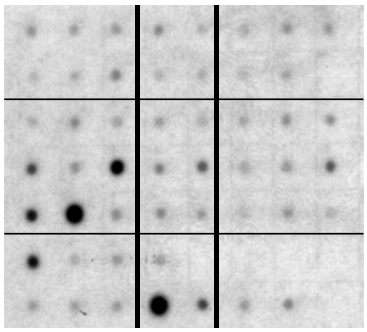
Three clones containing *COX1, COX3* and 16S ribosomal RNA were shown to form a complex with hMSH2. However, these interactions are probably artifacts as COX1 and COX3 are subunits of cytochrome *c* oxidase (Complex III) of the electron transport chain. It has not yet been determined if MMR is active in human mitochondria. In yeast a MutS homolog (Msh1) of the MMR pathway has been identified in mitochondria (Reenan & Kolodner, 1992). Our two-hybrid screenings with hMSH2 and hMLH1 did not indicate the presence of mitochondria specific MMR proteins, although we can not exclude that they can be found among the unidentified human cDNAs.

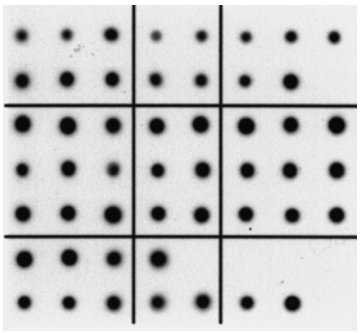
Two-hybrid screening with hMLH1 as a bait

The screening with hMLH1 was performed as described previously for hMSH2 (Rasmussen *et al*., 2000). The GAL4 DNA-BD was fused to full-length *hMLH1* and verified by sequencing. Again the sequence analysis of the hMLH1 interactors showed that the majority of the clones contained unidentified human cDNAs and proteins such as fibrinogen and α-antichymotrypsin (table 1.3B). Furthermore, we isolated three clones containing hPMS1, which is known to form a complex with hMLH1. To find hPMS1 as the interacting partner with hMLH1 at first surprised us, as hPMS2 has been shown to be approximately 10-fold more abundant in HeLa nuclear extract than hPMS1 (Räschle *et al*., 1999). However, the same authors demonstrated that the affinity of hMLH1 for hPMS1, measured in the yeast two-hybrid system, was greater than for hPMS2 (Räschle *et al*., 1999). Therefore, a greater affinity for hPMS1 could explain why we only isolated this gene in our two-hybrid screen.

Given that hPMS2 might compete with hPMS1 for the available hMLH1, we decided to determine the relative amounts of *hPMS1* in human tissues. We used a human RNA master blot (figure 1.3) and a human multiple tissue RNA master blot (figure 1.4) from CLONTECH to characterize the expression pattern of *hPMS1* (as described in Rasmussen *et al*., 2000). We found that hPMS1 is predominantly expressed in fetal liver and adult liver, but also in pancreas, kidney, testis and appendix (figure 1.3). The hMLH1-hPMS1 complex could therefore predominate in fetal liver and account for why we isolate hPMS1 and not hPMS2 when using the two-hybrid screen. It is tempting to speculate that hPMS1 is tissue specific and that the hMLH1-hPMS1 complex plays an important role in DNA repair in liver.

	1	2	3	4	5	6	7	8
A	whole brain	amygdala (brain)	caudate nucleus	cere-bellum	cerebral cortex	frontal lobe	hippo-campus	medulla oblongata
B	occipital lobe	putamen	substantia nigra	temporal lobe	thalamus	nucleus accumbeus	spinal cord	
C	heart	aorta	skeletal muscle	colon	bladder	uterus	prostate	stomach
D	testis	ovary	pancreas	pituitary gland	adrenal gland	thyroid gland	salivary gland	mammary gland
E	kidney	liver	small intestine	spleen	thymus	peripheral leukocyte	lymph node	bone marrow
F	appendix	lung	trachea	placenta				
G	fetal brain	fetal heart	fetal kidney	fetal liver	fetal spleen	fetal thymus	fetal lung	





hPMS1 β-actin

Figure 1.3. Expression profile of hPMS1. The RNA master blot (CLONTECH #7770-1) was hybridized with hPMS1 or β-actin control probes.

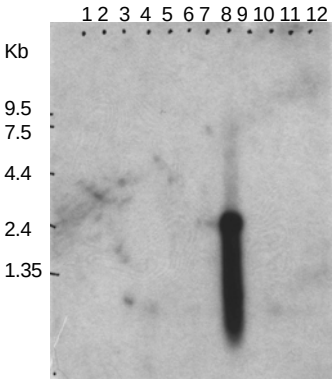


Figure 1.4. Hybridization of multiple tissue Northern blot with hPMS1 probe. Each lane contains following human tissues. Lane 1: brain. Lane 2: heart. Lane 3: skeletal muscle. Lane 4: colon. Lane 5: thymus. Lane 6: spleen. Lane 7: kidney. Lane 8: liver. Lane 9: small intestine. Lane 10: placenta. Lane 11: lung. Lane 12: peripheral blood leukocyte (CLONTECH #7780-1).

Importin α

In the hMSH2 and the hMLH1 two-hybrid screens we found interaction with an importin α homolog and importin-α, respectively. Importin-α is also known as importin 58/Srp1/Rch1/ Kap60/karyopherin α (Jans *et al*., 1998).

All passive and active transport into and out of the nucleus occurs through the nuclear pore complexes (NPCs) present in the nuclear envelope. Molecules smaller than approximately 60 kDa may passively diffuse through the NPCs into the nucleus, but import of larger molecules requires specific transport signals (Görlich, 1998). Targeting of many proteins to the nucleus is determined by the presence of a **N**uclear **L**ocalization **S**ignal (NLS), a short sequence containing one or two clusters of basic amino acid residues. (Köhler *et al*., 1999). An NLS-bearing protein, often termed the "cargo", is delivered to the nucleus by association with a heterodimer, formed by importin α and importin β (also called karyopherin- α and -β). Importin α recognizes the NLS, while importin β is responsible for docking to the NPC and translocation through the pore (Görlich, 1998; Jans *et al*., 1998; 2000). Translocation into the nucleus is terminated at the nuclear side of the NPC by disassembly of the trimeric NLS-protein/importin α/β complex. Association of importin β with the protein Ran, a Ras-related GTPase may trigger the dissociation (Jans *et al*., 2000).

Higher eukaryotes possess more than one form of importin-α (at least 6 forms for humans) (Köhler *et al*., 1999). The larger number of distinct importin α isoforms in higher mammals implies that there is specialization in their cellular role, and that different isoforms could bind unique target proteins. Consistent with this is the fact that neither any single human importin-α nor any triple combinations of three analyzed importin-α homologues were able to complement a *S. cerevisiae* SRP1 mutant (*S. cerevisiae* has only one gene for importin-α) (Nachury *et al*., 1998).

The hMSH2 human mismatch repair protein has a weak nuclear signal (Boulikas, 1997). A protein processing a single weak NLS is more likely to be retained in the cytoplasm after a mutation at the NLS peptide than a protein with two or more NLSs (Boulikas, 1997). Mutations at the weak NLS of the hMSH2 gene, or dysfunction of translocation proteins like importin-α may result in cytoplasmic retention that again could lead to dysfunction of the MMR pathway. Hampered translocation of the MMR proteins could thus be a possible explanation why so many HNPCC families fail to display mutations in the known MMR genes. We hope to do more research regarding translocation of MMR proteins in the future.

Potential NLSs for MMR proteins are listed in table 1.4 by using the PSORT software programs (Nakai *et al*., 1992; http://psort.nibb.ac.jp/).

Table 1.4 • Prediction of nuclear localization signal (NLS)

MMR protein	Potential NLSs	Reference
hMSH2	EKHEGKHQKLL at 422	(Boulikas *et al*., 1997)
hMSH3	RRKP at 3 RRKK at 76 RKKR at 77 KKRP at 78 KKRK at 704 KRKR at 1091 PVKKKVK at 87 PLIKKRK at 701 RRKKRPLENDGPVKKKV at 76 RKKRPLENDGPVKKKVK at 77 KKRPLENDGPVKKKVKK at 78 KRPLENDGPVKKKVKKV at 79	(PSORT; Nakai *et al*., 1992)
hMSH6	KKRR at 246 RKRK at 298 KRKR at 299 HRRR at 382 RRRP at 383 RKRKRMVTGNGSLKRKS at 298 KRKRMVTGNGSLKRKSS at 299 RKRMVTGNGSLKRKSSR at 300 KRMVTGNGSLKRKSSRK at 301	(PSORT; Nakai *et al*., 1992)
hMLH1	PRKR at 469 RKRH at 470 KRHR at 471 PRRR at 496 PRKRHRE at 469 PRRRIIN at 496	(PSORT; Nakai *et al*., 1992)
hPMS1	RPRK at 869 KRAIEQESQMSLKDGRK at 636	(PSORT; Nakai *et al*., 1992)
hPMS2	PNTKRFK at 574	(PSORT; Nakai *et al*., 1992)
hEXO1	KRPR at 418 KRKH at 775 PIKRKLI at 290	(PSORT; Nakai *et al*., 1992)

2. Repair of Mitochondrial DNA

2.1 Introduction

Within all mammalian cells there are two distinct genomes, one located in the nucleus (nDNA) and the other in the mitochondria (mtDNA). Although each human somatic cell has several hundred to thousand mitochondria (and 1-10 mtDNA copies per mitochondrion) the amount of the mtDNA is only roughly 1% of the total DNA in the cell due to the small size of mtDNA compared to nDNA (Bestwick, 1982; Wallace *et al*., 1998). The mammalian mtDNA retains only 22 tRNAs, 12S rRNA and 16S rRNA genes necessary for the mitochondrial protein synthesis as well as 13 polypeptide genes (Anderson *et al*., 1981). All 13 polypeptides are part of the 87 structural polypeptide subunits, all of which are components of the respiratory chain. The respiratory chain is composed of five multisubunit enzymes whose components are encoded by both the nuclear and mitochondrial genomes (Wallace, 1999).

Unlike nDNA mammalian mtDNA contains very few noncoding sequences, no introns and it is unprotected by histones. Therefore, damage to mtDNA can be expected to have greater impact on cell function than damage to nuclear DNA, as the probability of damaging coding sequences in mtDNA is much higher. Thus, it has been reported that the rate of point mutations is higher in mtDNA compared to nDNA in human tissues (Khrapko *et al*., 1997). In addition mtDNA point mutations and mtDNA rearrangement mutations (deletions and insertions) have now been recognized to play a critical role in numerous human disorders which prove the importance of maintaining the integrity of the mitochondrial genome (Kang, 1998; Kogelnik *et al*., 1998[2]; Pulkes & Hanna, 2001; Wallace, 1999).

For this reason DNA repair in mitochondria could be expected to be very efficient. However, early investigations of removal of UV-induced pyrimidine dimers in mtDNA led to the conclusion that mitochondria accumulate DNA damage because these organelles are deficient in repair activity (Clayton *et al*., 1974; Prakash *et al*., 1975). This finding, in combination with very limited interest in this research area, led to the belief that rather than repairing damage in mtDNA, cells simply destroyed the injured genomes and replaced them by replicating existing, undamaged mtDNA

[2]Variations in the human mitochondrial genomes are updated on http://www.gen.emory.edu/mitomap.html (Kogelnik *et al*., 1998).

(LeDoux *et al*., 1999). It has later been confirmed that UV-induced pyrimidine dimers are not repaired in mitochondria (LeDoux *et al*., 1992; Pascucci *et al*., 1997), which suggest absence of nucleotide excision repair. However, recent evidence showed that mitochondria are indeed able to repair their genomes (Croteau *et al*., 1999; Marcelino & Thilly, 1999). Unfortunately the knowledge about the repair mechanisms operating in the mitochondria is still limited.

To gain a better understanding of the molecular processes and components responsible for DNA repair in the human mitochondrion we have investigated the sub-cellular localization of O^6-methylguanine-DNA methyltransferase (MGMT) (Rasmussen *et al*., II).

2.2 Base Excision Repair

Several observations support that mitochondria have **B**ase **E**xcision DNA **R**epair (BER) pathways that are responsible for the removal of simple lesions in DNA (Croteau *et al*., 1999; Marcelino & Thilly, 1999). Removal of a damaged base by the BER pathway is initiated by DNA glycosylase that cleave the N-glycosylic bond between the base and the deoxyribose-phosphate backbone (Lindahl & Wood, 1999). The resulting abasic site is cleaved 5' by an apurinic/apyrimidinic (AP) endonuclease that generates a 3'OH group, which can be extended by a DNA polymerase, but not ligated before a 5' terminal deoxyribose-phosphate residue is removed. The removal of the abasic sugar residues is done by a lyase. Finally, the gap is filled by DNA polymerase and the ends rejoined by DNA ligase (Lindahl, 2000). Several distinct DNA glycosylases have been identified both in nuclei and mitochondria in human cells (table 2.1).

Repair of uracil

Uracil-DNA Glycosylase (UNG or UDG) removes uracil bases in DNA. An uracil base in DNA can occur as a result of either misincorporation of dUTP instead of dTTP into a newly synthesized DNA, or deamination of cytosine to uracil (Slupphaug *et al*., 1995). Deamination of cytosine to uracil, unless repaired before the next round of replication, will result in a GC $\rightarrow$ AT transition mutation (Slupphaug *et al*., 1995). Studies have demonstrated the presence of both nuclear- and mitochondrial-associated UNG (Anderson & Friedberg, 1980; Slupphaug *et al*., 1993; Otterlei *et al*., 1998). The human mitochondrial (hUNG1) and nuclear (hUNG2) forms have identical catalytic domains, but very different N-

terminal sequences. The two forms are both generated from the *hUNG* gene using two promoters, and making use of an exon specific for the N-terminal end of the nuclear form, and alternative splicing (Nilsen *et al*., 1997; Otterlei *et al*., 1998).

Repair of oxidative damage

Both purine and pyrimidine residues in DNA are sensitive to reactive oxygen species (ROS) (Croteau & Bohr, 1997). The most common purine lesion is 8-hydroxyguanine also called 7,8-dihydro-8-oxoguanine (8-oxoG) (Steenken & Jovanovic, 1997; Burrows & Muller, 1998). 8-oxoG is a highly mutagenic base derivative, which base-pairs with adenine as well as cytosine, causing G → T transversion mutations (Cheng *et al*., 1992). The mitochondrial respiratory chain produces superoxide (Wallace, 1999) which can be converted to hydroxyl radicals via hydrogen peroxide (Imlay & Linn, 1988). The hydroxyl radical is the main species of active oxygen that attacks the guanine base (Kasai *et al*., 1984). Because mtDNA is subjected to a relatively high amount of oxidative damage and because the strand containing 28 of the 37 mitochondrial genes is rich in guanine (Bianchi *et al*., 2001), it seems that mitochondria would need efficient DNA repair mechanisms to remove oxidative damage from its DNA.

Removal of 8-oxoguanine

An early study revealed that 8-oxoG can pair with all four normal bases (Kuchino *et al*., 1987) but it was later shown that 8-oxoG perferentially pairs with C or A during *in vitro* DNA synthesis (Shibutani *et al*., 1991). In *E. coli* two DNA glycosylases, encoded by *mutM* (*Fpg*) and *mutY* genes, function to prevent mutagenesis by removing 8-oxoG paired with cytosine and adenine (Bailly *et al*., 1989; Tchou *et al*., 1991; Michaels *et al*., 1992).

The human MutM homolog, hOGG1 (8-**O**xo**G**uanine DNA **G**lycosylase) excise 8-oxoG preferentially when it is paired with C, followed by 8-oxoG:T and 8-oxoG:G, but it has no detectable 8-oxoG:A-specific strand cleavage (Hazra *et al*., 1998). The glycosylase activity of hOGG1 is accompanied with apurinic/apyrimidinic (AP) lyase that cleaves the AP site via β-elimination (Hazra *et al*., 1998).

Human OGG1 localizes both to the nucleus and mitochondria (Takao *et al*., 1998; Nishioka *et al*., 1999). Seven alternatively spliced forms of *hOGG1* mRNAs have been identified (Nishioka *et al*., 1999). All these splice forms of hOGG1 contain a putative mitochondrial targeting signal (MTS) at the common N-terminal region. A nuclear localization signal (NLS) was only found in the C-terminal end of hOGG1-1a (Nishioka *et al*., 1999). Human

OGG1-1a, which has been found to have a weak MTS, localized predominantly to the nucleus. When the NLS is deleted, the protein is targeted to mitochondria (Nishioka *et al*., 1999). When a strong MTS is fused upstream to the *hOGG1-1a* gene it is selectively targeted to the mitochondria (Dobson *et al*., 2000).

Interestingly, results from mice liver have revealed that mitochondrial 8-oxoG glycosylase activity increased with age. In contrast no age-associated changes were found for nuclear 8-oxoG glycosylase activity or mtUDG activity (de Souza-Pinto *et al*., 2001). This result suggests that the mitochondrial OGG1 glycosylase is up-regulated during the aging process.

Removal of adenine paired with 8-oxoguanine

The **H**uman **M**ut**Y** **H**omolog, hMYH has been shown to excise adenine mispaired with guanine and 8-oxoG as well as 2-hydroxyadenine paired with 8-oxoG in double-stranded oligonucleotides (Ohtsubo *et al*., 2000). This glycosylase has been detected in nucleus and mitochondria (Takao *et al*., 1998; 1999; Ohtsubo *et al*., 2000; Boldogh *et al*., 2001). Boldogh *et al*. (2001) found that the levels of hMYH in the nucleus was increased 3- to 4-fold during progression of the cell cycle and reached maximum levels in S phase, suggesting a cell cycle-dependent regulation of expression and/or subcellular targeting. However, there was no evidence that the cytoplasmic or mitochondrial levels of hMYH decreased as nuclear levels increased (Boldogh *et al*., 2001).

It has been suggested that 8-oxoG:C is more efficiently repaired in mitochondria than 8-oxo:A. Miyako *et al*. (2000) detected inefficient cleavage of human mtDNA by the *E. coli* 8-oxoG:C specific MutM protein but could report that human mtDNA was cleaved by the 8-oxoG:A specific MutY protein, suggesting that 8-oxoG accumulates as an 8-oxoG:A pair but not as an 8-oxoG:C pair.

Removal of oxidized pyrimidines

Oxidized pyrimidines, such as thymine glycol, 5-hydroxycytosine and 5,6-dihydrouracil (DHU), are excised by the hNTH1 glycosylase (a homolog of *E. coli* endonuclease III, Nth) (Ikeda *et al*., 1998; Takao *et al*., 1998; Lindahl & Wood, 1999). Thymine glycol is only slightly mutagenic but it can block progression of both DNA and RNA polymerases (Stierum *et al*., 1999). The DHU DNA lesion derives from cytosine by deamination. Its opposite base should be guanine, but DHU is also able to mispair with adenine during DNA replication. hNTH1 cleaves A or G opposite DHU with the same rate (Ikeda *et al*., 1998). The human NTH1 has, similar to hOGG1, combined glycosylase and AP lyase activity and

this enzyme is located to both nucleus and mitochondria (Tomkinson *et al*., 1990; Takao *et al*., 1998; Mol *et al*., 1999).

Removal of 3-methyladenine

The N-methylpurine-DNA glycosylase, MPG (MDG, AAG, APNG) gene is coding for a human glycosylase, which removes 3-methyladenine (3-MeA) as well as 3-methylguanine (3-MeG), 7-methylguanine (7-MeG), N^6-ethenoadenine, hydroxanthine, guanine, and 8-oxoG (Pendlebury *et al*., 1994; Wyatt *et al*., 1999; Bouziane *et al*., 2000). The MPG activity has so far only been reported from nuclear extracts.

Table 2.1 • Enzymes identified in repair of mitochondrial DNA in human cells

Gene	Full Name	Substrates/Activity DNA glycosylases: major altered base released	AP lyase activity
Base excision repair (BER)			
hUNG1	Uracil-DNA glycosylase	U	no
hOGG1	8-oxoguanine DNA glycosylase 1	8-oxoG opposite C	yes
hMYH	MutY homolog (*E.coli*)	A opposite 8-oxoG or G	no
hNTH1	Endonuclease three homolog 1(*E.coli*)	T-glycol	yes
Other BER factors			
hMTH1	MutT homolog (*E.coli*)	Hydrolyzes 8-oxo-dGTP to 8-oxo-dGMP	
hLIG3	DNA Ligase III	Ligation	
hPOLG	Polymerase-gamma	Replication/repair polymerase	

Other BER factors

In cells, the deoxyribonucleotide pools are also subjected to oxidative damage. dGTP can be converted to 8-oxo-dGTP and incorporated into nascent DNA strands opposite adenine. The MutT enzyme in *E. coli* hydrolyzes 8-oxo-dGTP to 8-oxo-dGMP, and thereby prevents misincorporation of the damaged base into DNA (Maki & Sekiguchi, 1992). The human MutT homolog, hMTH1 (for ***mutT* h**omologue) is present in the cytoplasm as well as in the mitochondrial matrix (Kang *et al*., 1995; Nakabeppu, 2001). The *hMTH1* gene produces by alternative transcription inititation and splicing, seven different mRNAs. One of these mRNA's is imported into mitochondria when fused to green fluorescent protein, GFP (Nakabeppu, 2001). The human MTH1 protein has wide substrate specificity as it has been

reported to hydrolyze 8-oxo-dGTP, 8-oxoG, 8-oxo-dATP and 2-OH-dATP (Nakabeppu, 2001).

A major human AP endonuclease, hAPE1 (alternative titles; human apurinic endonuclease 1 (HAP1), apurinic/apyrimidinic exonuclease (APEX), apurinic/apyrimidinic endonuclease/ redox effector factor (APE/REF-1)) plays a central role in BER (Evans *et al.* 2000). It initiates repair by hydrolyzing AP sites in DNA produced either spontaneously or after removal of bases in DNA by DNA glycosylases (i.e. UNG). Alternatively, it can act as a 3'-phosphoesterase after the AP lyase reaction of DNA glycosylases/AP lyases (i.e. hOGG1) (Izumi *et al*., 2000). Takao *et al*. (1998) examined the subcellular localization of hAPE1 and found that it was only localized to the nucleus (Takao *et al*., 1998). Furthermore, Prieto-Alamo & Laval (1999) found no increased hAPE1 activity in the mitochondrial fraction of Chinese hamster ovary (CHO-9) cells transfected with a plasmid expressing the human APE1 compared to a 4.5 fold activity increase in nuclear fractions. However, the APE1 homolog in *S. cerevisiae*, the Apn1 enzyme has been shown to localize in both nucleus and mitochondria. The Pir1 (a cell wall protein) is required for the localization of Apn1 to mitochondria (Vongsamphanh *et al*., 2001). Results of two different studies have also shown that APE1 is present in mitochondria of rat thyroid FRTL-5 cells and rat pleural mesothelial cells (Fung *et al*., 1998; Tell *et al*., 2001). However, the contribution of the mitochondrial AP lyase activity, to repair of abasic sites, and the processing of 3'-unsaturated sugar-phosphate generated by e.i. hOGG1 AP lyase activity, still remain to be established in human mitochondria.

Human mitochondrial DNA is replicated by polymerase γ. Polymerase γ (POLG) is a heterodimer composed of a 140-kD catalytic subunit (POLG1) and a smaller accessory subunit (POLG2) (Schmitt & Clayton, 1993). Because polymerase γ is the only known DNA polymerase in human mitochondria, it is expected to participate in DNA replication and repair in this organelle (Longley *et al*., 1998). The human polymerase γ has been shown to posess both 3'→5' exonuclease proofreading activity and lyase activity (Schmitt & Clayton, 1993; Longley *et al*., 1998). The human polymerase γ fills single nucleotide gaps and produces a substrate that can be ligated after action of uracil-DNA glycosylase and AP-endonuclease (Longley *et al*., 1998).

A mitochondrial DNA ligase has been identified. The human DNA ligase III gene encodes both a nuclear and a mitochondrial protein and DNA ligase III plays an essential role in the maintenance of mtDNA in mammalian cells (Lakshmipathy & Campbell, 1999; 2001).

Mismatch repair

The DNA mismatch repair (MMR) removes errors in the newly synthesized DNA strand that are missed by the polymerase proofreading (see Analysis of human DNA Mismatch Repair). It is not yet known if there is an active post-replication MMR system in human mitochondria but it has been indicated that hMSH6 is not involved in repair of mismatches in the mtDNA. Human hMSH6 deficient lymphoblastoid cells have a higher spontaneous mutation rate in the nuclear genome but a similar spontaneous mutation rate in mitochondrial DNA when compared to a parental mismatch repair proficient cell line (Marcelino *et al.*, 1998; Marcelino & Thilly, 1999).

In yeast, a component of the MMR pathway (Msh1) has been identified in mitochondria (Reenan & Kolodner, 1992). The Msh1 protein is a homolog of the *E. coli* MutS mismatch binding protein (Reenan & Kolodner, 1992). Inactivation of the *MSH1* gene resulted in large scale mtDNA rearrangements suggesting that Msh1 is involved in repair of mtDNA (Reenan & Kolodner, 1992).

Direct repair

We have investigated the sub-cellular localization of O^6-methylguanine-DNA methyl-transferase (MGMT) in human mitochondria (Rasmussen *et al.*, II). Therefore, the MGMT protein will be described in greater detail in the next section.

2.3 Direct Repair: O^6-Methylguanine-DNA Methyltransferase

O^6-methylguanine (O^6-MeG) is generated endogenously by reactive cellular catabolites and *S*-adenosylmethionine, which normally acts as a methyl donor in the synthesis of 5-methylcytosine but occasionally causes methylation at other sites (Rossman & Goncharova, 1998; Lindahl & Wood, 1999).

Alkylating agents produce various kinds of alkylated purine and pyrimidine bases in DNA, 3-methyladenine (3-MeA), O^6-methylguanine (O^6-MeG) and O^4-methylthymine (O^4-MeT), among other adducts (Lindahl & Sedgwick, 1988). 3-MeA has been shown to block DNA replication (Larson *et al.*, 1985) and is excised by 3-methyladenine glycosylase (MPG). O^6-

MeG and O^4-MeT DNA lesions are mutagenic and carcinogenic (Loveless, 1969; Swann, 1990). However, O^4-MeT is formed less frequently than O^6-MeG. The proportion of alkylation at each site is ~ 0.1 % for O^4-MeT and 3-6% for O^6-MeG of total alkylation after reaction with Dimethylnitrosamine (DMN), Methylnitrosourea (MNU) or 1,2-Dimethylhydrazine (SDMH) (Singer & Dosanjh, 1990).

During DNA replication, O^6-MeG can pair with thymine leading to G:C → A:T transition mutations while O^4-MeT can pair with guanine, generating A:T → G:C transition mutations (Singer & Dosanjh, 1990; Dosanjh *et al*., 1991; 1993, Altshuler *et al*., 1996). Both prokaryotic and eukaryotic DNA alkyltransferases remove methyl groups from the O^6 position of the guanine. cDNAs coding for the repair protein from *E. coli* (***ada***, Sedgwick, 1983; ***ogt***, Potter *et al*., 1987), *S. cerevisiae* (***MGT1***, Xiao *et al*., 1991), mouse (Shiota *et al*., 1992), rat (Rahden-Staron & Laval, 1991) and human (***MGMT***, Tano *et al*., 1990; Rydberg *et al*., 1990; Hayakawa *et al*., 1990) have all been cloned and characterized**.** Furthermore, DNA methyltransferase has been identified in 28 different species (Pegg, 2000). Although the amino acid sequences of these proteins are homologous, there are only 8 absolutely invariant residues in these sequences (Gly^{109}, Asn^{137}, Pro^{144}, Cys^{145}, His^{146}, Arg^{147}, Lys^{165}, and Glu^{172}, numbered according to the human MGMT sequence) (Xu-Welliver *et al*., 2000).

MGMT function

Human O^6-**M**ethyl**G**uanine-DNA **M**ethyl**T**ransferase (MGMT, also known as AGT) repairs O^6-alkylguanine (O^6-MeG, O^6-ethylguanine & O^6-butylguanine) in double-stranded DNA in a single step. MGMT removes alkyl groups from the O^6 position of guanine to a cysteine acceptor residue in MGMT without removing the damaged base (Pegg, 2000). The cysteine residue is located at position Cys^{145} in MGMT in a highly conserved sequence -P**C**HR- (Pegg, 2000). It has been shown that mutation of the cysteine acceptor site leads to a complete loss of MGMT activity (Harris *et al*., 1992; Crone & Pegg, 1993; Hazra et al., 1997; Edara *et al*., 1999). Once bound to the cysteine acceptor site, the alkyl group permanently inactivates MGMT. Therefore, the number of O^6-alkylG that can be repaired is equal to the number of active MGMT molecules – MGMT is "suicidal" in the repair of O^6-alkylG residues in DNA (Pegg, 1990; 2000). Even though MGMT is not specific for methyl groups, the rate of the reaction decreases as the size of the adduct increases (Pegg & Byers, 1992).

Mutational analysis of the MGMT protein showed that deletion of 7 amino acids from the N terminus (codons 1-7) or 28 amino acids from the C terminus (codons 180-207) result in

specific activity comparable to that found for the full-length MGMT (Hazra *et al*., 1997). Mutant MGMT with deletion of codon 1-10 or 1-19 were shown to be active in protecting cells from a methylating agent *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG) suggesting that they are able to repair methylated DNA *in vivo* (Crone *et al*., 1996). However, the mutant MGMT proteins were less effective compared to the wild-type MGMT and highly unstable with half-lives of 48-90 min compared to a half-live of > 720 min for wild-type MGMT. Interestingly, we found that deletion of codons 2 to 18 resulted in a complete absence of MGMT in nucleus and accumulation in cytoplasm (Rasmussen *et al*., II). This suggests that deletion of the first 18 codons interfere with stability and translocation to nucleus. However, our results indicate that this mutant MGMT protein provide the same level of protection to killing by MNNG as MGMT wild-type (Rasmussen *et al*., II). It has been found by site-directed mutagenesis that the fully conserved residues Asn^{137}, His^{146}, Arg^{147} and Glu^{172} are critical for maintaining the MGMT stability. Proteins with mutations in these residues were highly unstable with half-lives of 15-60 min (Crone *et al*., 1996). Furthermore, the residues Arg^{128} and Tyr^{114} seemed to be involved in DNA binding and catalytic activity (Kanugula *et al*., 1995; Goodtzova *et al*., 1998).

Repair of O^4-methylthymine

Although *E. coli* Ogt has been shown to repair O^4-MeT it is still not fully established if the human MGMT can repair O^4-MeT (Sassanfar *et al*., 1991). The rate constants for MGMT acting on O^6-MeG is much higher than O^4-MeT (O^6-MeG = $1x10^9$ $M^{-1}min^{-1}$ and O^4-MeT = $1.8x10^5$ $M^{-1}min^{-1}$ (Zak *et al*., 1994). Repair of O^6-MeG by MGMT *in vivo* is 1000-5000-fold greater than for O^4-MeT (Samson *et al*., 1997; Encell & Loeb, 2000). However, one study has shown that human MGMT expressed in *E. coli* suppresses MNNG-induced A:T $\rightarrow$ G:C mutations indicating that MGMT repairs O^4-MeT DNA lesions (Kooistra *et al*., 1999). Other studies showed that expression of MGMT in a methyltransferase-deficient *E. coli* strain (lacking Ade and Ogt) failed to suppress A:T $\rightarrow$ G:C mutations, but actually increased O^4-MeT mutations (Samson *et al*., 1997, Edara *et al*., 1999). Samson *et al*. (1997) suggested that MGMT binds to O^4-MeT lesions but repairs it so slowly that it interferes with the repair of this lesion by NER and consequently increases the frequency of A:T $\rightarrow$ G:C mutations. The mammalian NER pathway has been shown to repair both O^6-MeG and O^4-MeT lesions (Bronstein *et al*., 1991; 1992, Huang *et al*., 1994, Kein *et al*., 1994). However, the ability to serve as substrates for NER increases with the size of adduct and is poor for methyl groups

(Pegg & Byers, 1992). It is still unknown whether NER is the predominant pathway for removal of O^4-MeT lesions in human cells or serves as a back-up repair system for removing O^6-MeG lesions in cells with limited MGMT activity.

The methylation tolerant phenotype

Even though MGMT is widely expressed, the intracellular MGMT levels vary between different tissues and also between different individuals. The liver and spleen have the highest MGMT activities and the nervous system and mammary gland the lowest (Myrnes *et al*., 1983; Yarosh *et al*., 1985; Pegg & Byers, 1992; Karran & Bignami, 1994). Most tumor cells express high level of MGMT, while about 15-20% of human tumor cell lines are deficient in MGMT expression (Sklar *et al*., 1981; Tsujimura *et al*., 1987; Yu *et al*., 1999). Cells lacking MGMT activity (Mex^- or Mer^- cells) are more sensitive to mutagenesis and killing by alkylating agents (Karran & Bignami, 1994).

Alkylating agents were among the first cytotoxic drugs used in the treatment of cancer and are still used clinically against a number of tumors (Kleibl & Margison, 1998). A class of anti-tumor alkylating agents as the chloroethylnitrosourea (CENU) is clinically used to treat a number of tumors, including brain neoplasms, malignant melanoma, advanced lymphomas, and gastrointestinal carcinomas (Kreklau *et al*., 1999). However, certain tumors are resistant to alkylating chemotherapeutic agents and this resistance are known to be correlated with the level of MGMT in the tumors (Pegg & Byers, 1992). Another mechanism of cellular resistance to methylating agents is an ability to ignore the presence of O^6-MeG lesions in DNA. This tolerance of DNA damage is due to the loss of MMR, even if MGMT is not expressed or expressed at low levels (Carethers *et al*., 1996; Karran & Hampson, 1996; Hampson *et al*., 1997; Umar *et al*., 1997; Dosch *et al*., 1998; Kawate *et al*., 1998, Humbert *et al*., 1999).

There is increasing evidence that tumor cells could acquire resistance to methylating drugs by loss of MMR activity. A lack of MMR results in poor ability of the cell to detect DNA damage and activate apoptosis, thus increasing the mutation rate throughout the genome (Fink *et al*., 1998). This implies that genotoxic and cytotoxic effects of O^6-MeG are mediated by MMR. *In vitro* studies have demonstrated that the mammalian hMSH2-hMSH6 complex can recognize O^6-MeG: T and O^6-MeG: C (Duckett *et al*., 1996; Ceccotti *et al*., 1996; Mu *et al*., 1997; Christmann & Kaina, 2000). Although, the mechanisms of MMR-mediated response to base damage are not known, two models have been proposed as explanations.

1. In one model, the MMR system recognizes the mismatched bases opposite the damaged base but since there is no perfect complementary match for O^6-MeG, the polymerase will again fail to find an ideal partner and incorporate thymine or cytosine opposite O^6-MeG on the newly synthesized strand. The repeated attempts to repair the mismatch will result in a methylated template strand that is single-stranded for much of the time and a newly synthesized strand that contains persisting DNA termini. The killing effect of the lesion is expected to come during the following cycle of DNA replication, when the replication fork arrives at a discontinuity and thus generates a double-strand break that will then trigger apoptosis (Galloway *et al*., 1995; Karran & Hampson, 1996; Kaina *et al*., 1997).
2. In the second model, MMR proteins function as the sensors of DNA damage, and generate a signal capable of activating apoptosis (Modrich, 1997; Fink *et al*., 1998).

The p53 protein functions as a transcription factor that regulates the expression of several genes involved in cell cycle control, DNA damage repair, and apoptosis. Therefore, p53 is the classic signal transduction molecule thought to respond to DNA breaks and gaps (Nelson & Kastan, 1994; Huang *et al*., 1996; Vogelstein *et al*., 2000). However, MNNG induced apoptosis seems to be p53-independent (Hickman & Samson, 1999). Moreover, Christmann & Kaina (2000) have shown that after exposure of cells to MNNG, hMSH2, hMSH6 and hPMS2 proteins are translocated from the cytoplasm into the nucleus, leading to an increased nuclear MMR protein level immediately after exposure. This translocation was independent of the p53 status of the cells and was first observed at high dosage level of the mutagen in cells expressing MGMT (>25 μM MNNG; 750 fmol/mg protein MGMT; Christmann & Kaina, 2000). In order to explore a possible link between p53 and MGMT we expressed the MGMT-GFP fusion protein in cell lines with different p53 status. One cell line was wild type for p53, one expressed very low levels of the p53 protein, and one cell line was completely deficient for p53 activity. We found no difference in subcellular localization of MGMT suggesting that MGMT translocation is independent of the p53 status. In all three cell lines the MGMT-GFP fusion protein was exclusively present in the nucleus (Rasmussen *et al*. II).

Treatment of human cells with MNNG concentrations that increased the mutation rate of nuclear genes showed no induction of mitochondrial mutations (Mita *et al*., 1988). Marcelino *et al*. (1998) observed that to induce MNNG mutations above a very high spontaneous background in mtDNA, one needed to treat MNNG-resistant (MT1) cells with an amount of MNNG (4 μM) that would kill all normal parental cells. Therefore, they

concluded that it is unlikely that *in vivo* mutations are induced in mtDNA by exogenous mutagens in normal human cells (Khrapko *et al*., 1997; Marcelino *et al*., 1998).

Mitochondrial MGMT activity

Two groups have reported mitochondrial MGMT activity in rat liver (Myers *et al*., 1988; Satoh *et al*., 1988). The kinetics of removal of O^6-MeG was similar in nuclear and mitochondrial DNA (Myers *et al*., 1988). Furthermore, they found very slow removal of O^6-butyl-2'-deoxyguanine in mtDNA but fast removal in nDNA which is consistent with the absence of NER in mitochondria (Myers *et al*., 1988). Satoh *et al*. (1988) reported the removal of O^6-ethyl-2'-deoxyguanosine (O^6-EtG) from mtDNA and nDNA in rat liver following exposure to *N*-ethyl-*N*-nitrosourea *in vivo*. Longer alkyl groups including ethyl- is known to be repaired by MGMT and NER (Pegg, 2000), indicating, that O^6-EtG is removed by an alkyltransferase mechanism in the mitochondria. Additionally, *N*-methyl-*N*-nitrosourea (MNU) induced mutations were repaired in mitochondria from Chinese hamster ovary (CHO) cells (LeDoux *et al*., 1992).

These results obtained in rodents suggest that an alkyltransferase mechanism is operating within the mitochondrion in mammalian species. However, extrapolation of results obtained with rodents to human cells may not be straightforward. Even MGMTs from mammalian species have different substrate specificity. For eksample rat MGMT repairs O^6-EtG much better than human MGMT (Liem *et al*., 1994). Mitochondrial MGMT activity has not yet been reported from human cells and the intracellular localization seems ambiguous.

MGMTs intracellular localization

Most immunostaining data have shown that MGMT is a nuclear protein (Ayi *et al*., 1992, Lim & Li, 1996; Belanich *et al*., 1996). However, there are reports on cytosolic appearance indicating that MGMT could be present in nucleus, cytoplasm and mitochondria (Ayi *et* al., 1992; Ishibashi *et al*., 1994a,b; Belanich *et al*., 1996). To access the actual localization of the MGMT protein we studied exogenous MGMT-GFP expressed as fusion protein in a human breast epithelial cell line (MCF12A) and detected the subcellular localization by fluorescence microscopy. We found that MGMT localized mainly in the nucleus. We also observed a weak cytoplasmic staining but no mitochondrial localization. Our results therefore suggest that MGMT is not present in mitochondria of human cells (Rasmussen *et al*., II). In order to test whether nuclear MGMT was recruited to mitochondria upon DNA damage of mtDNA by alkylating agents we treated cells expressing MGMT-GFP with

MNNG. The MGMT-GFP fusion protein was detected in nucleus but not in mitochondria indicating that nuclear MGMT is not recruited to mitochondria after DNA damage by MNNG (Rasmussen *et al*., II). This suggests that the alkyltransferase mechanism which seems to be operate within the mitochondrion in mammalian species is not MGMT in human breast epithelial cells.

3. Mitochondrial Dysfunction versus Genetic Stability of the Nuclear DNA

3.1 Introduction

Mitochondrial diseases are severely debilitating and characteristically complex in nature. They can affect any organ in the body at any age. Usually the mitochondrial diseases are inherited through the mother but they can also be sporadic or induced by the environment. Mitochondrial dysfunction is found in diseases as diverse as cancer, infertility, diabetes, heart diseases, blindness, deafness, kidney disease, liver disease, stroke, and migraine. Mitochondrial dysfunction is also involved in aging and neurodegenerative diseases such as Parkinson and Alzheimer dementia (Wallace, 1999; DiMauro & Schon, 2001). To date, there is no cure for mitochondrial diseases.

We used *Saccharomyces cerevisiae* as a model system to explore the role of mitochondrial dysfunction on genetic stability of the nuclear DNA (Rasmussen *et al*., III) as well as the role of mitochondrial activity in oxidative DNA damage and repair of the nuclear genome (Rasmussen *et al*., IV)

3.2 Mitochondrial Biology and Genetics

The mitochondria are surrounded by a double membrane, a smooth outer membrane, and a "highly folded" inner membrane (termed cristae). Depending on source and conformational state, cristae can vary from simple tubular structures to more complex lamellar structures that are connected to the inner boundary membrane (Frey & Mannella, 2000; Perkins & Frey, 2000). The inner membrane of the mitochondrion contains the proteins and enzymes of the electron transport chain (Complexes I-IV) and Complex V that are responsible for phosphorylation of ADP to ATP (adenosine diphosphate to adenosine trisphosphate). The inner compartment is called the matrix, which contains the many enzymes of Krebs cycle (the citric acid cycle), the pathway of fatty acid oxidation, and the mitochondrial DNA (Scheffler, 1999).

In mammalians, electron transfer from NADH donated to Complex I (NADH dehydrogenase or NADH:ubiquinone oxidoreductase) or electrons from succinate (donated to Complex II, succinate dehydrogenase) are transfered to coenzyme Q, also called ubiquinone or CoQ.

Regardless of whether CoQ receives its electrons from Complex I or II, it shuttles them to Complex III (ubiquinol:cytochome *c* oxidoreductase). From Complex III the electrons move through cytochrome c and Complex IV (cytochrome *c* oxidase or COX), which passes the electrons directly to oxygen to form water. As electrons transverse complex I, III, and IV, protons (H^+) are moved from the mitochondrial matrix across the inner membrane into the inter-membrane space. This creates an electrochemical proton gradient ($\Delta\Psi$) that is positive and acidic on the outside and negative and alkaline on the mitochondrial matrix side. The proton gradient across the inner-membrane is used to drive the condensation of ADP and P_i (inorganic phosphate) to generate ATP by allowing the protons to flow back through Complex V (ATP synthase or F_1F_0ATPase). Under normal circumstances, the inner membrane is impermeable to protons, leaving Complex V as the only route whereby protons can return to the matrix (figure 3.1). The ATP is exchanged for ADP across the mitochondrial inner membrane by the **A**denine **N**ucleotide **T**ranslocator, ANT (Wallace, 1999; Saraste, 1999). In fungi, Complex I is very similar to its counterpart in other eukaryotes except for *S. cerevisiae*, *Saccharomyces carlsbergii* and *Kluyveromyces lactis* which do not contain Complex I.

The mammalian Complex I is a NADH dehydrogenase involved in the oxidation of intra-mitochondrial NADH produced by the citric acid cycle, and its NADH-binding site faces the mitochondrial matrix. *S. cerevisiae* has three NADH dehydrogenases; Nde1 and Nde2 which both face the intermembrane space (referred to as external) and Ndi1 an internal NADH dehydrogenase like the mammalian Complex I (Joseph-Horne *et al*., 2001). Ndi1 is believed to be attached to the inner membranes on the matrix side (de Vries & Grivell, 1988; de Vries *et al*., 1992). It has been shown that when Ndi1 was expressed in a Complex I-deficient Chinese hamster cell, it restored the capacity for respiration and oxidative phosphorylation in these cells (Seo *et al*., 1998). Furthermore Ndi1 was incorporated into mitochondria in human embryonic kidney cells where Complex I was still present. Overexpression of Ndi1 in cells led to decreased coupling of NADH oxidation to ATP synthesis (ADP/Oxygen consumption ratio down from 2.4 to 1.8), while succinate oxidation was unchanged. These results suggest that the Ndi1 protein from *S. cerevisiae* complements the mammalian Complex I consisting of 43 peptides (Wallace, 1999). However, Ndi1 does not translocate protons, but only feeds electrons to the electron transport chain (Joseph-Horne *et al*., 2001). Apart from the lack of Complex I in *S. cerevisiae* the electron transport and proton transfer proceed via the same complexes as in mammalians (figure 3.1)

3.3 Reactive Oxygen Species

Mitochondria generate more than 80% of cellular energy in the form of ATP and are therefore regarded as the "powerhouse" of the cell (Kang *et al*., 1998). However, mitochondrial respiration is also the major endogenous source of **R**eactive **O**xygen **S**pecies (ROS), including superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO^\bullet$) (Cadenas & Davies, 2000). Under normal physiological conditions, *in vitro* evidence indicates that electrons "leak" from the electron transport chain, converting about 1-2% of oxygen molecules into O_2^-, a more reactive form of oxygen (Boveris & Chance 1973; Boveris, 1977; Papa, 1996; Loft & Poulsen, 1996). Research in ROS production from mitochondria indicate that Complex I (Robinson, 1998) and Complex III (Turrens *et al*., 1985; Nohl & Jordan 1986) play a major role in production of superoxides however O_2^- may also arise from Complex II (Ishii *et al*., 1998) in mammalian cells. Under normal metabolic conditions, Complex III seems to be the main site of O_2^- production (Finkel, 2001).

Superoxide itself can attack enzymes containing the catalytically iron-sulfur [4Fe-4S] tetranuclear cluster, such as Complex I, Complex II and the Krebs cycle enzyme aconitase, resulting in release of iron and decreased mitochondrial ATP production (Flint *et al*., 1993; Gardner, 1995, Melov *et al*., 1999; Cadenas & Davies, 2000). Hence, mitochondria are particularly sensitive to oxidative stress. As we are using *S. cerevisiae* as model system it should be noted that the Ndi1 is a polypeptide enzyme with no iron-sulfur cluster and therefore not attacked by O_2^- (de Vries & Grivell, 1988).

ROS is reduced by intracellular antioxidant enzymes including superoxide dismutase, glutathione peroxidase and catalase. Superoxide is simultaneously reduced and oxidized (dismutated) to form hydrogen peroxide and oxygen by superoxide dismutase (SOD). Eukaryotes, including *S. cerevisiae*, hold a manganese containing superoxide dismutase (MnSOD, product of the *SOD2* gene) in the matrix of the mitochondria, and a copper-and zinc containing form (CuZnSOD, product of the *SOD1* gene) in the nuclear and cytoplasmic matrixes. Further, mammalians have an extracellular CuZn superoxide dismutase (SOD3) (Crapo *et al*., 1992; Lindenau *et al*., 2000; Srinivasan *et al*., 2000).

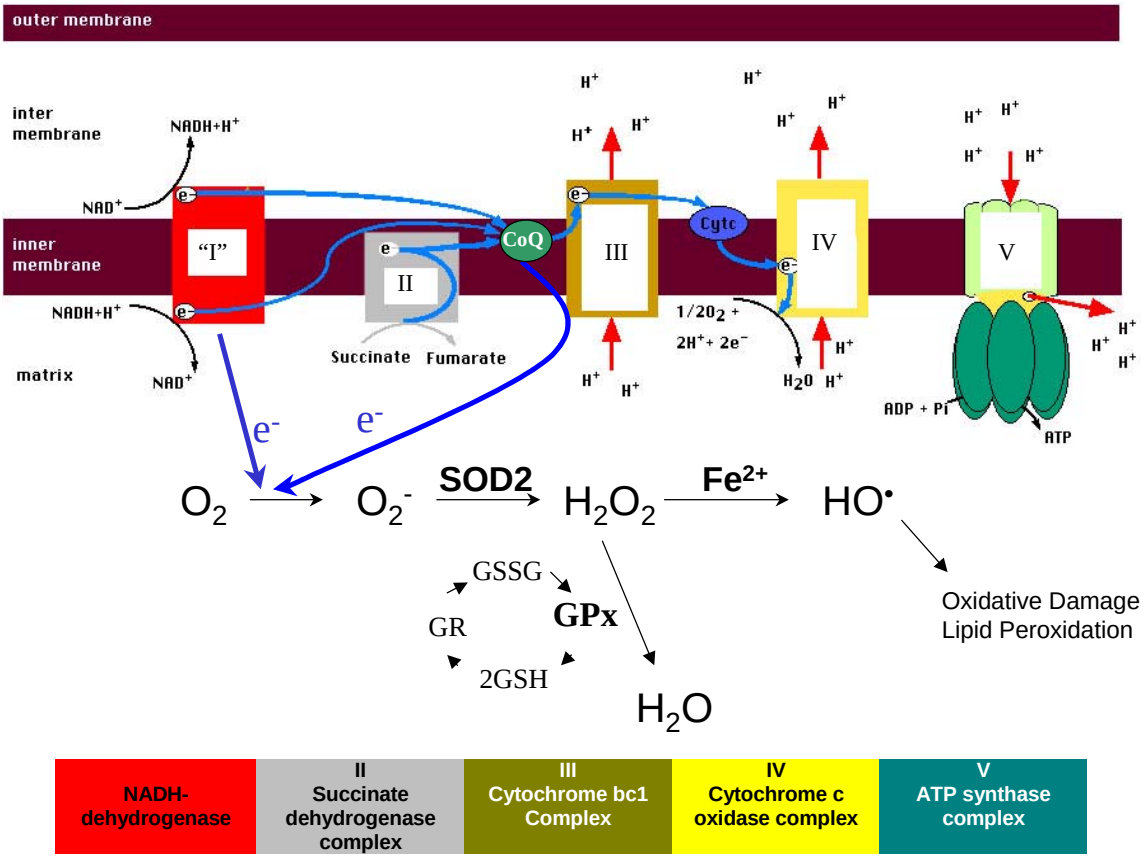


Figure 3.1. Mitochondrial respiratory chain and reactions involved in removal of ROS in mitochondria The respiratory enzyme complexes involved in oxidative phosphorylation are NADH-dehydrogenase (Complex I in mammalian and Ndi1 in *S. cerevisiae*); succinate dehydrogenase (Complex II); ubiquinol:cytochrome *c* oxidoreductase (Complex III); cytochrome *c* oxidase (Complex IV); ATP synthase (Complex V) in both mammalian and *S. cerevisiae*. Complex I and II in mammalian cells and Complex II in *S. cerevisiae* contains 4Fe-4S centers. When the respiratory chain is inhibited, the electrons accumulate in the early stages of the respiratory chain (Complex I and coenzyme Q), were they can be donated to molecular oxygen to give superoxide. Superoxide (O_2^-) is converted to hydrogen peroxide (H_2O_2) by mitochondrial superoxide dismutase (SOD2). H_2O_2 is converted to water (H_2O) by glutathione peroxide (GPx1 in mammalian). Ferrous ions (Fe^{2+}), released e.i. during O_2^- attack of enzymes with 4Fe-4S centers, can participate in a Fenton reaction and convert H_2O_2 into hydroxyl radical ($HO^\bullet$) (Wallace, 1999; Joseph-Horne *et al*., 2001). GSH (reduced glutathione); GR (glutathione reductase); GSSG (oxidized glutathione).

Hydrogen peroxide produced by SOD2 in the mitochondria is reduced to water (H_2O) by glutathione peroxidase (GPx), in a reaction that converts reduced glutathione (GSH) to oxidized glutathione (GSSG) (figure 3.1). Five GPx isoenzymes have been found in mammalian cells. GPx1, which are located in the mitochondria and cytosol and Gpx4 (or PHGPx, a phospholipid hydroperoxide) are found in most tissues. Cytosolic Gpx2 and extracellular Gpx3 are poorly detected in most tissues except for the gastrointestinal tract and kidney, respectively (de Haan *et al*., 1998). Hydrogen peroxide can also be reduced to

H_2O by catalase (CAT), located primarily in peroxisomes (especially in liver) and in cytoplasm (erythrocyte) (Bai & Cederbaum, 2001). Catalase has only been found in mitochondria of rat heart (Radi *et al*., 1991). In the presence of transition metal ions (Fe^{2+}, Cu^{+}) hydrogen peroxide can be converted by the Fenton reaction to the highly damaging hydroxyl radical, which can cause degradation of most biological macromolecules, e.g. peroxidation of lipids, oxidation of sugars and of protein thiols, DNA base damage, and strand breakage of nucleic acids (Bai *et al*., 1999).

3.4 Effects of Mitochondrial ROS Production

Inhibition of mitochondrial oxidative phosphorylation

Inhibition of mitochondrial oxidative phosphorylation in mice not only reduces energy production, but also increases mitochondrial ROS production. Mitochondria from normal mice have an increased H_2O_2 production after antimycin A inhibition (Esposito *et al*., 1999). Antimycin A is not a direct producer of H_2O_2 but an inhibitor of Complex III. Thus, the inhibition results in a substantial increase in superoxide production (Raha *et al*., 2000) which is converted into H_2O_2 by SOD2.

Also, mitochondria from adenine nucleotide translocator *Ant1* (-/-) mice were shown to have the same high level of H_2O_2 as normal mitochondria treated with antimycin A. This suggests that the absence of ANT1 blocks the exchange of ADP and ATP across the mitochondrial inner membrane, thus inhibiting oxidative phosphorylation which is resulting in increased oxidative stress. Furthermore, tissues with high oxidative stress (skeletal muscle and heart) were showed to increase the level of ROS detoxification enzymes, SOD2 and GPx protein in *Ant1* (-/-) mice. Although the antioxidant activities were increased, a very high level of mtDNA rearrangement was observed in the *Ant1* (-/-) mice heart but not in skeletal muscle. It was suggested that the different levels of mtDNA rearrangement in the two tissues were a result of lower induction of SOD2 in the heart than in skeletal muscle (Esposito *et al*., 1999).

Cytosolic superoxide dismutase (sod1) mutants

By analyzing null *SOD1* and *SOD2* mutations in different species it has been suggested that ROS could be involved in limiting life span. Yeast cells deficient in the *SOD1* gene are viable but grow poorly in oxygen and the mutation frequencies are increased in nDNA in *sod1Δ* mutant cells compared to the wild type parental strain (Gralla & Valentine, 1991;

Longo *et al*., 1997, Rasmussen *et al*., IV). It has been shown that SOD1 activity in motor-neurons is an important factor in ageing and lifespan of *Drosophila* (Parkes *et al*., 1998). *Sod1* null mutants of *Drosophila* have a very short lifespan (the adult lifespan is shortened by 85-95%). Overexpression of human SOD1 in motor neurons of a wild type *Drosophila* extended the adult life span by up to 40% (Parkes *et al*., 1998).

Mutations in the *SOD1* gene are also linked to Familial Amyotrophic Lateral Sclerosis (FALS) a disorder resulting from degeneration and death of motor neurons. 15-20% of FALS patients have been found to harbour mutations in *SOD1*. Transgenic mice that express a mutant *SOD1* allele develop a motor neuron degeneration that parallels most aspects of ALS (Gurney *et al*., 1994; Bruijn *et al*., 1997). Whereas, knockout mice deficient in the entire *SOD1* gene live to adulthood and show no overt motor defect (Reaume *et al*., 1996). This result demonstrates that the loss of SOD1 function is not, by itself, sufficient to kill motor neurons *in vivo*. It has been suggested that FALS is a consequence of reduction in SOD1 dismutation activity leading to oxidative damage (loss-of-function) or a gain-of-function as the mutant SOD1 protein seems to increase the ability to generate hydroxyl radicals (Yim *et al*., 1996; Bogdanov *et al*., 1998; Liu *et al*., 1999). The results obtained with *SOD1-/-* knockout mice suggest that FALS SOD1 acquires or enhances toxic property.

Mitochondrial superoxide dismutase (sod2) mutants

Yeast *sod2Δ* mutants have a less dramatic phenotype than *sod1Δ* mutants when grown in glucose media although *sod2Δ* mutants are oxygen-sensitive and grow poorly in carbon sources that require respiration for their metabolism (Liu *et al*., 1992; Longo *et al*., 1996).
In contrast to *sod2Δ* mutants in yeast, an acute toxicity of mitochondrial O_2^- has been found in *SOD2-/-* mice. While mice, deficient in cytosolic SOD1 activity were viable (Reaume *et al*., 1996), inactivation of the mitochondrial SOD2 was lethal early in life. Two models of *SOD2* knockout mice have been reported. *SOD2*^{tm1Cje} mutant mice in which exon 3 has been deleted (Li *et al*., 1995) and *SOD2*^{m1BCM} mutants which are missing exon 1 and 2 (Lebovitz *et al*., 1996). The homozygous *SOD2* ^{tm1Cje} mutant mice die within the first 10 days of life with a dilated cardiomyopathy, accumulation of lipid in liver and skeletal muscle, and metabolic acidosis (Li *et al*., 1995). The homozygous *SOD2*^{m1BCM} mutant mice survived up to 18 days and showed motor disturbance, central nervous system

injury, and extensive mitochondrial injury within cells with requirements of high levels of oxidative metabolism, including cardiac myocytes, neurons, hepatocytes, and hematopoietic cells (Lebovitz *et al*., 1996).

In the *SOD2* ^{tm1Cje} (-/-) mutant mice, the mitochondria's O_2^- toxicity was shown to result in dramatic reductions in the activities of mitochondrial enzymes containing iron-sulfur centers, including the Krebs cycle enzyme aconitase and respiratory chain enzymes of Complex I and II. Hence, the dilated cardiomyopathy observed in *SOD2* (-/-) mice (Li *et al*., 1995) was caused by blocking of the Krebs cycle and respiratory chain giving energy starvation in the heart (Melov *et al*., 1999). The increased mitochondrial oxidative stress of the *SOD2* (-/-) mice also resulted in high levels of oxidative damage to DNA. However, it was not determined whether it was nDNA, mtDNA damage or both (Melov *et al*., 1999).

Measurement of the lipid peroxidation levels in heterozygous *SOD2* ^{tm1Cje} (+/-) mutants and *SOD2* (+/+) control mice revealed that young *SOD2* (+/-) and control animals had the same levels of lipid peroxidation in the liver mitochondria. However, lipid peroxidation peaked for *SOD2* (+/-) at middle age and declined in old *SOD2* (+/-) animals, whereas lipid peroxidation of *SOD2* (+/+) mice first peaked at old age (Kokoszka *et al*., 2001). Apoptosis seemed to be able to explain the loss of mitochondrial lipid peroxidation from middle-age to old age in *SOD2* (+/-) mice. TUNEL staining showed that old *SOD2* (+/-) mouse livers had three times more apoptotic hepatocytes than old *SOD2* (+/-) mouse livers. It was therefore suggested that cells with a high number of damaged mitochondria was destructed by apoptosis (Kokoszka *et al*., 2001).

It has also been demonstrated that *Caenorhabditis elegans* life span was increased 54% by treatment with SOD/catalase mimetics. In addition, treatment with these compounds could restore a *mev-1* mutant life span to normal. The *mev-1* gene, encodes the cytochrome b subunit of succinate dehydrogenase (Complex II) and the mutant increases mitochondrial ROS production which shortens the worms life span by 37% (Melov *et al*., 2000).

The just mentioned models showed that ROS generation by mitochondria can be damaging to mitochondria themself but also to the length of life span of the animal. High ROS production can increase the percentage of mutant mtDNA, which decreases the mitochondrial energetic capacity and increases ROS production as well as the propensity for apoptosis. The tissues most sensitive to mitochondrial dysfunction are brain, heart, skeletal muscle, endocrine system and kidney (Wallace, 1999).

ROS generates a variety of DNA lesions including modified bases, abasic sites, and single strand breaks. If left unrepaired, these damages may contribute to a number of degenerative processes, including aging and cancer. Association to cancer has been established because one of the major base lesions formed upon oxidative attack to DNA, 7,8-dihydro-8-oxoguanine (8-oxoG), was present at higher level in both lung and breast tumor tissue compared to normal tissue (Malins *et al*., 1991; Olinski *et al*., 1992). However, an important question is whether ROS generated in mitochondria contributes to induce mutations in nDNA?

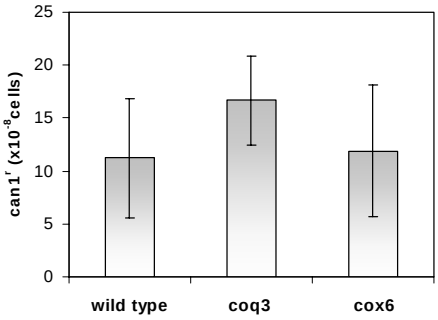
3.5 Mitochondrial Dysfunction Contributes to nDNA Mutations

Hydroxyl radicals are extremely unstable with an estimated half-live of only 10^{-9} seconds (Pryor, 1986) whereas H_2O_2 is freely diffusible and relatively long-lived and may therefore efflux out of the mitochondria, and into the cytoplasm (Finkel & Holbrook, 2000; Bai & Cederbaum, 2001). Diffusion of H_2O_2 across the mitochondrial membrane has been suggested by Bai *et al.* (1999). They showed that catalase overexpressed in the mitochondria of HepG2 cells protected the cells from cytotoxicity of H_2O_2. Similarly, overexpression of cytosolic catalase protected HepG2 cells from cytotoxicity of antimycin A. Bai *et al*. (1999) showed that H_2O_2 diffuses into the mitochondria and that damage to the mitochondria could be an important factor contributing to H_2O_2 toxicity. Similarly, cytosolic catalase protected the cells against antimycin A induced H_2O_2 toxicity, suggesting that mitochondrial produced H_2O_2 diffused into the cytosol.

We used the yeast *S. cerevisiae* as a model system to investigate a potential link between mitochondrial activity and genomic instability. We generated strains that were either impaired in mitochondrial activity due to mutations in the mitochondrial genome (rho^-) or strains absolutely deficient in mitochondrial activity due to lack of mitochondrial genome (rho^0). We assayed these strains for spontaneous mutations and found that spontaneous mutation frequencies, measured as nuclear mutational events, were significantly higher in both rho^- and rho^0 strains compared to the wild type strains (Rasmussen *et al*., III)

To investigate whether the increased spontaneous mutation frequencies observed in the rho^0 and rho^- strains were a result of defective respiratory function in these cells, we measured the effect of disrupting the respiratory chain function in *S. cerevisiae* using various

mitochondrial inhibitors. We demonstrated that all drugs caused an increase in nuclear spontaneous mutation frequencies but that antimycin A had the most profound impact. This indicates that mitochondrial ROS production can cause nuclear mutations. Further, our results show that mitochondrial function and more specifically complex III activity is critical for maintaining genomic integrity (Rasmussen *et al*., III). The results correspond with *in vitro* studies indicating that Complex III may be responsible for more than 80% of ROS produced in *S. cerevisiae* (Chance *et al*., 1979). However, not all types of inhibition of the respiratory chain result in mutations in nDNA. We observed that mutations in *COQ3* and *COX6* did not affect spontaneous mutation frequencies and we therefore concluded that inhibition of these specific gene products is not involved in mitochondrial-mediated mutagenesis (figure 3.2).



• **Spontaneous mutation frequency**

Strain	***can1*^{r} (x10^{-8})**	**Fold increase**
wild type Hansen BY4741	11.2 ± 5.6	1
coq3Δ	16.7 ± 4.2	1.5
cox6Δ	11.9 ± 6.2	1.1

Figure 3.2. Mutation frequencies in *S. cerevisiae* Hansen BY4741 wild type, *coq3Δ*, and *cox6Δ* (Research Genetics, Inc., Huntsville, AL). The *COQ3* gene encodes an *O*-methyltransferase required for two steps in the biosynthetic pathway of ubiquinone (coenzyme Q) - *coq3* mutants do not synthesize coenzyme Q (Jonassen & Clarke, 2000). The *cox6* gene encodes subunit VI of Complex IV. *S. cerevisiae* cytochrome *c* oxidase is composed of nine different subunits. The three largest (cox1, cox2, and cox3) are encoded by mitochondrial genes the other six are encoded by nuclear genes (de Vries and Marres, 1987; Foury *et al*., 1998). Strains deficient in cox6 activity show residual cytochrome *c* activity corresponding to 7% of the activity found in wild type mitochondria (de Vries and Marres, 1987).

It has been demonstrated that respiration plays a role in ROS production as well as for viability in SOD deficient yeast strains. *S. cerevisiae* deficient in Sod2 activity failed to grow normally in hyperoxia (doubling time 10.8 h.). In contrast, *sod2* null mutants with complete absence of electron transport (rho^{0}) grew normally in hyperoxia (doubling time 2.8h.). In addition, the viability was partially restored by mutations (*cox6*) which disrupt the electron transport chain (doubling time 5.6 h.). Lack of respiration (*coq3* mutant) also prevents viability loss of *sod1* null mutants and *sod1sod2* double null mutants (Guidot *et al*., 1993; Longo *et al*., 1996).

We examined *S. cerevisiae sod* mutant strains and found that deficient sod activity increases nuclear spontaneous mutation frequencies and that this mutator phenotype is suppressed by inactivation of mitochondrial activity (*sod1Δ* rho^0 or *sod2Δ* rho^0). These data indicate that mitochondrial dysfunction either decreases oxidative damage to nuclear DNA by preventing formation of ROS and/or increase repair of oxidative DNA damage (Rasmussen *et al*. IV). Measurement of intra-cellular levels of O_2^- and H_2O_2 in strains deficient in mitochondrial activity (rho^0 and rho^-) showed lower levels of both O_2^- and H_2O_2 in rho^0 and rho^- cells (Rasmussen *et al*. III). These results showed that inactivation of mitochondrial function decreases intracellular levels of ROS and suggest decreasing mutagenesis caused by ROS. Furthermore, gene expression analysis showed that mitochondrial dysfunction did not increase repair gene expression in *sod1*rho^0 cells compared to *sod1* cells (Rasmussen *et al*. IV). Gene expression analysis also revealed that genes involved in detoxification are repressed in rho^0 compared to wild type cells (Table 3.1).

Table 3.1 • Expression of genes involved in detoxification in rho^0 and rho^+ cells

Probe Set Name	ORF	Gene name	Abs Call	Fold Change in rho^0	Descriptions
Genes repressed in rho^0					
7907_at	YPL163C	*SVS1*	P	2.4	Serine and threonine rich protein.
7432_at	YBL064C		P	2.7	Homolog to thiol-specific antioxidant
10634_at	YKL062W	*MSN4*	P	2.8	zinc finger protein
4074_at	YIR038C	*GTT1*	P	2.9	Glutathione transferase
8410_at	YOR163W	*DDP1*	P	3.1	Strong similarity to S.pombe SPAC13G6.14 protein
4924_at	YGR088W	*CTT1*	P	5.9	Cytoplasmic catalase T
10581_at	YKL026C	*GPX1*	A	5.3	Strong similarity to glutathione peroxidase
Genes induced in rho^0					
8723_at	YOL158C	*ENB1*	P	2.2	Similarity to subtelomeric encoded proteins
4483_s_at	YHR053C	*CUP1-1*	P	2.5	Copper-binding metallothionein
8400_at	YOR153W	*PDR5*	P	3.5	Multidrug resistance transporter
7109_at	YBR244W	*GPX2*	P	7.9	Probable glutathione peroxidase (EC 1.11.1.9)

The Abs Call = the Absolute Call; The transcript is present (P) or absent (A) for rho^0. When the transcript is absent, is the fold change an approximation and calculated using the noise level.

We used DNA microarray hybridization to analyse the expression of genes known to be involved in detoxification of ROS in rho^0 and wild type cells (table 3.1). We found that the cytoplasmic catalase (*CTT1*), glutathione transferase (*GTT1*), and glutathione peroxidase (*GPX1*) genes were repressed in rho^0 cells. In contrast, glutathione peroxidase (*GPX*2) was induced. It has previously been shown that several oxidative agents such as H_2O_2 and O_2^- generating agents (Inoue *et al*., 1999) induce the expression of the GPX2 gene. Our gene expression analysis showed that the expression of the transcription factor Msn4 encoded by

the *MSN4* gene was repressed in rho^0 cells. Msn4 and Msn2 proteins regulate the expression of gene products that are induced by stress conditions through the stress response element (STRE) promoter sequence (Gasch *et al*., 2000; Causton *et al*., 2001). Msn4 and Msn2 proteins have been shown to bind upstream of the STRE element (Görner *et al*., 1998). A known target for this transcription factor is the *CTT1* gene (Gasch *et al*., 2000). Our results indicate that rho^0 cells experience stress although the nature of this stress is unknown at this time.

These data indicate that absence of mitochondrial respiration is an important factor for the viability as well as for suppression of nuclear spontaneous mutations in *sod1*rho^0 mutant cells. Our results suggest that rho^0 cells have a lower production of ROS which results in lower nuclear spontaneous mutation frequency even when Sod1 or Sod2 are absent.

Since the level of ROS is lower in rho^0 cells one would expect that rho^0 cells had lower spontaneous mutation frequencies than wild type but this is not the case. Interestingly we show that rho^0 cells had (2.6 fold) statistically significant higher spontaneous mutation frequencies than wild type. Gene expression analysis showed that *REV1* expression is upregulated in rho^0 strains. The *REV1* gene encodes a deoxycytidyl transferase activity involved in error-prone translesion DNA synthesis (TLS) that inserts dCMP opposite an abasic site or a strand break in the template, thereby producing a terminus that can be extended by polymerase zeta (Pol ξ). Polymerase ξ is composed of two subunits encoded by *REV3* and *REV7*. TLS is mutagenic because Rev1 preferentially inserts cytosine opposite an abasic site in yeast (Lawrence and Hinkle, 1996; Nelson *et al*., 1996; Lawrence and Maher, 2001). We examined *rev1*rho^0, *rev3*rho^0 and *rev7*rho^0 strains to determine if inactivation of TLS could eliminate the mutator phenotype observed in rho^0 cells. Our results show that mitochondrial-mediated mutator phenotype depend on functional Rev1, Rev3 and Rev7 proteins and that the mutator phenotype of rho^0 cells is suppressed by preventing Rev1/Rev3/Rev7-dependent translesion synthesis (Rasmussen *et al*., III).

It has been shown that 3-methyladenine DNA glycosylase encoded by *MAG1* in *S. cerevisiae*, which removes a variety of alkylated bases and generates abasic sites, increases spontaneous mutation frequency by 600-fold when overexpressed in *S. cerevisiae*. This mutator phenotype is also dependent on the Rev1/Rev3/Rev7 TLS bypass pathway (Glassner *et al*., 1998).

Our genetic evidence therefore suggests that mitochondrial dysfunction contributes to spontaneous DNA damage, which is fixed in the genome as mutation by error-prone Rev1/Rev3/Rev7 TLS pathway, which results in cell survival but genetic instability.

Interestingly, one of the profound features of cancer cells is their defective mitochondrial function. A role for mitochondria in tumorigenesis was hypothesized when it was found that most tumors up-regulate glycolysis and therefore seems to be more dependent upon glycolysis for energy production, than mitochondrial oxidative phosphorylation (Warburg, 1956).

Furthermore, cancer cell lines (bladder, head, neck, colorectal and pancreatic) and lung primary tumors have been observed to have high frequency of homoplasmic mutations of mtDNA. The mitochondrial mutations are present in each of the hundreds of mitochondrial genomes in the tumor cell and in virtually all of the cells of the tumor (Polyak *et al*., 1998; Fliss *et al*., 2000; Jones *et al*., 2001). Homoplasmic mutations of mtDNA indicate that a single cell with a mutant mitochondrial genome has acquired a selective growth advantage during tumor evolution, allowing it to become the predominant cell type in the tumor cell population. Homoplasmy also indicates that each mutant mitochondrial genome has a replicative advantage in the particular mitochondria in which it occurred, and that this mitochondrion has selectively proliferated over other mitochondria in the same cell (Polyak *et al*., 1998; Jones *et al*., 2001).

Our studies using *S. cerevisiae* with depleted mitochondria suggest that cancer cells by losing their mitochondrial function create a mutator phenotype (Rasmussen *et al*., II). Figure 3.3 provides a model for how mitochondrial dysfunction generates a mutator phenotype in two different ways:

1. Inhibition of the respiratory chain increases mitochondrial ROS production. Mitochondrial reactive oxygen species damage the mtDNA and the nDNA if mitochondrial H_2O_2 diffuses to the nucleus and is converted to $HO^{\bullet}$ which can cause DNA damage.
2. Mitochondrial dysfunction leads to nuclear DNA damage. The DNA damage activates the Rev1/Rev3/Rev7 error-prone translesion DNA synthesis pathway that contributes to cell survival, but at the expense of higher spontaneous mutation frequency.

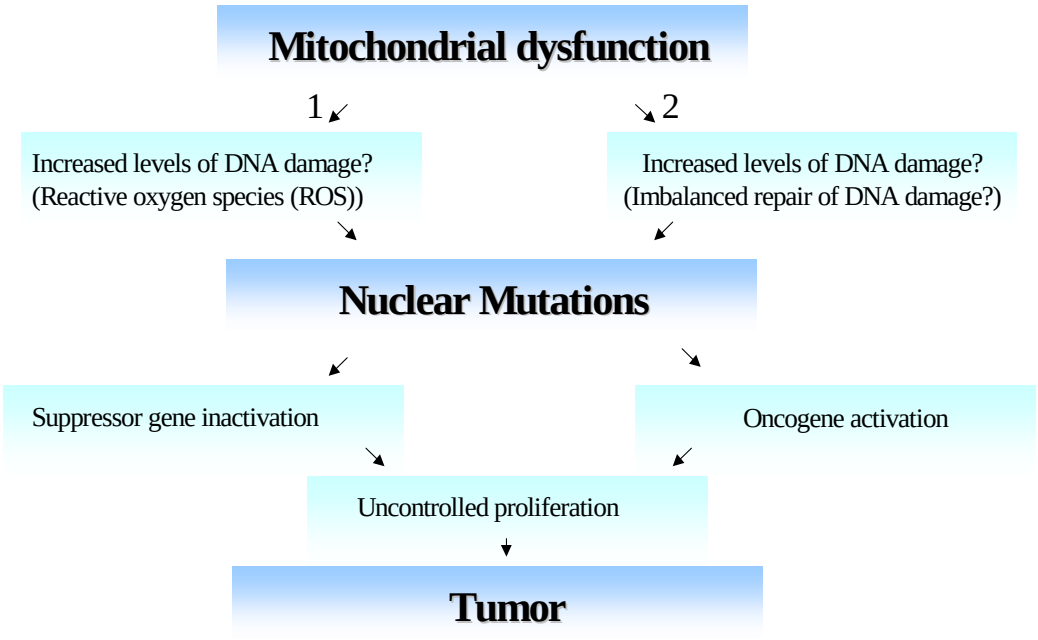


Figure 3.3. Mitochondrial dysfunction creates a mutator phenotype. Our results suggest that mitochondrial dysfunction can create a mutator phenotype in at least two ways. 1) Mitochondrial dysfunction caused by inhibition of the respiratory chain increases mitochondrial ROS production and if the ROS level is higher than the endogenous antioxidant capacity, ROS might difusse as H_2O_2 to the nucleus. In nucleus H_2O_2 can be converted to $HO^{\bullet}$ which causes DNA damage. If the DNA damage is left unrepaired mutations in nDNA will occur and may lead to mutations that enhances cancer risk. 2) Mitochondrial dysfunction leads to nuclear DNA damage. The nature of this spontaneous nuclear DNA damage is unknown at this time, but might be caused by imbalanced repair as seen when Mag1 is overexpressed (Glassner *et al*., 1998). However, the DNA damage activates the Rev1/Rev3/Rev7 TLS pathway, which contributes to cell survival, but at the expense of higher spontaneous mutation frequency, which may lead to enhanced cancer risk.

Section two

Paper I

Lene Juel Rasmussen, Merete Rasmussen, Byung-In Lee, **Anne Karin Rasmussen**, David M. Wilson III, Finn Cilius Nielsen, and Hanne Cathrine Bisgaard. Identification of factors interacting with hMSH2 in the fetal liver utilizing the yeast two-hybrid system. In vivo interaction through the C-terminal domains of hEXO1 and hMSH2 and comparative expression analysis. **Mutation Research** (2000) 460, 41-52.

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Identification of factors interacting with hMSH2 in the fetal liver utilizing the yeast two-hybrid system
In vivo interaction through the C-terminal domains of hEXO1 and hMSH2 and comparative expression analysis

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Abstract

Mutations in DNA mismatch repair (MMR) genes have been shown to segregate with Hereditary Nonpolyposis Colorectal Cancer (HNPCC). However, because many HNPCC families fail to display mutations in known MMR genes, we argued that changes in other components of the MMR pathway may be responsible. The increasing number of proteins reported to interact in the MMR pathway suggests that larger complexes are formed, the composition of which may differ among cell types and tissues. In an attempt to identify tissue-specific MMR-associated factors, we employed the yeast two-hybrid system, using the human hMSH2 as bait and a human fetal liver library as prey. We demonstrate that hMSH2 interacts with a human 5′–3′ exonuclease 1 (*hEXO1 / HEX1*) and that this interaction is mediated through their C-terminal domains. The hMSH6 protein does not interact with hEXO1 in the two-hybrid system. Dot-blot analysis of multiple tissue RNA revealed that *hMSH2* and *hEXO1* are coexpressed at high levels in fetal liver as well as in adult testis and thymus. Northern blot analysis also revealed that *hEXO1 / HEX1* is highly expressed in several liver cancer cell lines as well as in colon and pancreas adenocarcinomas, but not in the corresponding non-neoplastic tissue.

Keywords: Mismatch repair; hMSH2; hEXO1; Two-hybrid; Cancer

1. Introduction

Changes in the efficiency of DNA repair and recombination activities can be associated with predisposition to cancer [1]. The finding that Hereditary

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Nonpolyposis Colorectal Cancer (HNPCC) families frequently harbor mutations in DNA mismatch repair (MMR) genes has generated widespread interest in this research area [2–4]. At the time of writing, germline mutations in at least four genes, *hMSH2*, *hMLH1*, *hPMS1* and *hPMS2*, all homologs of bacterial components involved in MMR, have been found in HNPCC patients. Although no biochemical role has to date been proposed for hPMS1, the other three proteins have been shown to participate in MMR [3]. In addition, MMR proteins might play important roles in other pathways of DNA metabolism such as DNA recombination and cell cycle checkpoint signaling [4].

The development of an assay to study MMR in cell-free extracts of bacteria has led to a detailed understanding of the mechanism underlying the repair process and, on the basis of these in vitro studies, a model for MMR has been proposed [5], reviewed in Refs. [4,6]. Based on homology between eukaryotic and prokaryotic MMR proteins, it has been hypothesized that the mechanism underlying human MMR is similar. Subsequently, it has been shown that eukaryotic MMR proteins interact with polypeptides involved in DNA replication, DNA recombination, and DNA nucleotide excision repair (NER) [7–17]. A number of these genes have been inactivated giving rise to complex phenotypes, such as defects in MMR and recombination or in NER and recombination [18–20]. These findings suggest that DNA repair proteins are components of larger protein complexes, and that at least some of these proteins function in several distinct processes. The increasing number of polypeptides reported to interact with proteins in the MMR pathway suggests that these complexes are composed of a larger number of subunits, and also that the composition of such complexes differs among cell types and tissues.

In the present communication, we have employed the yeast two-hybrid system in an attempt to identify proteins that interact with the human MMR protein hMSH2 in vivo, using hMSH2 as bait. We chose a human fetal liver cDNA library as prey since we expected MMR-associated proteins to be highly expressed in an organ containing rapidly proliferating cells. We report that hMSH2 interacts with the gene products of both *hMSH6* and *hEXO1 / HEX1*. We were unable to detect binding between hMSH6 and hEXO1 in the two-hybrid assay. Our results suggest that the interaction between hMSH2 and hEXO1 is mediated via the C-terminal domains of these proteins. Expression analysis showed that high levels of this exonuclease are indeed coexpressed with *hMSH2* in fetal liver as well as in adult testis and thymus. Coexpression, although at lower levels, is also found in colon, small intestine, bone marrow, placenta, fetal kidney, fetal spleen and fetal thymus. Further expression analysis revealed high levels of *hEXO1* transcripts in liver cancer cell lines and in colon and pancreas adenocarcinomas, but not in the corresponding non-neoplastic tissue.

2. Material and methods

2.1. Human cell lines

The cell lines HepG2 (human hepatocellular carcinoma), SK-Hep1 (human liver adenocarcinoma), Huh-7 (human hepatocellular carcinoma), Chang (human liver), and WRL68 (human liver embryo) were purchased from European Collection of Cell Cultures (Wiltshire, UK). All cell lines were maintained as monolayer cultures in DMEM (Gibco, Life Technologies) supplemented with 10% FBS (Gibco, Life Technologies).

2.2. Plasmids

Plasmid pLJR105 was constructed by inserting the human *hMSH2* coding sequence into the *Nco*I site of the pAS2 binding domain vector (CLONTECH). The *hMSH2* gene was obtained on a 2.9-kbp fragment from pCite-1-hMSH2 [21] kindly provided by Dr. Josef Jiricny (Institute of Medical Radiobiology of the University of Zurich, Switzerland). The hMSH2 bait was sequenced to confirm that the hMSH2 protein was in frame with the GAL4 binding domain. Plasmid pLJR112 contains a C-terminal truncation of the *hMSH2* gene and was constructed by deleting a 1-kb fragment between the *Blp*I (bp 1796 in *hMSH2*) and *Sal*I (located in the vector) sites in pLJR105. This truncation eliminates the C-terminal 374 amino acids of the hMSH2 protein and includes elimination of the conserved region. The HEX1 (C-terminal) and HEX1 (N-terminal) plasmids

were constructed by inserting either a 1182- or a 1355-bp fragment into the *Nco*I and *Bam*HI sites of the pACT2 activation domain vector (CLONTECH). The fragments were amplified by PCR using primers containing additional *Nco*I or *Bam*HI sites. Primer pairs 5′-GGCACCATGGGGATACAGGGAT-3′, 5′-CGGGATCCTCACTTCAATTGTGGGGCATCT-3′ and 5′-GGCACCATGGCCTGCCCATT-CAAGAAGT-3′, 5′-CGGGATCCTCAGAATTTTT-TAAATCCAA-3′ were used for amplification of the N-terminal and C-terminal parts of the hEXO1a/HEX1 protein, respectively.

2.3. Two-hybrid techniques

The GAL4-hMSH2 (pLJR105) bait plasmid was transformed into *S. cerevisiae* strain Y190 (*MATα, ura3-52, his3-200, lys2-801, ade2-101, trp1-901, leu2-3, 112, gal4Δ, gal80Δ, cyh^{r}2, LYS2*::*GAL1*$_{UAS}$-*HIS3*$_{TATA}$-*HIS3, URA3*::*GAL1*$_{UAS}$-*GAL1*$_{TATA}$-*lacZ* [22]. The resulting strain was subsequently transformed with a human fetal liver matchmaker cDNA library (CLONTECH, #HL4029AH) and hMSH2 interactors were selected on synthetic dextrose minimal medium (SD) [23] lacking tryptophan (to maintain pLJR105), leucine (to maintain the GAL4 activation domain plasmids), and histidine (to identify peptides capable of assembling a functional GAL4 transcription factor) and supplemented with 25 mM 3-amino-1, 2, 4-triazole (3-AT) (SD-HIS-TRP-LEU + 25 mM 3-AT). 3-AT is a competitive inhibitor of the *S. cerevisiae* His3 protein and is used to lower the background growth of the reporter strain due to leaky expression of the *HIS3* gene. The plates were incubated at 30°C for 7–10 days and a total of 53 positive clones, Y1–Y53, were screened for β-galactosidase activity on SD-TRP-LEU + 60 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) plates to verify positive interactions. These clones were rescued for the GAL4 binding domain plasmid (pLJR105) by repeated growth on SD-LEU plates. The rescued clones were tested for lack of *lacZ* expression on SD-LEU + X-gal plates to eliminate false positive clones.

2.4. DNA sequencing

DNA sequencing was performed directly on yeast colonies grown on SD-HIS-TRP-LEU plates by touching the colony with a sterile pipette tip. The pipette tip was rinsed in incubation buffer (1.2 M sorbitol; 100 mM sodium phosphate, pH 7.4; 2.5 mg/ml zymolase) and incubated at 37°C for 5 min. Sequence inserts were amplified by PCR using the MATCHMAKER AD LD-Insert Screening Amplimer Set (CLONTECH). The PCR amplified fragments were purified using a Qiaquick PCR purification kit (Qiagen) and sequenced. Database searches were performed using the BLAST algorithm http://www.ncbi.nlm.nih.gov/BLAST/).

2.5. Northern blot analysis

Human RNA Master Blot containing 100–500 ng poly(A)$^+$RNA/dot (Human RNA Master Blot) was purchased from CLONTECH. For detection of transcripts in human cells aliquots (20 μg) of total RNA (RNAqueous™, Ambion) were run on 1% agarose/0.2 M formaldehyde gels and immobilized onto nylon membranes. All probes were labeled by random primer extension (Rediprime DNA labeling system, Amersham Pharmacia Biotech) and [α-^{32}P]dCTP (Amersham Pharmacia Biotech) using a PCR product as template. For the detection of exonuclease homologs, the blots were probed with either a 914-bp fragment containing the N-terminal region of *hEXO1a/HEX1* (N-terminal probe) or a 946-bp fragment containing the C-terminal region of *hEXO1a/HEX1* (C-terminal probe). These probes were generated by PCR primers 5′-GGTGCCA-CATGCGATCTCTGAG-3′ and 5′-TCCAATCC-CACGCAGTGATGAC-3′ (HEX1 N-terminal probe) and 5′-GAGTGTAAGCACTCCACCTAGG-3′ and 5′-CCCGGCTTGTTCTCGGCATTAT-3′ (HEX1 C-terminal probe). For the detection of *hMSH2*, the blots were probed with a 1054-bp fragment containing the N-terminal region, which shows limited homology to other known MutS homologs. This probe was generated by PCR primers 5′-GTGCGCTTCTT-TCAGGGCATGC-3′ and 5′-TTATTCAGCAAGG-CAGCCAGAG-3′ (*hMSH2* probe). All membranes were hybridized with 1×10^6 cpm/ml of ^{32}P-labeled probes. The Human RNA Master Blot was prehybridized for 30 min at 65°C and hybridized overnight at 65°C in ExpressHyb® solution according to the manufacturer (CLONTECH). All other membranes were prehybridized for 30 min at 68°C and hy-

bridized for 90 min at 68°C in QuikHyb® Hybridization Solution (Stratagene). Blots were washed twice at room temperature for 15 min and once at 60°C with wash solution (1× SSC, 0.1% SDS). Images were obtained using autoradiography. *GAPDH* and β-actin probes provided by the manufacturer (CLONTECH) were prepared as described above and used to correct for differences in loading.

2.6. β-galactosidase assays

β-galactosidase specific activity was assayed on three separate transformants of each strain. In brief, colonies were inoculated into 5 ml of SD-LEU-TRP medium and grown overnight at 30°C with vigorous shaking. The next day, 2 ml of overnight cultures were inoculated into 2 ml of YPD medium and the cultures grown for 3–5 h at 30°C with vigorous shaking. Samples were prepared and assayed for β-galactosidase activity essentially as described by CLONTECH (Protocol # PT1020-1, www.clontech.com).

3. Results

3.1. Identification of hMSH2-interacting protein partners

To define the mechanism of human MMR and to identify missing components of this complex process, we set up a two-hybrid screen in *S. cerevisiae*, using as bait GAL4 fused to the entire open reading frame of human *hMSH2* and as prey GAL4 activation-tagged human fetal liver matchmaker cDNA library. Our initial screen identified 53 (Y1–Y53) clones as interactors. Sequence analysis showed that the majority of the clones contained either yet unidentified human cDNAs or proteins such as γ-globulin, fibrinogen, albumin, haptoglobin, repeat regions, ferritin, and α-chymotrypsin but that Y5, Y10, Y47 and Y50 contained a carboxyl-terminal region of the human homologue of *S. cerevisiae* exonuclease EXO1, termed *hEXO1b* [10, 11] (Fig. 1). We also isolated two clones containing hMSH6, which is known to form a complex with hMSH2 [4], confirming to us that hMSH2 is able to form a complex with human MMR proteins in yeast.

The DNA sequences of the *hEXO1* clones we isolated (Y5, Y10, Y47 and Y50) showed a perfect match to the 3′-end of *hEXO1b*, which is dissimilar to the *hEXO1a/HEX1* sequence (Fig. 1). Furthermore, we observed several DNA sequence polymorphisms in the *hEXO1* clones that could reflect human population variation (Fig. 1). The C to T base pair change (bp 2488) in Y5, Y10 and Y47 causes a proline (P) to leucine (L) codon change, whereas the G to A (bp 1279) in Y50 causes an arginine (R) to histidine (H) codon change. The Y50 clone contains also an A to G base pair change (bp 1983), which results in a lysine (K) to glutamic acid (E) codon change. Furthermore, Y50 contains a deletion of CAG (bp 1486) resulting in a deletion of an alanine (A) residue. All the clones we isolated appear to interact equally well with hMSH2 in the two-hybrid assay, indicating that these amino acid residues are not crucial for hMSH2-hEXO1 interaction. The Y5, Y10 and Y47 clones contained an additional 127-bp sequence (Fig. 1), which corresponds to 59171–59048 bp of the genomic DNA sequence (Genbank AC004783). This sequence is located upstream of the first exon (Exon 1) in *hEXO1a/HEX1* [12]. For simplicity, we have named this 127-bp DNA sequence for Exon 0. We do not think that Y5, Y10 and Y47 represent true splice variants of *hEXO1b* since we were only able to detect transcripts corresponding to full-length *hEXO1* using Northern blot analysis (data not shown).

3.2. Interaction domains of hMSH2 and hEXO1

It has been shown that purified full-length hEXO1b interacts with hMSH2, in vitro, in an immunoprecipitation assay [10]. We used two-hybrid analysis to characterize the interaction between hMSH2 and hEXO1 in vivo and to define the region of association. We fused the full-length *hEXO1a/HEX1* and *hEXO1b* cDNAs to the GAL4 activation domain in pACT2 and tested for interaction with our GAL4–hMSH2 binding domain plasmid (pLJR105). We were unable to detect protein–protein interactions with any of our full-length exonuclease 1 constructs in the two-hybrid assay (Fig. 2A). However, when we switched the inserts around and instead fused *hMSH2* to the GAL4 activation domain in pACT2, and *hEXO1b* and

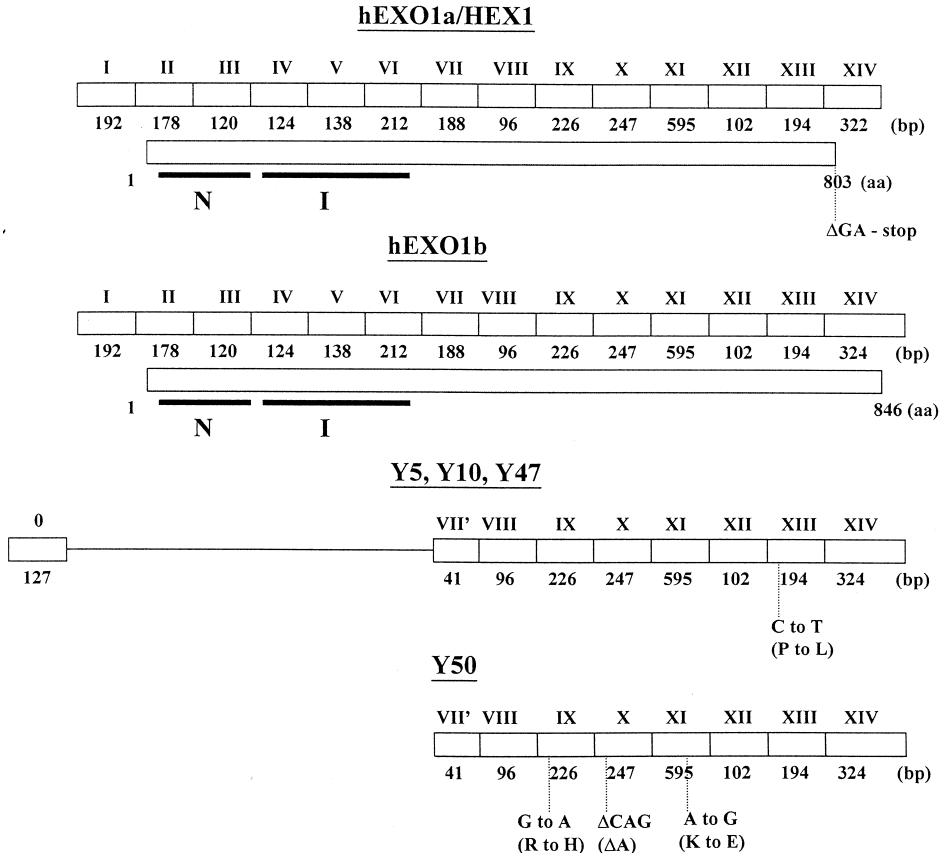

Fig. 1. Exonuclease 1 gene structures. The full-length *hEXO1a/HEX1* and *hEXO1b* [10–12] coding regions comprise 13 exons (II–XIV). The shorter variants named Y5, Y10, Y47 and Y50 comprise eight exons (VII′–XIV). These clones contain only a part of exon VII that we refer to as exon VII′. Solid lines represent exon sequences containing the N and I regions thought to be required for exonuclease activity. Shaded boxes in *hEXO1a/HEX1* and *hEXO1b* represent the predicted coding regions. ΔGA to stop indicates the two base pairs deletion of GA that causes a frameshift in *hEXO1a/HEX1* compared with *hEXO1b*. The Y5, Y10 and Y47 clones contain an additional 127-bp DNA sequence (exon 0) corresponding to 59171–59048 bp of the genomic DNA sequence (Genbank AC004783).

hEXO1a/HEX1 to the GAL4 binding domain in pAS2, we could detect an interaction in the two-hybrid assay (Fig. 2A, Table 1). One reason for this discrepancy could be that the GAL4 (activation domain)-hEXO1 fusion proteins are structurally different from the native proteins and therefore unable to interact with the GAL4 (binding domain)–hMSH2 fusion protein. This would also explain why we failed to isolate any full-length cDNAs of *hEXO1b* or *hEXO1a/HEX1* in our initial two-hybrid screen.

We constructed truncated peptides of hEXO1a/HEX1 such that the protein was divided into two regions containing either the 451 amino acids N-terminal or the 388 amino acids C-terminal part of full-length hEXO1a/HEX1 (Fig. 1). We also constructed a C-terminal truncation of hMSH2 to delete the conserved region of the protein that is necessary for interaction with hMSH3 and hMSH6 [24]. We did not observe any interaction between hMSH2 and the N-terminal region of hEXO1 in the two-hybrid test (Fig. 2B). Neither did we observe interaction between the hMSH2 N-terminal region and any of the exonuclease clones we isolated in the two-hybrid screen (data not shown). However, we cannot rule out that the N-terminal region of hEXO1 makes contact to hMSH2 in the native protein. Interestingly, we did observe an interaction between hMSH2 and Y5 (hEXO1b C-terminal), as well as

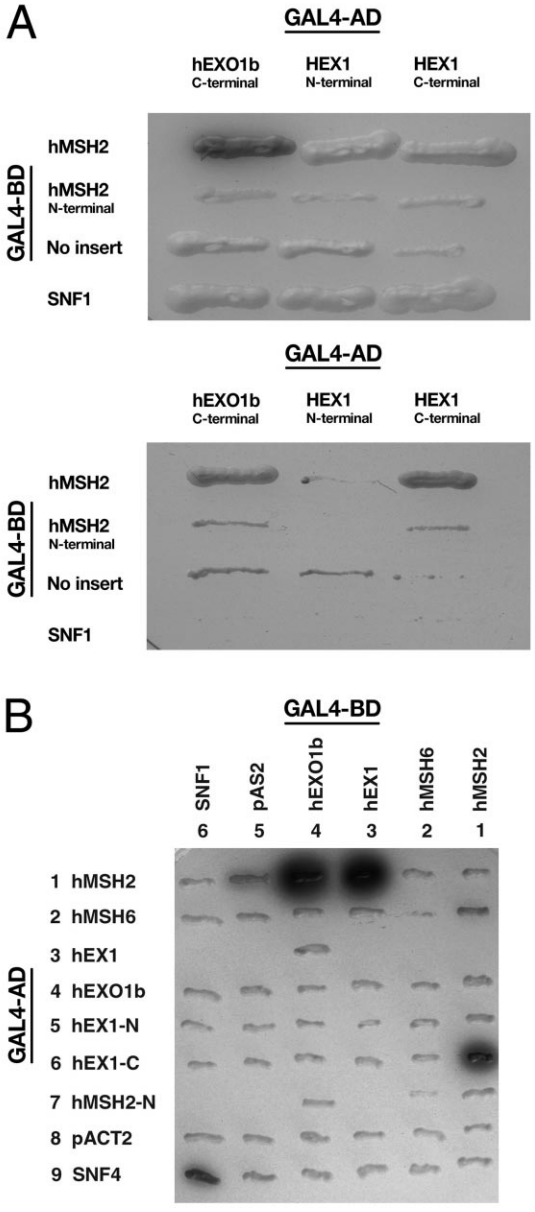

Fig. 2. Interaction of hMSH2 with hEXO1a/HEX1 and hEXO1b (Y5). (A) Strains containing various plasmids were streaked in horizontal rows on either SD-LEU-TRP + X-gal (upper panel) or SD-LEU-TRP-HIS + 45 mM 3-AT (lower panel) plates. (B) Strains containing various plasmids were streaked on SD-LEU-TRP + X-gal plate. The plasmids contained in each strain tested are indicated at the top of each column and at the left of each row. No insert: pAS2, SNF4: pACT2 containing SNF4 (control), and SNF1: pAS2 containing SNF1 (control). BD: binding domain. AD: activation domain.

Table 1
Specific β-galactosidase activity assay for protein interactions in the two-hybrid assay

	Specific β-galactosidase activity[a]
Y5 (AD) + hMSH2 (BD)	6.3
Y5 (AD) + hMSH2-N (BD)	0
HEX1-N (AD) + hMSH2 (BD)	0
HEX1-N (AD) + hMSH2-N (BD)	0
HEX1-C (AD) + hMSH2 (BD)	0.3
HEX1-C (AD) + hMSH2-N (BD)	0
hMSH2 (AD) + HEX1 (BD)	17.7
hMSH2 (AD) + hEXO1b (BD)	12.8
SNF4 (AD) + SNF1 (BD)	3.1
pACT2 (AD) + pAS2 (BD)	0

[a] Specific β-galactosidase activity was determined as described in Materials and methods.

between hMSH2 and hEXO1a/HEX1 (C-terminal region) (Fig. 2B, Table 1). Our Y5 (hEXO1b C-terminal) clone did not contain the full-length *hEXO1b* cDNA, but instead contained an insert encoding the carboxyl half of hEXO1b protein beginning at amino acid 302. These data argue that the carboxyl-terminal region of hMSH2 contacts the carboxyl-terminal domains of both hEXO1a/HEX1 and hEXO1b. Thus, the hMSH2-interacting region is outside the N and I regions (Fig. 1) thought to be required for exonuclease activity [12,25,26]. Our results complement the finding that *S. cerevisiae* yMSH2 interacts with the carboxyl-terminal region of yEXO1 outside the N and I regions [16]. *hEXO1a/HEX1* differs from *hEXO1b* by two nucleotides (2616-AG-2617) at the boundary of exon 14 (Fig. 1). This frame-shift results in the truncation of the hEXO1a/HEX1 polypeptide by 43 amino acids. We show that hMSH2 interacts with both forms of human exonuclease 1, suggesting that the interacting domain is located between exons 8 and 13 (Fig. 1). Interestingly, from data shown in Fig. 2 it appears that hMSH6 does not interact with hEXO1 in our two-hybrid assay.

3.3. Expression of exonuclease and hMSH2 in human tissues

We used a human RNA master blot to characterize the expression pattern of *hEXO1* and to look for

tissues predominately expressing the C-terminal transcript of *hEXO1*. One of the DNA probes contained only the C-terminal region of *hEXO1a/HEX1* and should detect all *hEXO1* splice variants identified to

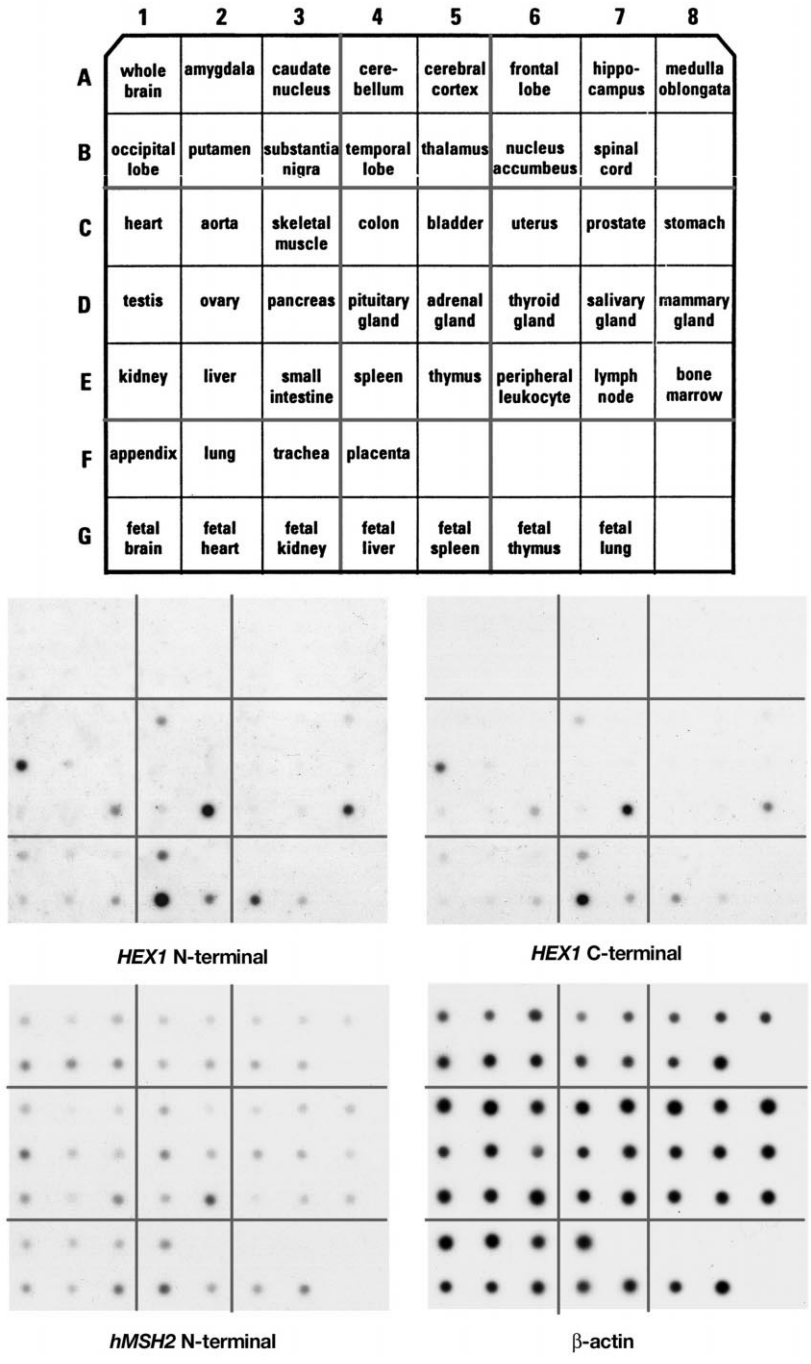

Fig. 3. Expression profiles of human exonuclease 1 and *hMSH2*. The RNA master blot was hybridized with *hMSH2*, *hEXO1a/HEX1* N-terminal, *hEXO1a/HEX1* C-terminal or β-actin control probes.

date. The second probe contained the N-terminal region of *hEXO1a/HEX1*, which would be specific for full-length *hEXO1a/HEX1* and *hEXO1b* mRNAs, but would not detect the C-terminal transcripts (Fig. 1). We reasoned that by comparing the expression patterns obtained with these two probes, we would be able to identify tissues that express only C-terminal fragment. Since we did not observe any differences in expression results, it would appear that no tissue preferentially expresses the C-terminal part of hEXO1 (Fig. 3). We do not know whether the C-terminal part of hEXO1 is naturally present and has a specific function in the cell or whether its occurrence is a result of inefficient DNA synthesis during the construction of the matchmaker cDNA library, but presume the latter to be true based on the fact that shorter transcripts were not observed. Both probes detected transcripts prominently expressed in fetal liver, adult testis and thymus, but we also observed significant expression in adult colon, small intestine, bone marrow, placenta, fetal kidney, fetal spleen and fetal thymus (Fig. 3). Interestingly, we did not detect any expression of *hEXO1* mRNA in adult liver, spleen, and kidney, indicating a specific role for hEXO1 in the development of these tissues.

Since hMSH2 interacts with exonuclease 1 (Fig. 2), we compared the expression patterns of *hMSH2* with that of *hEXO1* to identify tissues where the exonuclease could play a role in DNA repair and/or recombination. We found that *hMSH2* is expressed in all tissues investigated (Fig. 3). Fig. 3 shows that *hMSH2* and *hEXO1* are coexpressed at higher levels in fetal liver, adult testis, thymus, and at lower levels in bone marrow, placenta, fetal spleen, fetal kidney and fetal thymus.

3.4. Expression of exonuclease and hMSH2 in human liver cancer cell lines

We have shown that *hEXO1* is expressed in the fetal liver but not in the adult liver (Fig. 3). We thus examined the expression of the *hMSH2* and *hEXO1* genes in various human liver cell lines (Fig. 4): HepG2 (human hepatocellular carcinoma), SK-Hep1 (human liver adenocarcinoma), Huh-7 (human hepatocellular carcinoma), Chang (human liver), and WRL68 (human liver embryo). As expected, both *hEXO1* and *hMSH2* were highly expressed in the fetal liver but not in the adult liver (Fig. 4). One reason for failing to detect any *hMSH2* expression in the adult liver in this experiment could be that the blot shown in Fig. 3 contains poly(A)$^+$ mRNA while the blot in Fig. 4 contains total RNA. However, since *hMSH2* is preferentially expressed in proliferating cells [27–29], high transcript levels in the normally quiescent adult liver would be unexpected. When the *hEXO1* probes were used, we could only detect a single transcript of approximately 3 kb, which corresponds to full-length *hEXO1* (Fig. 4 and data not shown). This result suggests that *hEXO1* mRNA is unlikely spliced to any of the shorter forms identified in the two-hybrid screens and that the C-terminal clones likely do not exist. A 3-kb transcript was detected using the *hMSH2* probe, which corresponds to the full-length *hMSH2* cDNA. Inter-

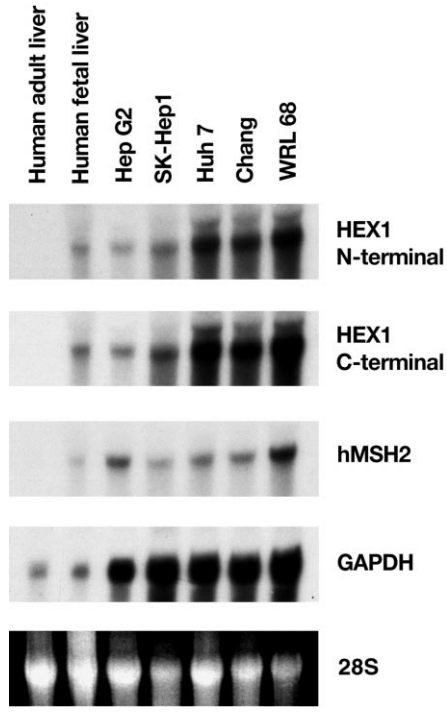

Fig. 4. Expression of human exonuclease 1 and *hMSH2* in liver cancer cell lines. Membranes containing total RNA isolated from human adult liver, fetal liver, and various liver cell lines were probed with *hMSH2*, *hEXO1a/HEX1* N-terminal domain, *hEXO1a/HEX1* C-terminal domain and *GAPDH* control probes. The lower panel of the figure shows the 28S ribosomal RNA band of the ethidium bromide stained gel. The human liver cell lines are described in Materials and methods.

estingly, we find that both *hEXO1* and *hMSH2* are highly expressed in the liver cancer cell lines HepG2, SK-Hep1 and Huh7 as well as in the fetal-like Chang and WRL68 cells.

3.5. Expression of exonuclease and hMSH2 in tumor tissue

Our results revealed that *hEXO1* is expressed preferentially in tissue containing proliferating cell populations as well as in cell lines established from human liver tumors (Figs. 3 and 4). This led us to investigate the expression of *hEXO1* in human tumors from colon and pancreas. Interestingly, we found that *hEXO1* expression is indeed upregulated in samples isolated from colon and pancreas adenocarcinomas compared to normal tissue (Fig. 5). These results support the idea of *hEXO1* being preferentially expressed in proliferating cells. Interestingly, we found that *hMSH2* expression is upregulated in the colon tumors but not in the pancreas tumors (Fig. 5). These results may imply a defect in MMR activity in the pancreas tumors due to the low levels of hMSH2 protein.

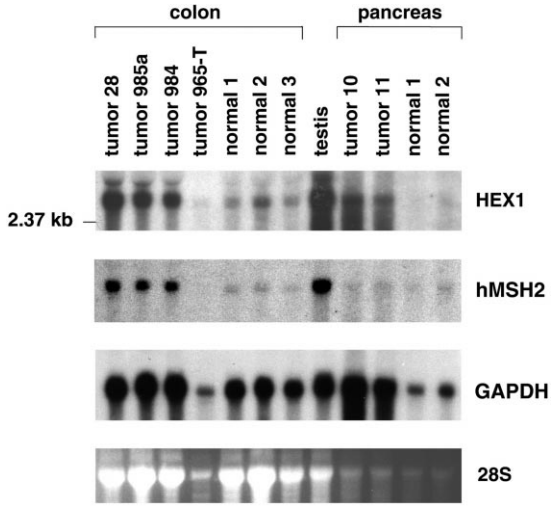

Fig. 5. Expression of human exonuclease 1 and *hMSH2* in colon and pancreas adenocarcinomas. Membranes containing total RNA isolated from human adenocarcinomas and corresponding non-neoplastic tissue were probed with *hMSH2*, *hEXO1a / HEX1* C-terminal domain and *GAPDH* control probes. The lower panel of the figure shows the 28S ribosomal RNA band of the ethidium bromide stained gel. Colorectal and pancreatic adenocarcinoma and normal colon and pancreatic tissue were collected during surgery and immediately frozen in liquid nitrogen. All tumors were verified by histological examination. Normal tissues from colon and pancreas were also examined histologically and found to be free of tumor cells. The local ethics committee approved the use of tumor and normal tissue.

4. Discussion

Eukaryotic proteins are often components of larger complexes. It is therefore easy to imagine that the disruption of a particular protein–protein interaction might affect several different cellular processes. In addition, many eukaryotic genes show tissue-specific expression and it is conceivable that the composition of protein complexes varies among different cell types and tissues. In agreement with this model, it has been shown that mice carrying disruptions of specific MMR genes display a variety of phenotypes ranging from tissue-specific tumorigenesis to sterility [20,30–35]. This is consistent with the evidence that implicates MMR proteins in processes other than postreplicative MMR. Homologous and homeologous recombination, transcription coupled repair or cell cycle control following DNA damage are examples of such pathways [2,4,6]. It is possible, even likely, that MMR proteins will interact with different partners in each of these alternative pathways. Identification of these partners will help elucidate the mechanisms of these complex processes.

In an attempt to identify tissue-specific MMR-associated factors, we employed the yeast two-hybrid system, using the human hMSH2 as bait and a human fetal liver library as prey. We demonstrate that hMSH2 interacts with hEXO1 through their C-terminal domains. Another MutS homolog hMSH6 does not interact with hEXO1 in the two-hybrid system. It has previously been shown that the human exonuclease 1 is expressed in a wide variety of tissues [11]. Our comparative expression analysis revealed that *hMSH2* and *hEXO1* are coexpressed in the fetal liver, adult testis and thymus as well as in colon, small intestine, and in the fetal tissues of kidney and spleen but not in the corresponding adult tissues. *hEXO1 / HEX1* is highly expressed in several liver cancer cell lines as well as in colon and pancreas adenocarcinomas, but not in the corresponding non-neoplastic tissue.

4.1. Interaction between hMSH2 and human exonuclease 1

The human MMR protein hMSH2 forms heterodimers with hMSH3 or with hMSH6, which function in the substrate recognition step during MMR. In the subsequent step, these bound heterodimers interact with the hMLH1/hPMS2 complex. Although the following events of the MMR process are not clear, it is anticipated based on the bacterial model that the four-protein complex will recruit DNA helicase and exonuclease activities, which mediate the displacement and the degradation of the mismatch-containing strand, respectively. The position of the strand break and the initiation site of the exonucleolytic process with respect to the mismatch would determine the nature of the exonuclease. Thus, repair of mismatches with upstream-positioned strand-discrimination nicks will require a 5′–3′ exonuclease such as EXO1 [16]. Conversely, MMR involving downstream nicks would require 3′–5′ exonucleases to mediate the degradation process. In the present study, we show that hMSH2 interacts with the human 5′–3′ exonuclease hEXO1. We are currently investigating whether the hMSH2/hEXO1 complex functions in MMR or other pathways such as recombination, where hEXO1 has already been shown to play a role [11,16].

4.2. Comparative expression analysis of hEXO1

Tumorigenesis in HNPCC patients is primarily restricted to the proximal colon and endometrium. We found that the *hMSH2* and *hEXO1* genes are coexpressed, although at low levels, in colon and small intestine. Due to the likely redundancy between exonucleases in the MMR process, it would appear unlikely to anticipate that mutations in the *hEXO1* gene will segregate with HNPCC. Human population studies monitoring the association of *hEXO1* gene defects with cancer or the construction of a *mEXO1* knockout mouse should clarify if this exonuclease plays any role in carcinogenesis. Our expression profiles of *hMSH2* and *hEXO1* also revealed that these genes are coexpressed in human testis and bone marrow, suggesting a role in meiosis and hematopoiesis likely due to their function in recombination [36]. Along these lines, we have also shown that both *hEXO1* and *hMSH2* are highly expressed in the fetal liver; a site of hematopoiesis during fetal development. However, in addition to supporting hematopoiesis during embryonic development, the fetal liver is also characterized by rapid division of fetal hepatocytes. We have shown that *hEXO1* and *hMSH2* are not expressed in normal adult liver where cell turnover is normally very slow. However, both genes are highly expressed in the liver cancer cell lines HepG2, SK-Hep1 and Huh7 as well as in the fetal-like Chang and WRL68 cells. Furthermore, we showed that *hEXO1* is highly expressed in colon and pancreas adenocarcinomas but not in the corresponding non-neoplastic tissues. These results suggest a role for hEXO1 in cellular processes other than meiosis and hematopoiesis [36]. Such processes could be MMR, recombinational repair or replication, which are expected to be active in proliferating cells such as fetal hepatocytes and cancer cells. Current studies are examining for potential associations of hEXO1 with factors from these cellular activities. The fact that *hEXO1* is specifically expressed in liver cancer cells as well as in colon and pancreas adenocarcinomas could make the expression of this gene a useful marker to diagnose these cancers.

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References

[1] C. Lengauer, K.W. Kinzler, B. Vogelstein, Genetic instabilities in human cancers, Nature 396 (1998) 643–649.
[2] J. Jiricny, Eukaryotic mismatch repair: an update, Mutat. Res. 409 (1998) 107–121.
[3] J. Jiricny, Replication errors: cha(lle)nging the genome, EMBO J. 17 (1998) 6427–6436.
[4] P. Modrich, R. Lahue, Mismatch repair in replication fidelity, genetic recombination, and cancer biology, Annu. Rev. Biochem. 65 (1996) 101–133.

[5] P. Modrich, Mechanisms and biological effects of mismatch repair, Annu. Rev. Genet. 25 (1991) 229–253.
[6] L.J. Rasmussen, L. Samson, M.G. Marinus, Dam-directed DNA mismatch repair, in: M.F. Hoekstra, J.A. Nickoloff (Eds.), DNA Damage and Repair: Biochemistry, Genetics and Cell Biology vol. 1 Humana Press, Clifton, NJ, 1998, pp. 205–228.
[7] M.E. Budd, J.L. Campbell, A yeast replicative helicase, Dna2 helicase, interacts with yeast FEN-1 nuclease in carrying out its essential function, Mol. Cell. Biol. 17 (1997) 2136–2142.
[8] R.E. Johnson, G.K. Kovvali, L. Prakash, S. Prakash, Requirement of the yeast RTH1 5′ to 3′ exonuclease for the stability of simple repetitive DNA, Science 269 (1995) 238–239.
[9] D.X. Tishkoff, N. Filosi, G.M. Gaida, R.D. Kolodner, A novel mutation avoidance mechanism dependent on *S. cerevisiae* RAD27 is distinct from DNA mismatch repair, Cell 88 (1997) 253–263.
[10] C. Schmutte, R.C. Marinescu, M.M. Sadoff, S. Guerrette, J. Overhauser, R. Fishel, Human exonuclease 1 interacts with the mismatch repair protein hMSH2, Cancer Res. 58 (1998) 4537–4542.
[11] D.X. Tishkoff, N.S. Amin, C.S. Viars, K.C. Arden, R.D. Kolodner, Identification of a human gene encoding a homologue of *Saccharomyces cerevisiae EXO1*, an exonuclease implicated in mismatch repair and recombination, Cancer Res. 58 (1998) 5027–5031.
[12] D.M. Wilson III, J.P. Carney, M.A. Coleman, A.W. Adamson, M. Christensen, J.E. Lamerdin, Hex1: a new human Rad2 nuclease family member with homology to yeast exonuclease 1, Nucleic Acids Res. 26 (1998) 3762–3768.
[13] R.E. Johnson, G.K. Kovvali, S.N. Guzder, N.S. Amin, C. Holm, Y. Habraken, P. Sung, L. Prakash, S. Prakash, Evidence for involvement of yeast proliferating cell nuclear antigen in DNA mismatch repair, J. Biol. Chem. 271 (1996) 27987–27990.
[14] Y.-L. Lin, M.K.K. Shivji, C. Chen, R. Kolodner, R.D. Woods, A. Dutta, The evolutionarily conserved zinc finger motif in the largest subunit of human replication protein A is required for DNA replication and mismatch repair but not for nucleotide excision repair, J. Biol. Chem. 273 (1998) 1453–1461.
[15] A. Umar, A.B. Buermeyer, J.A. Simon, D.C. Thomas, A.B. Clark, R.M. Liskay, T.A. Kunkel, Requirement for PCNA in DNA mismatch repair at a step preceding DNA resynthesis, Cell 87 (1996) 65–73.
[16] D.X. Tishkoff, A.L. Boerger, P. Bertrand, N. Filosi, G.M. Gaida, M.F. Kane, R.D. Kolodner, Identification and characterization of *Saccharomyces cerevisiae EXO1*, a gene encoding an exonuclease that interacts with MSH2, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 7487–7492.
[17] P. Bertrand, D.X. Tishkoff, N. Filosi, R. Dasgupta, R.D. Kolodner, Physical interaction between components of DNA mismatch repair and nucleotide excision repair, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 14278–14283.
[18] D. Melton, A.M. Ketchen, F. Nunez, S. Bonatti-Abbondandolo, A. Abbondandolo, S. Squires, R. Johnson, Cells from ERCC1-deficient mice show increased genome instability and a reduced frequency of S-phase-dependent illegitimate chromosome exchange but a normal frequency of homologous recombination, J. Cell Sci. 111 (1998) 395–404.
[19] G. Weeda, I. Donker, J. de Witt, H. Morreau, R. Janssens, C.J. Vissers, A. Nigg, H. van Steeg, D. Bootsma, H. Hoeijmakers, Disruption of mouse *ERCC1* results in a novel repair syndrome with growth failure, nuclear abnormalities and senescence, Curr. Biol. 7 (1997) 427–439.
[20] N. de Wind, M. Dekker, A. Berns, M. Radman, H. te Riele, Inactivation of the mouse *Msh2* gene results in mismatch repair deficiency, methylation tolerance, hyperrecombination, and predisposition to cancer, Cell 82 (1995) 321–330.
[21] F. Palombo, P. Gallinari, I. Iaccarino, T. Lettieri, M. Hughes, A. D'Arrigo, O. Truong, J.J. Hsuan, J. Jiricny, GTBP, a 160-kilodalton protein essential for mismatch-binding activity in human cells, Science 268 (1995) 1912–1914.
[22] J.W. Harper, G.R. Adami, N. Wei, K. Keyomarski, S.J. Elledge, The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases, Cell 75 (1993) 805–816.
[23] M.D. Rose, F. Winston, P. Hieter, Methods in Yeast Genetics: A Laboratory Course Manual, Cold Spring Harbor Lab. Press, Plainview, NY, 1990.
[24] S. Guerrette, T. Wilson, S. Gradia, R. Fishel, Interaction of human hMSH2 with hMSH3 and hMSH2 with hMSH6: explanation of mutations found in hereditary nonpolyposis colorectal cancer, Mol. Cell. Biol. 18 (1998) 6616–6623.
[25] F.A. Digilio, A. Pannuti, J.C. Lucchesi, M. Furia, L.C. Polito, Tosca: a Drosophila gene encoding a nuclease specifically expressed in the female germline, Dev. Biol. 178 (1996) 90–100.
[26] P. Fiorentini, K.N. Huang, D.X. Tishkoff, R.D. Kolodner, L.S. Symmington, Exonuclease 1 of *Saccharomyces cerevisiae* functions in mitotic recombination in vivo and in vitro, Mol. Cell. Biol. 17 (1997) 2764–2773.
[27] F.S. Leach, K. Polyak, M. Burrell, K.A. Johnson, D. Hill, M.G. Dunlop, A.H. Wyllie, P. Peltomäki, A. de la Chapelle, S.R. Hamilton, K.W. Kinzler, B. Vogelstein, Expression of the human mismatch repair gene *hMSH2* in normal and neoplastic tissues, Cancer Res. 56 (1996) 235–240.
[28] G. Marra, C.L. Chang, L.A. Laghi, D.P. Chauhan, D. Young, C.R. Boland, Expression of human MutS homolog 2 (hMSH2) protein in resting and proliferating cells, Oncogene 13 (1996) 2189–2196.
[29] T.M. Wilson, A. Ewel, J.R. Duguid, J.N. Eble, M.K. Lescoe, R. Fishel, M.R. Kelley, Differential cellular expression of the human MSH2 repair enzyme in small and large intestine, Cancer Res. 55 (1995) 5146–5150.
[30] S.M. Baker, C.E. Bronner, L. Zhang, A.W. Plug, M. Robatzek, G. Warren, E.A. Elliott, J. Yu, T. Ashley, N. Arnheim, R.A. Flavell, R.M. Liskay, Male mice defective in the DNA mismatch repair gene PMS2 exhibit abnormal chromosome synapsis in meiosis, Cell 82 (1995) 309–319.
[31] S.M. Baker, A.M. Plug, T.A. Prolla, C.E. Bronner, A.C. Harris, X. Yao, D.-M. Christie, C. Monell, N. Arnheim, A.

Bradley, T. Ashley, R.M. Liskay, Involvement of mouse Mlh1 in DNA mismatch repair and meiotic crossing over, Nat. Genet. 13 (1996) 336–342.

[32] W. Edelmann, P.E. Cohen, M. Kane, K. Lau, B. Morrow, S. Bennett, A. Umar, T. Kunkel, G. Cattoretti, R. Chaganti, J.W. Pollard, R.D. Kolodner, R. Kucherlapati, Meiotic pachytene arrest in *MLH1*-deficient mice, Cell 85 (1996) 1125–1134.

[33] T.A. Prolla, S.M. Baker, A.C. Harris, J.-L. Tsao, X. Yao, C.E. Bronner, B. Zheng, M. Gordon, J. Reneker, N. Arnheim, D. Shibata, A. Bradley, R.M. Liskay, Tumour susceptibility and spontaneous mutation in mice deficient in Mlh1, Pms1 and Pms2 DNA mismatch repair, Nat. Genet. 18 (1998) 276–279.

[34] A.H. Reitmair, R. Schmits, A. Ewel, B. Bapat, M. Redston, A. Mitri, P. Waterhouse, H.-W. Mittrücker, A. Wakeham, B. Liu, A. Thomason, H. Griesser, S. Gallinger, W.G. Ballhausen, R. Fishel, T.W. Mak, *MSH2* deficient mice are viable and susceptible to lymphoid tumours, Nat. Genet. 11 (1995) 64–70.

[35] A.H. Reitmair, M. Redston, J.C. Cai, T.C.Y. Chuang, M. Bjerknes, H. Cheng, K. Hay, S. Gallinger, B. Bapat, T.W. Mak, Spontaneous intestinal carcinomas and skin neoplasms in Msh2-deficient mice, Cancer Res. 56 (1996) 3842–3849.

[36] B.-I. Lee, M. Shannon, L. Stubbs, D.M. Wilson III, Expression specificity of the mouse exonuclease 1 (*mExo1*) gene, Nucleic Acids Res. 27 (1999) 4114–4120.

Manuscript II

Anne Karin Rasmussen, Keshav K. Singh, and Lene Juel Rasmussen. Characterization of O^6-MeG DNA Methyltransferase (MGMT) protein in repairing human mitochondrial DNA. **Manuscript in preparation.**

Characterization of O^6-MeG DNA Methyltransferase (MGMT) protein in repairing human mitochondrial DNA

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Abstract

DNA repair is essential for maintaining the integrity of the genetic material. A number of DNA repair mechanisms have been well characterized for the nuclear DNA. However, little is known about DNA repair in mitochondria. Mutations in mitochondrial DNA (mtDNA) are involved in the pathogenesis of a variety of diseases including cancer, heart disease, diabetes, and a number of degenerative diseases. Furthermore, accumulation of mutations in mtDNA is a common feature of aging cells. Human mtDNA is extremely rich in guanine, therefore, repair of modified guanine is important for proper mitochondrial function. Certain intracellular or exogenous DNA damaging agents are known to frequently modify guanine to O^6-MeG in DNA. Generation of O^6-MeG is mutagenic because it can mispair with thymine resulting in G:C $\rightarrow$ A:T transition mutations. The DNA repair protein O^6-Methylguanine-DNA Methyltransferase (MGMT) removes methyl groups from the O^6 position of guanine (O^6-MeG) in the nuclear DNA. In order to gain understanding of the role of MGMT in mitochondria, we investigated (1) whether MGMT contains mitochondrial localization signals and is transported to mitochondria, (2) whether MGMT, when expressed in mitochondria can repair O^6-MeG DNA lesions in this organelle, and (3) whether MGMT when expressed in mitochondria can protect cells from apoptosis induced by the DNA damaging agents which modify guanine in mtDNA.

Introduction

Mammalian cells contain two distinct genomes: a nuclear (nDNA) and a mitochondrial (mtDNA). Despite each human somatic cell contains 100 to 1000 mitochondria, that each contains 1 to 10 mitochondrial genomes, the amount of the mtDNA is only estimated to account for 1% of total DNA in the cell (Wallace *et al*., 1998; Bestwick, 1982).

In contrast to nDNA the mammalian mitochondrial genome contains few noncoding sequences, no introns and it is not protected by histones. Thus, damage to mtDNA is expected to have great impact on the function of this organelle. Accordingly, it has been reported that the rate of point mutations is elevated in mtDNA compared to nDNA (Khrapko *et al*., 1997). Point mutations in and genomic rearrangements of mtDNA have been recognized to play a critical role in numerous human disorders underscoring the importance of maintaining the integrity of the mitochondrial genome (Pulkes and Hanna, 2001; Wallace, 1999; Kogelnik *et al*., 1998[3]).

Given the importance of maintaining the mitochondrial genome intact for organelle activity, efficient DNA repair systems in mitochondria would be expected. Even so, early investigations showed that UV-induced pyrimidine dimers are not removed from mtDNA, a result that led to the conclusion that mitochondria are deficient in DNA repair (Clayton *et al*., 1974; Prakash *et al*., 1975). These findings encouraged the model that rather than repairing DNA damage in mtDNA, cells simply eliminate damaged genomes and replace them by replicating existing undamaged mitochondrial genomes (LeDoux *et al*., 1999). It was later confirmed that UV-induced pyrimidine dimers are not repaired in mitochondria (LeDoux *et al*., 1992; Pascucci *et al*., 1997) suggesting that mitochondria do not have a functional nucleotide excision repair (NER) pathway. However, recent research has shown that at least base excision repair (BER) activities are operational in mitochondria showing that this organelle does indeed repair damaged mtDNA (Croteu *et al*., 1999; Sawyer *et al*., 1999). But the knowledge about the mechanisms underlying DNA repair in the mitochondria is still limited.

DNA repair alkyltransferases, responsible for removing methyl groups from the O^6 position of guanine, have been identified in both prokaryotic and eukaryotic organisms. The genes coding for these DNA repair alkyltransferases have been cloned from *Escherichia coli* (***ada***, Sedgwick, 1983; ***ogt***, Potter *et al*., 1987), *Saccharomyces cerevisiae* (***MGT1***,

Xiao *et al*., 1991), mouse (***MGMT***, Shiota *et al*., 1992), rat (***MGMT***, Rahden-Staron and Laval, 1991) and human (***MGMT***, Tano *et al*., 1990; Rydberg *et al*., 1990; Hayakawa *et al*., 1990). To date most research has focused on the role of DNA repair alkyltransferases in repair of nDNA but mitochondrial MGMT activity has been reported in rat liver (Myers *et al*., 1988; Satoh *et al*., 1988).

A mitochondrial DNA repair alkyltransferase has been partially purified from rat liver mitochondria and it was shown that this protein is similar in size to the 23 kDa DNA repair protein found in nuclei (Myers *et al*., 1988). DNA damage caused by the methylating agent *N*-methyl-*N*-nitrosourea (MNU) is repaired in mitochondria from Chinese Hamster Ovary (CHO) cells (LeDoux *et al*., 1992) suggesting that an alkyltransferase repair mechanism is active in mammalian mitochondria. However, mitochondrial MGMT activity has not been identified in human cells and the intracellular localization seems ambiguous.

To gain a better understanding of the molecular processes and components responsible for DNA repair of methylation damage in the human mitochondrion we have investigated the localization of human MGMT. We found that full-length MGMT localized to the nucleus when expressed as a GFP fusion protein. This nuclear localization was independent of p53 status and we did not observe any MGMT protein in mitochondria in cells treated with MNNG. Our results suggest that the N-terminal region (codons 2-18) is required for directing the MGMT protein to nucleus. Expression of the MGMT protein lacking codons 2-18 in the mitochondria protected the cells from the cytotoxic effect of MNNG but had no effect on survival of cells treated with the oxidizing agent Menadione.

[3]Variations in the human mitochondrial genomes are updated on http://www.gen.emory.edu/mitomap.html (Kogelnik *et al*., 1998).

Materials & Methods

Cell Cultures

Human MCF12A (breast, mammary gland, normal, non-tumorigenic epithelial) cells were obtained from ATCC. MCF12A cells were maintained in DMEM/F12 growth medium (Gibco/Life Technologies) supplemented with 10% Horse Serum (Gibco/Life Technologies), 1% Penicillin-Streptomycin (Gibco/Life Technologies), 100 ng/ml Cholera Toxin (Sigma), 500 ng/ml Hydrocortisone (Sigma), 10 μg/ml Insulin (Sigma), and 20 ng/ml EGF (Epidermal Growth Factor, PeproTech) at 37^{o}C in a humidified 95% air-5% CO_2 atmosphere. HCT116 p53-/- (colorectal carcinoma) human cells were obtained from Dr. Bert Vogelstein (Johns Hopkins University) and maintained in McCoy's 5A medium (Gibco/Life Technologies) supplemented with 10% fetal bovine serum (Gibco/Life Technologies), 1% Penicillin-Streptomycin (Gibco/Life Technologies) at 37^{o}C in a humidified 95% air-5% CO_2 atmosphere. HeLa (HSL2, cervical carcinoma) cells were maintained as previously described (Singh *et al*., 1999) in DMEM (Gibco/Life Technologies) supplemented with 10% FBS (Gibco/Life Technologies), 1% Penicillin-Streptomycin (Gibco/Life Technologies) and 4 ng/ml uridine (Gibco/Life Technologies) at 37^{o}C in a humidified 95% air-5% CO_2 atmosphere.

Construction of plasmids

A DNA sequence of *MGMT* lacking the terminal 23 amino acids (codon 185 to 207) was cloned into the *Eco47*III and *Sac*I sites of pEGFP-N2 (Clontech). Oligonucleotides were used to PCR amplify a MGMT fragment (ΔMGMT) lacking codons 2 to 18 and 185 to 207. The 5' primer: $^{5'}$GA**AGATCT**ATGCTGGAG CTGTCTGGTT$^{3'}$ contains a *Bgl*II site and the 3' primer, $^{5'}$TT**GAGCTC** CCTCCCAAGCCTG$^{3'}$ contains a *Sac*I site. The ΔMGMT fragment was cloned into the *Bgl*II and *Sac*I sites of the pEGFP-N2 vector. The pShooter vectors pEF/myc/mito and pEF/myc/mito/GFP (control) (Invitrogen) containing the mitochondrial targeting sequence (MSVLTPLLLRGLTGSARRLPVPRAKIHSL) are designed to express and target a recombinant protein to the mitochondria in mammalian cells. Plasmid pEF/myc/mito-MGMT plasmid was constructed by inserting the complete coding sequence of human *MGMT* into the *Xho*I and *Not*I sites of pEF/myc/mito. The fragment containing full-length MGMT was amplified by PCR using the primers: (5' primer) $^{5'}$CCG**CTCGAG**ATGGACAAGGATTGTGA$^{3'}$ containing a *Xho*I site and (3'

primer) $^{5'}$AA**GCGGCCGC**TCAGTTTCGGCCAGCA$^{3'}$ containing a *Not*I site. The DNA sequences of all constructs were confirmed by DNA sequencing.

Transfection

Cell cultures were transfected using either Lipofectamine2000 or Lipofectin (Gibco/LifeTechnologies) according to manufacturer's guidelines. For fluorescence microscopy exponentially growing cells were counted and diluted, and 3 x 10^5 cells were seeded in 2 ml culture medium in glass bottom dishes (BioSoft International, Amsterdam, The Netherlands) one day prior to treatment. On the day of treatment cells were transfected with 3.5 μg DNA of each construct and incubated at 37^{o}C in a humidified 95% air-5% CO_2 atmosphere for 24 hours before they were assayed for MGMT-GFP expression. Alternatively, exponentially growing cells were counted and diluted, and 7 x 10^4 cells were seeded in 2 ml culture medium without serum in tissue culture dishes containing coverslips one day prior to treatment. On the day of treatment cells were transfected with 2 μg DNA and incubated at 37^{o}C in a humidified 95% air-10% CO_2 atmosphere for 48 hours before they were assayed. In these experiments the growth medium was changed every 18 hours.

MitoTracker staining

For detection of mitochondria, MitoTracker Red (CMXros, Molecular Probes, Eugene, OR) was added to the growth medium of the transfected cells. Cells grown in glass bottom dishes were stained with 500 nM of MitoTracker Red for 20 min at 37^{o}C in a humidified 95% air-5% CO_2 atmosphere. Cells grown on coverslips were stained with 50 nM of MitoTracker Red for 30 min in a humidified 95% air-5% CO_2 atmosphere before fixation with 3.7% formaldehyde in Hank's balanced salt solution. Cells were treated with 20 μM MNNG (*N*-methyl-*N*'-nitro-*N*-nitrosoguanidine) (Sigma) for 2 or 3 hours. After 1 hour and 45 min of MNNG treatment the growth medium was changed to medium containing both MNNG and MitoTracker Red and incubated further. The cells were visualized using a LSM 510 confocal laser scanning microscope (Zeiss) or a Zeiss-Axiovert 135 TY Inverted microscope equipped with PXL camera (SENSYS Phptometrics).

Cell proliferation assay

The Cell Titer 96TN AQueous nonradioactive cell proliferation assay kit (Promega) was used to assay cell survival. Exponentially growing cells were counted, diluted, and seeded

in 100 μl of culture medium in 96-well microtiter plates at a cell density of 1 x 10^4 cell/well one day prior to treatment. On the day of treatment cells were transfected with 0.32 μg DNA/well in 125 μl growth medium Cells were transfected with either pEF/myc/mito/GFP (control), pEF/myc/mito-MGMT, pEGFPN2 (control), MGMT-GFP or ΔMGMT-GFP. Cells were incubated for 2-3 days at 37^{o}C in a humidified 95% air-5% CO_2 atmosphere before treatment with 0, 13.6, 54.4, 108.8, and 217.6 μM MNNG or 100, 400 or 1000 μM Menadione (Sigma). Cells were incubated for 3 days and on the third day cell survival was evaluated by the cell proliferation assay according to manufacturer's guidelines. The data represent an average of two experiments where each data point represents an average of ten independent measurements.

Results

The human MGMT protein localizes to nucleus

DNA repair alkyltransferases have been identified in both nuclear and cytoplasmic fractions of eukaryotic cells (Ayi *et al*., 1992; Lee *et al*., 1992; Brent *et al*., 1993; Ishibashi *et al*., 1994a,b; Lim and Li, 1996). Cytoplasmic appearance of MGMT indicates that this repair protein could be present in mitochondria. To access the actual localization of the MGMT protein we studied exogenous MGMT-GFP expressed as fusion protein in human breast cell line MCF12A and detected the subcellular localization by fluorescence microscopy. We found that MGMT protein is mainly localized in the nucleus (Figure 2), which is supported by most of the immunostaining analysis reported (Ayi *et al*., 1992, Lim and Li, 1996; Belanich *et al*., 1996). However, we observed no mitochondrial localization concluded from the results of Mito Tracker Red staining of the cells (Figure 2). We did not observe any difference in subcellular localization of MGMT when we compared fixed cells (Figure 2B) with viable cells (Figure 2C). Our control experiment expressing the GFP alone showed the expected diffuse localization (Figure 2A). These results reveal that the human MGMT, when expressed as a GFP fusion protein, localizes in the nucleus. The results presented in Figure 2 also show that the codons 184 to 207 of the C-terminal part of MGMT is not required for proper nuclear localization of the protein as these were deleted in our construct.

We deleted codons 2 to 18 of MGMT in order to investigate the importance of this region in subcellular localization of MGMT. The ΔMGMT-GFP construct, lacking codons 2 to 18 of the N-terminal region and codons 184 to 207 of the C-terminal region of MGMT, was exogenously expressed in MCF12A cells. Interestingly, we found that deletion of codons 2 to 18 resulted in weak nucleus staining and an accumulation of ΔMGMT in cytoplasm (Figure 2D). We observed weak fluorescence with several fluorescence forming dots within the cytosol. However, the granular dots was not identical with the mitochondrial Mito Tracker Red staining, although we can not exclude that any cytoplasmic ΔMGMT protein is located in mitochondria because of the uniform weak fluorescence around the granular dots (Figure 2D). Our data suggest the N-terminal domain or more specifically codons 2 to 18 are essential for proper nuclear localization of MGMT.

The localization of MGMT is independent of p53 status

The p53 protein has been detected inside the mitochondria but the majority of mitochondrial-associated p53 is found on the surface of this organelle (Sansome *et al*., 2001). Furthermore, the p53 protein has been shown to reduce transcription of the *MGMT* gene in human fibroblasts (Harris *et al*. 1996). Both p53 and MGMT play roles in DNA damage recognition and repair. The p53 protein is believed to detect DNA damage and activate one or more cell cycle checkpoints that allow repair of damaged DNA. In contrast, there has been no evidence so far that MGMT is involved in activation of cell cycle checkpoints but a role in repair of DNA damage is well established. Previous studies by Balanich *et al*. (1996) have shown that MGMT in HT29 colon cancer cells were localized to nucleus and cytoplasm (cell lines defective in p53, Gayet *et al*., 2001), indicating that MGMT could be present in nucleus, cytoplasm and mitochondria in p53 defective cells. In order to explore a possible link between p53 and MGMT we expressed the MGMT-GFP fusion protein in two cell lines differing in p53 status. One cell line is completely deficient in p53 activity (HCT116 p53-/-) and the other expresses very low levels of the p53 protein (HeLa HSL2). We found no difference in subcellular localization of MGMT (Figure 3) suggesting that MGMT translocation is independent of the p53 status. In both cell lines the MGMT-GFP fusion protein was exclusively present in the nucleus.

Alkylating agents have no effect on the localization of MGMT

It has been shown that mtDNA is damaged by alkylating agents and that O^6Me-G DNA lesions are repaired in mitochondria (Satoh *et al*., 1988; LeDoux *et al*., 1992). However, our data suggest that MGMT locates to the nucleus and no MGMT protein is detected in mitochondria of untreated cells (Figure 2). In order to test if nuclear MGMT is recruited to mitochondria upon DNA damage of mtDNA by alkylating agents we treated cells expressing MGMT-GFP with MNNG. MCF12A cells were treated with 20 μM MNNG for 3 hours and cells were analyzed by fluorescence microscopy. The MGMT-GFP fusion protein was detected in nucleus but not in mitochondria (Figure 4) indicating that nuclear MGMT is not recruited to mitochondria after DNA damage by MNNG.

Effect of MGMT expression in protecting cells from alkylating agents

Research from other groups has shown that O^6-Me-G and O^6-Et-G lesions are repaired in mitochondria (Satoh *et al*., 1988; LeDoux *et al*., 1992). Our data suggest that MGMT does

not play a role in repair of O^6Me-G DNA lesions in mitochondria. We constructed a set of plasmids in order to (1) investigate the cytotoxic effect of alkylation damage in cells expressing MGMT in mitochondria and (2) investigate the role of the cytoplasmic ΔMGMT protein in protecting cells after alkylation damage. MCF12A cells were transfected with the pEF/myc/mito-MGMT construct, which directs the fusion protein to mitochondria, as well as the pEF/myc/ΔMGMT-GFP and pEF/myc/MGMT-GFP constructs. Surprisingly, expression of mito-MGMT increased sensitivity to killing by MNNG whereas expression of ΔMGMT-GFP provided the same level of protection to killing by MNNG as MGMT-GFP (Figure 5). We also treated cells with Menadione, which introduce oxidative damage to DNA. These lesions are not repaired by MGMT serving as a control for the effects on cell survival observed after transfection with the plasmids and MNNG treatment are specific for alkylation damage. We did not observe any difference in cell survival after treatment with Menadione (Figure 6). Our results indicate that the presence of MGMT in mitochondria increases sensitivity to MNNG. Conversely the cytoplasmic ΔMGMT protein protects cells from killing by MNNG at high doses.

Discussion

MGMT is a small protein of 207 amino acids and a molecular weight of 21.7 kDa (Hayakawa *et al*., 1990; Rydberg *et al*., 1990). It has been proposed that small non-nuclear proteins, with a molecular weight less than 40-60 kDa can passively equilibrate between the cytosol and the nucleus (Weis, 1998). The small size of the MGMT protein and its DNA binding property may allow apparent mobilization to nuclear DNA by passive diffusion and DNA binding. However, when bacterial methyltransferase (*ada;* 39 kDa) is expressed in mammalian cells it fails to accumulate in the nucleus of NIH-3T3 cells (Dumenco *et al*., 1989). Therefore, it seems unlikely that the "diffusion" model of the entry into the nucleus for this protein operates. It has been suggested that the sequence -**K**LL**K**VV**K**- (codons 101-107) shares some homology to the nuclear location signal PKKKRKV (Ayi *et al*., 1992; Weis, 1998). However, mutants of this basic –KLLKVVK- region of MGMT were DNA binding and repair deficient but entirely nuclear (Lim and Li, 1996). MGMT expressed as β-gal fusion protein, which is too large (130 kDa) for the passive diffusion into the nucleus, was shown to be present in the nucleus (Lim and Li, 1996). We show that a MGMT-GFP fusion protein localizes to the nucleus suggesting that MGMT can target the GFP into the nucleus. We also show that the MGMT protein does not localize to mitochondria when fused to GFP.

Comparison of the structures and sequences of bacterial (Ada) with the human MGMT proteins reveals that the similarity between these proteins is highest in the C-terminal region (MGMT amino acids 92 to 174 and Ada amino acids 94 to 175)(Wibley *et al*., 2000). A divergent N-terminal region could therefore explain nuclear-targeting differences between Ada and MGMT. It has been suggested that the residues Glu45 to Gly55 form a "hydrophobic handle" that may serve to anchor MGMT to other proteins (Wibley *et al*., 2000). The conformation of residues Glu45 to Gly55 is stabilized by the first β-strand (residues Lys8 to Ser14) in MGMT. We showed that a deletion of the first 18 amino acids is sufficient to keep MGMT in the cytoplasm. Mutant proteins of MGMT carrying deletions of either codons 1 to 10 or 1 to 19 were active in protecting cells from killing by MNNG (Crone *et al*., 1996). These results suggest that these mutant proteins are active in repair of alkylation DNA damage *in vivo*. However, it was shown that the mutant MGMT proteins were less effective in repair compared to wild type MGMT as well as highly unstable with half-lives of 48-90 min compared to a half-live of > 720 min for the

wild type MGMT. We found that the ΔMGMT-GFP construct lacking codons 2 to 18 of the N-terminus resulted in weak fluorescence from nuclear and several bright granular dots in the cytosol, indicating degradation in lysosome and therefore a shorter half-live than MGMT-GFP. Interestingly, it has been shown that both N- and C-terminal deletions of the MGMT protein (deletion of codons: 1-30; 1-60; 1-90; 1-120; 1-150; 1-180, 165-180; 59-207 or 31-207) affect the nuclear localization of MGMT (Lim and Li, 1996). In summary these observations indicate that the structure of MGMT is of great importance for subcellular localization.

We re-evaluated possible targeting signal for nucleus and mitochondria by using the PredictNLS, MitoProt and PSORT software programs[4]. PredictNLS finds experimentally known nuclear localization signals in a given protein. MitoProt calculates the N-terminal protein region that can support a Mitochondrial Targeting Sequence (MTS) and the cleavage site (Claros and Vincens, 1996) and PSORT finds both MTS and NLS sequences (Nakai *et al*., 1992). Neither of these programs identified any nuclear localization signal or Mitochondrial Targeting Sequence (MTS) in the MGMT protein (data not shown).

The majority of proteins, which are synthesized in the cytoplasm and imported to the mitochondria, contain a MTS in the N-terminal region of the protein. The sequence is diverse in both length (20 to 60 amino acid residues) and composition. However, the major feature appears to be residues with abundant positive charges, very little if any negative charges, and frequent hydroxylated residues which can form an amphipathic a-helix (Neupert, 1997). A fraction of mitochondrial proteins lacks this targeting sequence, for instance, APE1 which has been shown to locate to rat mitochondria (Tell *et al*., 2001). In fact a large number of outer and inner membrane proteins, as well as proteins that reside in the intermembrane space, have internal targeting signals and no MTS (Neupert, 1997). The conclusion of the re-evaluation of possible targeting signals is, therefore, that MGMT seems to lack both MTS and experimentally known nuclear signals.

Overexpression of the simple glycosylase MPG (AAG) increases sensitivity to alkylating agents as a result of accumulation of cytotoxic AP sites (Limp-Foster and Kelley, 2000; Coquerelle *et al*., 1995). This is supported by the finding that overexpression of MPG in the human breast carcinoma cells does not alter the activities of the proteins immediately downstream in the BER pathway (i.e. APE1) (Kreklau *et al*., 2001). Increased sensitivity to alkylating agents is also observed when MPG is overexpressed in the mitochondria

[4] PredictNLS (http://cubic.bioc.columbia.edu/predictNLS) & MitoProt (www.mips.biochem.mpg.de/cgi-bin/proj/medgen/mitofilter) & PSORT (http://psort.nibb.ac.jp)

(personal communication with Dr. Mark R. Kelley, Department of Pediatrics - Hematology/Oncology, Indiana University School of Medicine, Indianapolis, IN, USA, submitted to JBC). It was concluded that overexpression of MPG results in imbalance of the proteins involved in BER. MGMT is a direct repair protein and it is unlikely to cause imbalance in downstream components of a pathway. However, it has been suggested that MGMT binds to O^4-MeT DNA lesions, but the repair is slow, resulting in hindrance of NER of this lesion (Samson *et al*., 1997). This could explain the increased sensitivity to MNNG treatment in cells overexpressing the mito-MGMT protein in mitochondria (Figure 6). However; so far no NER activity has been identified in mitochondria. Alternatively, MGMT blocks the mitochondrial DNA replication by binding to O^4-MeT DNA lesions.

In summary, we have shown that MGMT when fused to GFP does not localize to mitochondria but to the nucleus. This nuclear localization is independent of the p53 status of the cells. We show that deletion of the first 18 amino acids is sufficient to keep MGMT in the cytoplasm and overexpression of MGMT in mitochondria increases sensitivity to the alkylating agent MNNG.

References

Ayi TC, Loh KC, Ali RB, Li BF. Intracellular localization of human DNA repair enzyme methylguanine-DNA methyltransferase by antibodies and its importance. Cancer Res. 1992;52(23):6423-30.

Belanich M, Randall T, Pastor MA, Kibitel JT, Alas LG, Dolan ME, Schold SC Jr, Gander M, Lejeune FJ, Li BF, White AB, Wasserman P, Citron ML, Yarosh DB. Intracellular Localization and intercellular heterogeneity of the human DNA repair protein O(6)-methylguanine-DNA methyltransferase. Cancer Chemother Pharmacol. 1996;37(6):547-55.

Bestwick RK, Moffett GL, Mathews CK. Selective expansion of mitochondrial nucleoside triphosphate pools in antimetabolite-treated HeLa cells. J Biol Chem. 1982;257(16):9300-4.

Brent TP, von Wronski MA, Edwards CC, Bromley M, Margison GP, Rafferty JA, Pegram CN, Bigner DD. Identification of nitrosourea-resistant human rhabdomyosarcomas by *in situ* immunostaining of O^6-methylguanine-DNA methyltransferase. Oncol Res. 1993;5(2):83-6.

Claros MG, Vincens P. Computational method to predict mitochondrially imported proteins and their targeting sequences. Eur J Biochem. 1996;241(3):779-86.

Clayton DA, Doda JN, Friedberg EC. The absence of a pyrimidine dimer repair mechanism in mammalian mitochondria. Proc Natl Acad Sci U S A. 1974;71(7):2777-81.

Coquerelle T, Dosch J, Kaina B. Overexpression of N-methylpurine-DNA glycosylase in Chinese hamster ovary cells renders them more sensitive to the production of chromosomal aberrations by methylating agents--a case of imbalanced DNA repair. Mutat Res. 1995;336(1):9-17.

Crone TM, Goodtzova K, Pegg AE. Amino acid residues affecting the activity and stability of human O^6-alkylguanine-DNA alkyltransferase. Mutat Res. 1996;363(1):15-25.

Croteau DL, Stierum RH, Bohr VA. Mitochondrial DNA repair pathways. Mutat Res. 1999;434(3):137-48. Review.

Dumenco LL, Warman B, Hatzoglou M, Lim IK, Abboud SL, Gerson SL. Increase in nitrosourea resistance in mammalian cells by retrovirally mediated gene transfer of bacterial O^6-alkylguanine-DNA alkyltransferase. Cancer Res. 1989;49(21):6044-51.

Gayet J, Zhou XP, Duval A, Rolland S, Hoang JM, Cottu P, Hamelin R.
Extensive characterization of genetic alterations in a series of human colorectal cancer cell lines. Oncogene. 2001;20(36):5025-32.

Harris LC, Remack JS, Houghton PJ, Brent TP. Wild-type p53 suppresses transcription of the human O^6-methylguanine-DNA methyltransferase gene. Cancer Res. 1996;56(9):2029-32.

Hayakawa H, Koike G, Sekiguchi M. Expression and cloning of complementary DNA for a human enzyme that repairs O^6-methylguanine in DNA. J Mol Biol. 1990;213(4):739-47.

Ishibashi T, Nakabeppu Y, Sekiguchi M. Artificial control of nuclear translocation of DNA repair methyltransferase. J Biol Chem. 1994;269(10):7645-50.

Ishibashi T, Nakabeppu Y, Kawate H, Sakumi K, Hayakawa H, Sekiguchi M. Intracellular localization and function of DNA repair methyltransferase in human cells. Mutat Res. 1994;315(3):199-212.

Khrapko K, Coller H, Andre P, Li XC, Foret F, Belenky A, Karger BL, Thilly WG. Mutational spectrometry without phenotypic selection: human mitochondrial DNA. Nucleic Acids Res. 1997;25(4):685-93.

Kogelnik AM, Lott MT, Brown MD, Navathe SB, Wallace DC. MITOMAP: a human mitochondrial genome database--1998 update. Nucleic Acids Res. 1998;26(1):112-5.

Kreklau EL, Limp-Foster M, Liu N, Xu Y, Kelley MR, Erickson LC. A novel fluorometric oligonucleotide assay to measure O(6)-methylguanine DNA methyltransferase, methylpurine DNA glycosylase, 8-oxoguanine DNA glycosylase and abasic endonuclease activities: DNA repair status in human breast carcinoma cells overexpressing methylpurine DNA glycosylase. Nucleic Acids Res. 2001;29(12):2558-66

LeDoux SP, Wilson GL, Beecham EJ, Stevnsner T, Wassermann K, Bohr VA. Repair of mitochondrial DNA after various types of DNA damage in Chinese hamster ovary cells. Carcinogenesis. 1992;13(11):1967-73.

LeDoux SP, Driggers WJ, Hollensworth BS, Wilson GL. Repair of alkylation and oxidative damage in mitochondrial DNA. Mutat Res. 1999;434(3):149-59. Review.

Lee SM, Rafferty JA, Elder RH, Fan CY, Bromley M, Harris M, Thatcher N, Potter PM, Altermatt HJ, Perinat-Frey T, et al. Immunohistological examination of the inter- and intracellular distribution of O^6-alkylguanine DNA-alkyltransferase in human liver and melanoma. Br J Cancer. 1992;66(2):355-60.

Lim A, Li BF. The nuclear targeting and nuclear retention properties of a human DNA repair protein O^6-methylguanine-DNA methyltransferase are both required for its nuclear localization: the possible implications. EMBO J. 1996;15(15):4050-60.

Limp-Foster M, Kelley MR. DNA repair and gene therapy: implications for translational uses. Environ Mol Mutagen. 2000;35(2):71-81. Review.

Myers KA, Saffhill R, O'Connor PJ. Repair of alkylated purines in the hepatic DNA of mitochondria and nuclei in the rat. Carcinogenesis. 1988;9(2):285-92.

Nakai K, Kanehisa M. A knowledge base for predicting protein localization sites in eukaryotic cells, Genomics. 1992; 14, 897-911.

Neupert W. Protein import into mitochondria. Annu Rev Biochem. 1997;66:863-917. Review.

Pascucci B, Versteegh A, van Hoffen A, van Zeeland AA, Mullenders LH, Dogliotti E. DNA repair of UV photoproducts and mutagenesis in human mitochondrial DNA. J Mol Biol. 1997;273(2):417-27.

Potter PM, Wilkinson MC, Fitton J, Carr FJ, Brennand J, Cooper DP, Margison GP. Characterisation and nucleotide sequence of *ogt*, the O^6-alkylguanine-DNA-alkyltransferase gene of *E. coli*. Nucleic Acids Res. 1987;15(22):9177-93.

Prakash L. Repair of pyrimidine dimers in nuclear and mitochondrial DNA of yeast irradiated with low doses of ultraviolet light. J Mol Biol. 1975;98(4):781-95.

Pulkes T, Hanna MG. Human mitochondrial DNA diseases. Adv Drug Deliv Rev. 2001;49(1-2):27-43. Review.

Rahden-Staron I, Laval F. cDNA cloning of the rat O^6-methylguanine-DNA-methyltransferase. Biochem Biophys Res Commun. 1991;177(2):597-602.

Rydberg B, Spurr N, Karran P. cDNA cloning and chromosomal assignment of the human O^6-methylguanine-DNA methyltransferase. cDNA expression in *Escherichia coli* and gene expression in human cells. J Biol Chem. 1990;265(16):9563-9.

Samson L, Han S, Marquis JC, Rasmussen LJ. Mammalian DNA repair methyltransferases shield O^4MeT from nucleotide excision repair. Carcinogenesis. 1997;18(5):919-24.

Sansome C, Zaika A, Marchenko ND, Moll UM. Hypoxia death stimulus induces translocation of p53 protein to mitochondria. Detection by immunofluorescence on whole cells. FEBS Lett. 2001;488(3):110-5.

Satoh MS, Huh N, Rajewsky MF, Kuroki T. Enzymatic removal of O^6-ethylguanine from mitochondrial DNA in rat tissues exposed to N-ethyl-N-nitrosourea *in vivo*. J Biol Chem. 1988;263(14):6854-6.

Sawyer DE, Van Houten B. Repair of DNA damage in mitochondria. Mutat Res. 1999;434(3):161-76. Review.

Sedgwick B. Molecular cloning of a gene which regulates the adaptive response to alkylating agents in *Escherichia coli*. Mol Gen Genet. 1983;191(3):466-72.

Shiota S, von Wronski MA, Tano K, Bigner DD, Brent TP, Mitra S. Characterization of cDNA encoding mouse DNA repair protein O^6-methylguanine-DNA methyltransferase and high-level expression of the wild-type and mutant proteins in *Escherichia coli*. Biochemistry. 1992;31(7):1897-903.

Singh KK, Russell J, Sigala B, Zhang Y, Williams J, Keshav KF. Mitochondrial DNA determines the cellular response to cancer therapeutic agents. Oncogene. 1999;18(48):6641-6.

Tano K, Shiota S, Collier J, Foote RS, Mitra S. Isolation and structural characterization of a cDNA clone encoding the human DNA repair protein for O^6-alkylguanine. Proc Natl Acad Sci U S A. 1990;87(2):686-90.

Tell G, Crivellato E, Pines A, Paron I, Pucillo C, Manzini G, Bandiera A, Kelley MR, Di Loreto C, Damante G. Mitochondrial localization of APE/Ref-1 in thyroid cells. Mutat Res. 2001;485(2):143-52.

Wallace DC, Brown MD, Melov S, Graham B, Lott M. Mitochondrial biology, degenerative diseases and aging. Biofactors. 1998;7(3):187-90. Review.

Wallace DC. Mitochondrial diseases in man and mouse. Science. 1999;283(5407):1482-8. Review.

Weis K. Importins and exportins: how to get in and out of the nucleus. Trends Biochem Sci. 1998;23(5):185-9. Review.

Wibley JE, Pegg AE, Moody PC. Crystal structure of the human O(6)-alkylguanine-DNA alkyltransferase. Nucleic Acids Res. 2000;28(2):393-401.

Xiao W, Derfler B, Chen J, Samson L. Primary sequence and biological functions of a *Saccharomyces cerevisiae* O^6-methylguanine/O^4-methylthymine DNA repair methyltransferase gene. EMBO J. 1991;10(8):2179-86.

Legends to figures

Figure 1. Nucleotide sequence of human *MGMT* cDNA. Letters in bold indicate the sequences deleted in full-length and truncated MGMT expression plasmids. A highlighted box marks the cysteine acceptor residue surrounded by the 3 conserved amino acids.

Figure 2. Subcellular localization of MGMT in MCF12A cells. (A) GFP-control, MCF12A transfected with pEGFP-N2 vector. **(B)** Detection of fixed cells transfected with MGMT fused to GFP. **(C)** Detection of viable cells transfected with MGMT fused to GFP. **(D)** Detection of viable cells transfected ΔMGMT-GFP construct lacking codon 2-18 of the N-terminus.

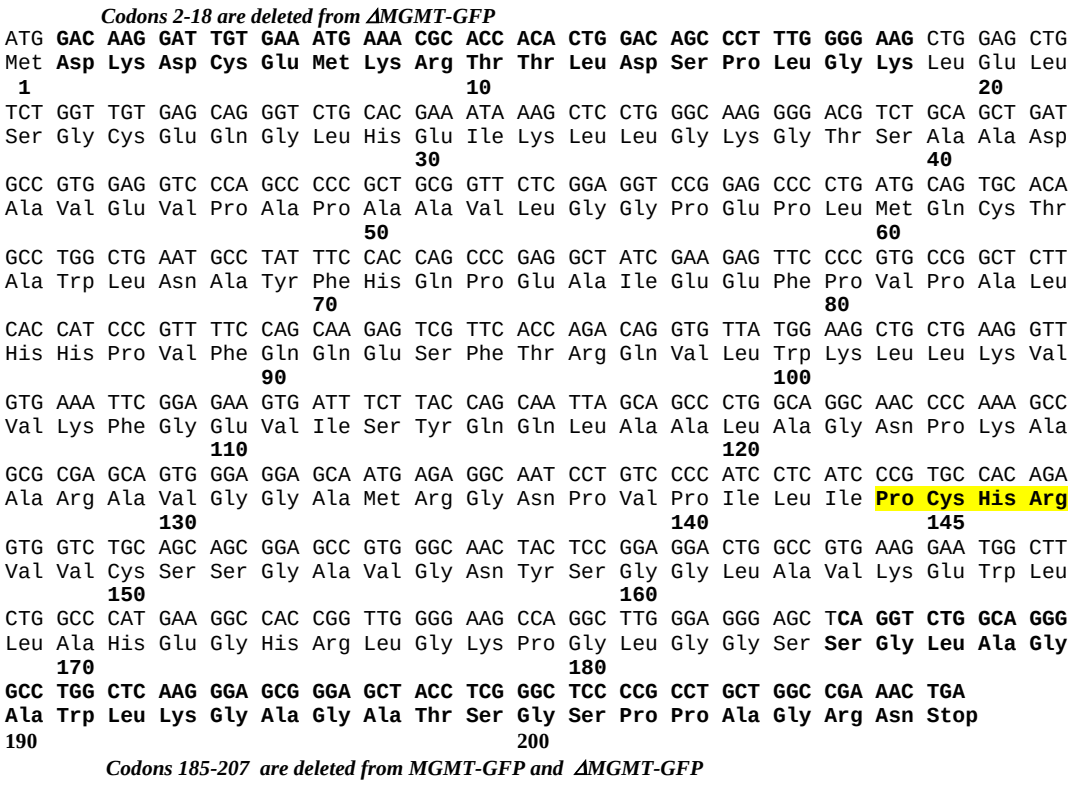
Figure 3. Subcellular localization in HeLa and HTC116 (p53-/-) cells. (A) Detection of HeLa cells transfected with fusion product of MGMT and GFP. **(B)** Detection of HTC116 (p53-/-) cells transfected with MGMT-GFP.

Figure 4. Localization of MGMT in MCF12A cells after 20 μM MNNG treatment for 3 hours.

Figure 5. Survival following methylation. MCF12A cells transfected with Mito-MGMT, MGMT-GFP and ΔMGMT-GFP were treated with different doses of MNNG (0, 13.6, 54.4, 108.8 or 217.6 μM) before the viability of the cells were determined. Each data point represents an average number of ten measurements from one representative experiment.

Figure 6. Survival following oxidative damage. MCF12A cells transfected with Mito-MGMT, MGMT-GFP and ΔMGMT-GFP were treated with different doses of Menadione (0, 100, 400 or 1000 μM) before the viability of the cells was determined. Each data point represents an average number of ten measurements from one representative experiment.

Figure 1


Codons 2-18 are deleted from ΔMGMT-GFP
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Met Asp Lys Asp Cys Glu Met Lys Arg Thr Thr Leu Asp Ser Pro Leu Gly Lys Leu Glu Leu
1 10 20
TCT GGT TGT GAG CAG GGT CTG CAC GAA ATA AAG CTC CTG GGC AAG GGG ACG TCT GCA GCT GAT
Ser Gly Cys Glu Gln Gly Leu His Glu Ile Lys Leu Leu Gly Lys Gly Thr Ser Ala Ala Asp
30 40
GCC GTG GAG GTC CCA GCC CCC GCT GCG GTT CTC GGA GGT CCG GAG CCC CTG ATG CAG TGC ACA
Ala Val Glu Val Pro Ala Pro Ala Ala Val Leu Gly Gly Pro Glu Pro Leu Met Gln Cys Thr
50 60
GCC TGG CTG AAT GCC TAT TTC CAC CAG CCC GAG GCT ATC GAA GAG TTC CCC GTG CCG GCT CTT
Ala Trp Leu Asn Ala Tyr Phe His Gln Pro Glu Ala Ile Glu Glu Phe Pro Val Pro Ala Leu
70 80
CAC CAT CCC GTT TTC CAG CAA GAG TCG TTC ACC AGA CAG GTG TTA TGG AAG CTG CTG AAG GTT
His His Pro Val Phe Gln Gln Glu Ser Phe Thr Arg Gln Val Leu Trp Lys Leu Leu Lys Val
90 100
GTG AAA TTC GGA GAA GTG ATT TCT TAC CAG CAA TTA GCA GCC CTG GCA GGC AAC CCC AAA GCC
Val Lys Phe Gly Glu Val Ile Ser Tyr Gln Gln Leu Ala Ala Leu Ala Gly Asn Pro Lys Ala
110 120
GCG CGA GCA GTG GGA GGA GCA ATG AGA GGC AAT CCT GTC CCC ATC CTC ATC CCG TGC CAC AGA
Ala Arg Ala Val Gly Gly Ala Met Arg Gly Asn Pro Val Pro Ile Leu Ile Pro Cys His Arg
130 140 145
GTG GTC TGC AGC AGC GGA GCC GTG GGC AAC TAC TCC GGA GGA CTG GCC GTG AAG GAA TGG CTT
Val Val Cys Ser Ser Gly Ala Val Gly Asn Tyr Ser Gly Gly Leu Ala Val Lys Glu Trp Leu
150 160
CTG GCC CAT GAA GGC CAC CGG TTG GGG AAG CCA GGC TTG GGA GGG AGC TCA GGT CTG GCA GGG
Leu Ala His Glu Gly His Arg Leu Gly Lys Pro Gly Leu Gly Gly Ser Ser Gly Leu Ala Gly
170 180
GCC TGG CTC AAG GGA GCG GGA GCT ACC TCG GGC TCC CCG CCT GCT GGC CGA AAC TGA
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190 200
Codons 185-207 are deleted from MGMT-GFP and ΔMGMT-GFP

Figure 2

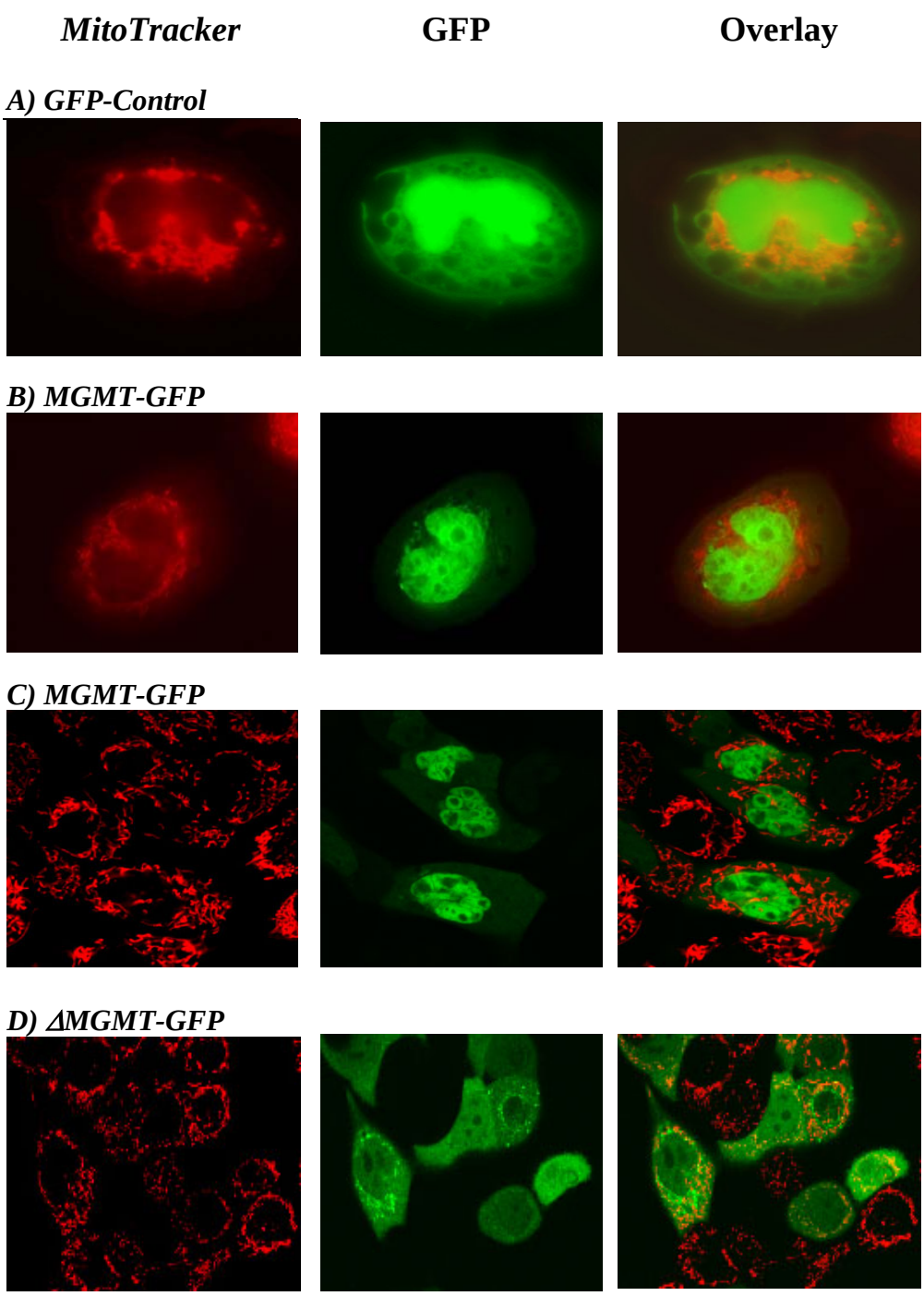

MitoTracker
GFP
Overlay
A) GFP-Control
B) MGMT-GFP
C) MGMT-GFP
D) ΔMGMT-GFP

Figure 3

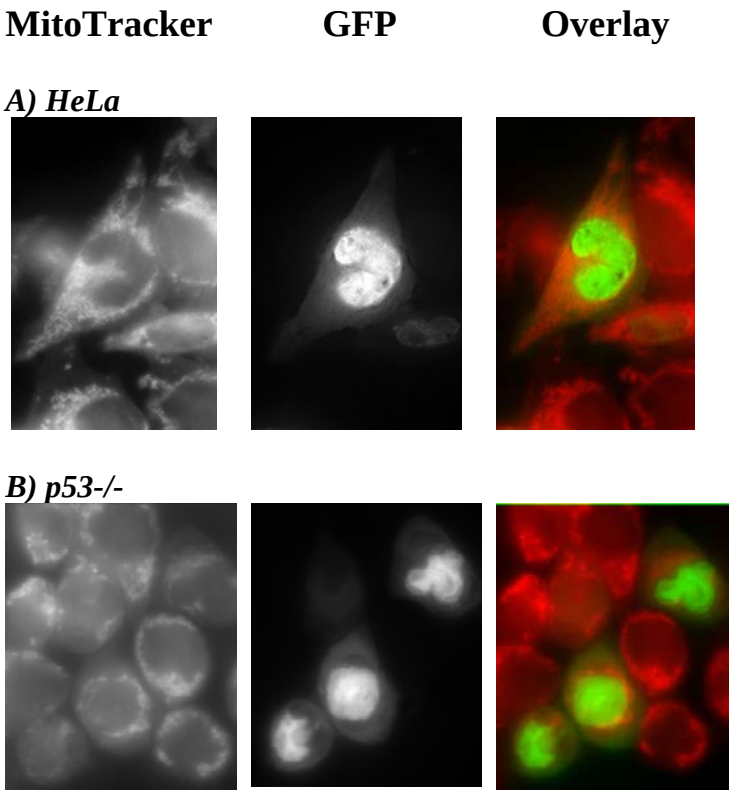


Figure 4

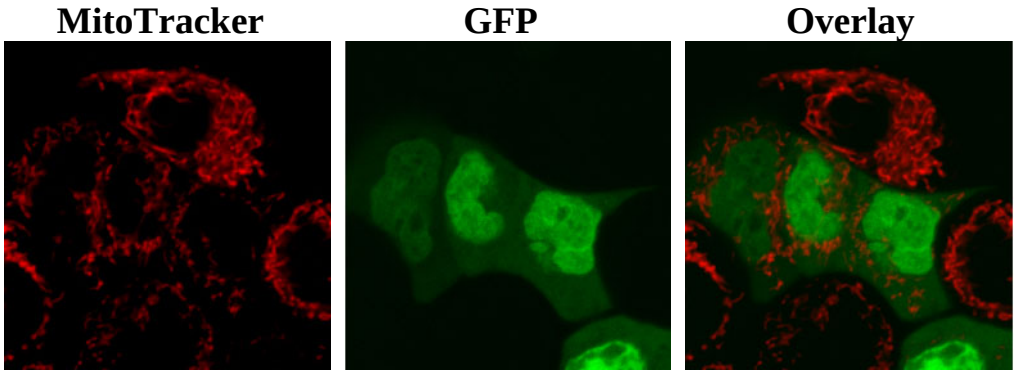


Figure 5

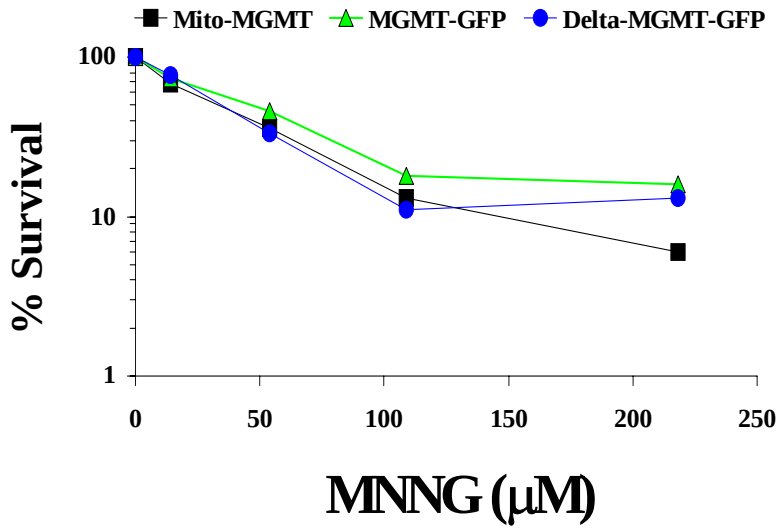
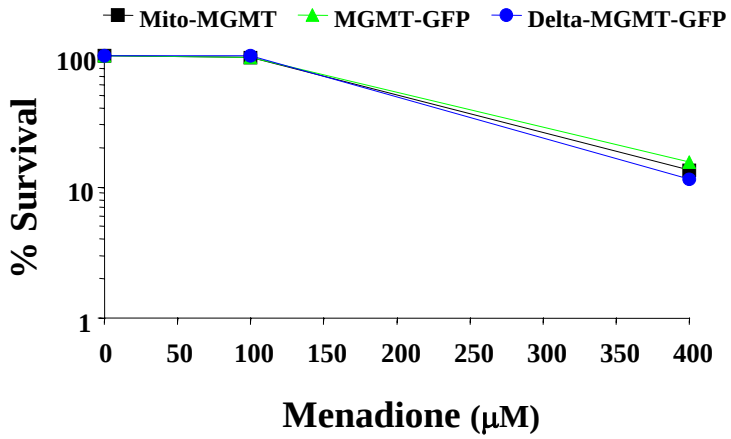


Figure 6



Manuscript III

Anne Karin Rasmussen, Aditi Chatterjee, Lene Juel Rasmussen and Keshav K. Singh. Mitochondria as determinant of genetic stability in *Saccharomyces cerevisiae*. **Nature Genetics (submitted).**

Mitochondria as determinant of genetic stability in *Saccharomyces cerevisiae*

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Accumulation of mitochondrial mutations is a consistent phenotype of cancer cells (1-2). Another hallmark of cancer cells is the generation of a mutator phenotype, which results in a rapid accumulation of mutations that, drives tumor development (3). To date, it is not clear how mitochondrial dysfunction impacts on the genetic stability of nuclear genome. Using *Saccharomyces cerevisiae* as a model organism we analyzed the consequences of disrupting mitochondrial function on genetic stability of the nuclear genome. In strains of yeast lacking mitochondrial DNA (rho^0), and those with mitochondrial mutations (rho^-), we tested the instability of the nuclear genome by measuring the frequency of canavanine resistant colonies. We demonstrate that mitochondrial dysfunction induces mutations in the nuclear genome. We also demonstrate that *REV1*, *REV3* or *REV7* gene products, implicated in error-prone translesion DNA synthesis (TLS) (4) mediate the genetic instability of the nuclear genome arising as a result of mitochondrial dysfunction. Our results in the yeast model provide a direct link between mitochondrial dysfunction and genomic instability, which has important implications in human cancer and aging.

Besides generating ATP, mitochondria perform multiple functions that are essential to the cell (5). Mitochondrial dysfunction is one of the most common and profound phenotypes of many human cancers (6-14). Mitochondrial dysfunction is also involved in aging, adaptive mutation, pathogenesis of many childhood and adult mitochondrial diseases (7, 16-20). Aging studies and studies conducted in human cancer cells have reported the accumulation of mutations in mitochondrial DNA (mtDNA) (2, 5, 11-12, 16-19). In this paper, we investigated the effect of mitochondrial dysfunction on the integrity of the nuclear genome. We generated yeast strains that were either impaired in mitochondrial activity due to mutations in the mitochondrial genome (rho^-) or strains deficient in mitochondrial functions due to the absence of the entire mitochondrial genome (rho^0) (Figure 1). To these cells, we applied a mutation detection assay that measures the stability of the nuclear genome (21). The *CAN1* gene of *S. cerevisiae* encodes a transmembrane amino acid transporter that renders the cell sensitive to lethal arginine analogue, canavanine. Any inactivating mutation in this gene results in a canavanine resistant phenotype (CAN^R) (15). We calculated the frequency of canavanine resistant colonies as a measure of spontaneous nuclear mutational events in rho^0 and rho^- strains. We found that, compared to wild type cells, nuclear mutational events were significantly higher in both rho^- and rho^0 strains (Figure 2A).

Both rho^0 and rho^- cells lack proper mitochondrial function. Therefore, one possible explanation for the increased spontaneous mutation frequencies observed in the rho^0 and rho^- strains is that these cells exhibit defective respiratory function. To test if respiratory dysfunction played a role in increased rate of mutagenesis in rho^- and rho^0 cells, we disrupted mitochondrial respiration with Oligomycin, Antimycin A, and potassium cyanide, inhibitors of mitochondrial function (22). These inhibitors influence different steps in mitochondrial-mediated electron transport. Oligomycin binds directly to mitochondrial ATP synthase and inhibits both electron transfer and ATP production (complex V). Antimycin A is a specific inhibitor of the quinone reduction site, binds to the bc1 complex and blocks electron flow at complex III. Potassium cyanide blocks electron flow at complex IV (22). Analysis of nuclear mutational events in the strain treated with

each of these inhibitors showed that mutation frequency increased in cells exposed to the mitochondrial inhibitors (Figure 2B). Of all the mitochondrial inhibitors used in this study, Antimycin A had the most profound effect indicating that complex III activity is critical for maintaining genomic integrity (Figure 2B). It is noteworthy, that of all the mitochondrial inhibitors, only Antimycin A treatment induces many of the same genes induced in rho^0 cells (23). Together, our results provide evidence that mitochondrial dysfunction generates a mutator phenotype in the nuclear genome.

It has been hypothesized that mitochondrial dysfunction could increase the level of reactive oxygen species (ROS) (1, 18-19). Elevated level of reactive oxygen species could lead to loss of genomic integrity. Therefore, we measured superoxide and hydrogen peroxide levels in rho^0 and rho^- cells. Our results show that both complete loss of the mitochondrial genome and mutations in mtDNA result in lower levels of superoxide (O_2^-) and H_2O_2 (Figure 3 A and B). Thus, ROS does not appear to play a major role in the mitochondria-mediated mutagenesis. Mitochondria are intimately involved in pyrimidine biosynthesis (24). It is conceivable that impairment of nucleotide biosynthesis due to mitochondrial dysfunction contributes to mutagenesis of the nuclear genome.

Yeast cells are known to modulate the expression of nuclear genes in response to mitochondrial dysfunction (25). In fact, mitochondria-to-nucleus communication has been studied extensively in yeast cells lacking mitochondrial function (25-26). Previous studies have demonstrated that up-regulation of certain nuclear genes involves activation and nuclear translocation of heterodimeric basic helix-loop-helix factors Rtg1 and Rtg3p in response to mitochondrial dysfunction. A related protein Rtg2 is also involved in mitochondria-to-nucleus communication (25). To identify genes, whose expression profiles change as a result of impairment of mitochondrial function, we conducted microarray analysis (data not shown). We compared gene expression of cells lacking mitochondrial function (rho^0) with that of wild type cells (rho^+). Interestingly we found that among other genes, the expression of *REV1*, which is involved in error-prone translesion DNA synthesis (TLS) (27), was upregulated. TLS enables cells to repair DNA lesions that escape the vigilance of the generally

efficient DNA repair systems (27). TLS occurs when the replication machinery, upon encountering a lesion, has, or some how acquires, the ability to copy the damaged template directly by incorporating a nucleotide opposite the modified base. TLS is potentially mutagenic because it often incorporates incorrect nucleotides (27). TLS has significance in tumorigenesis since mutations induced during replication of damaged nucleotides are believed to cause cancer (3).

In *S. cerevisiae*, the three proteins, Rev1, Rev3 and Rev7 constitute the major components of TLS. The *REV1* gene product possesses deoxycytidyl transferase activity whereas Rev3 and Rev7 proteins are the subunits of DNA polymerase zeta (27). We examined whether these three gene products play a role in determining the mitochondria-mediated mutator phenotype. We determined mitochondria-mediated mutator phenotype in strains with null mutations in *REV1, REV3 or REV7* genes. Our results clearly showed that the mutator phenotype is eliminated in cells lacking *REV1, REV3, or REV7* genes (Figure 4). These results strongly suggest that the mitochondria-mediated mutator phenotype is dependent on functional Rev1, Rev3 and Rev7 proteins. The *REV1, REV3* and *REV7* genes are conserved between yeast and humans. Based on our observations in yeast, it is conceivable that human Rev1p, Rev3p and Rev7p proteins are involved in mitochondria-mediated mutagenesis.

The data presented in this paper supports the following conclusion for the role of mitochondrial dysfunction in genetic instability. Mitochondrial dysfunction leads to spontaneous nuclear DNA damage. The nature of this spontaneous nuclear DNA damage is unknown at this time. In order to avoid cell death due to spontaneously damaged nuclear DNA, cells activate the error-prone TLS pathway that contributes to cell survival but genetic instability.

Mitochondrial dysfunction and genetic instability are both characteristic features of cancer cells (1-3, 32). Mitochondrial dysfunction due to accumulation of mutations in mtDNA is also a key feature of aging (16-19). Our studies using a yeast model system suggest that mitochondria contain some intrinsic properties that control the mutator phenotype associated with cancer cells. Our study provides, for the first time, a link between cancer and aging and mitochondrial dysfunction.

Methods

Media and Strains. Growth media were prepared as described in (29). The *S. cerevisiae* strains used in this study are derived from RKY3109 (15) provided by R. D. Kolodner (Ludwig Institute for Cancer Research, La Jolla, CA).

Generation of rho$^-$ and rho^0 strains. Strains with mitochondrial mutations (rho$^-$) or strains lacking mtDNA (rho^0) were generated by treatment of strains with ethidium bromide (29). The mutants were selected as cells unable to form colonies on yeast extract-peptone-glycerol (YPG) plates. In rho^0 cells the loss of mtDNA was verified by DAPI (4,6-diamidino-2-phenylindole) staining and rho$^-$ status was verified by genetic crossing with rho^0 cells.

Generation of yeast *rev* null mutants. Mutations in *REV1, REV3* and *REV7* genes (rho^0*rev1,* rho^0*rev3* and rho^0*rev1*) were introduced by one-step gene deletion/replacement in YAKR144 (table 1). The plasmids pSF3-*REV1*Δ::*URA3*, pYPG101-*REV3Δ::URA3* and pYPG102-*REV7Δ::URA3* used to generate the *S. cerevisiae REV* null mutants. The null plasmids were obtained from Dr. C. Lawrence (Department. of Biochemistry and Biophysics, University of Rochester, NY).

Determination of spontaneous mutation frequency. Mutation frequencies were determined using the fluctuation test method essentially as described in (30). Briefly, for each strain five to seven independent cultures were grown in yeast extract-pentose-dextose (5 ml, YPD). Each experiment was repeated at least twice. Appropriate dilutions were plated on YPD plates to determine the number of viable cells. The frequency of forward mutation at the *CAN1* gene locus was determined from the number of canavanine resistant colonies that grew on selective minimal dropout plates (SD/-Arg + 60 μg/ml canavanine). The colonies were counted after incubation at 30^{o}C for 3-5 days. The data were analyzed by using the method of the median (30).

Inhibition of respiratory activity. Wild type strain (RKY3109) was grown in YPD media overnight before plating the cells on YPD plates and SD/-Arg + canavanine plates containing either water (control), oligomycin (5 μg/ml), Antimycin A (0.02 mM), potassium cyanide (0.5 mM). After incubation at 30°C for 3-5 days the colonies were counted and the data were analyzed by the method of the median (30).

Flow cytometric analysis of intracellular levels of free radicals. To determine superoxide anion generation, dihydroethidium (DHE, Molecular Probes, Eugene, OR) was added to early exponential phase cultures of either wild type (RKY3109), rho^- (YAKR145) or rho^0 (YAKR144). The cultures were grown in YPD medium to A_{600} = 0.4 - 0.5. The cells were washed twice in water and the cells were incubated in 1 ml 50 mM Tris pH 7.5 containing 40 μg/ml DHE for 2 hrs before they were analyzed by flow cytometry. The H_2O_2-sensitive dye 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA, Molecular Probes) was used to measure peroxide production in the cells. CM-H_2DCFDA staining was conducted by adding the dye (10 μM) to the growth medium (1 ml) and incubated for 20 hrs. For sample analysis, an aliquot (100 μl) of the CM-H_2DCFDA-stained yeast cultures were suspended in 900 μl of 50 mM Tris pH 7.5 before they were analyzed by flow cytometry (31).

Acknowledgement

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References

1. Wallace DC. Mitochondrial diseases in man and mouse. Science 283:1482-1488 (1999)

2. Bianchi NO, Bianchi MS, Richard SM. Mitochondrial genome instability in human cancers. Mutat Res 488:9-23 (2001)

3. Loeb LA. A mutator phenotype in cancer. Cancer Res 61:3230-3239 (2001)

4. Lawrence CW and Maher VM. Mutagenesis in eukaryotes dependent on DNA polymerase zeta and Rev1p. Phil Trans R. Soc London 356:41-46 (2001)

5. Singh KK Mitochondrial DNA mutation in aging disease and cancer. Springer, New York, NY USA (1998).

6. Warburg O. On the origin of cancer cells. Science 123:309-314 (1956).

7. Warburg O. Metabolism of tumours. Translated by F. Dickens, Constable, London (1930)

8. Pedersen PL. Mitochondrial events in the life and death of animal cells: a brief overview. J Bioenerg Biomembr 31:291-304 (1999)

9. Duchesne, J. A unifying biochemical theory of cancer, senescence and maximal life span. J Theor Biol; 66:137-145 (1977).

10. Hartung, J. Might cancer be a failed response to renegade mitochondria? J Theor Biol 94:173-178 (1982).

11. Polyak K, Li Y, Zhu H, Lengauer C, Willson JK, Markowitz SD, Trush MA, Kinzler KW, Vogelstein B.Somatic mutations of the mitochondrial genome in human colorectal tumours. Nat Genet 20:291-293 (1998).

12. Fliss MS, Usadel H, Caballero OL, Wu L, Buta MR, Eleff SM, Jen J, Sidransky D. Facile detection of mitochondrial DNA mutations in tumors and bodily fluids. Science 2000 287:2017-2019 (2000).

13. Baggetto, L.G. Role of mitochondria in carcinogenesis. Europ J Cancer 1992; 29:156-169 (1992).

14. McCabe, E.R. Role of mitochondria in oncogenesis. Biochem Med Metab Biol 47:105-107 (1992).

15. Cotton, D.W., Rogers, S. Aging, cancer, and mitochondrial deterioration. Lancet 341:281-282 (1993).

16. Richter, C. Oxidative damage to mitochondrial DNA and its relationship to aging. Int J Biochem Cell Biol; 27:647-653 (1995).

17. Shigenaga, M.K., Hagen, T.M., Ames, B.N. Oxidative damage and mitochondrial decay in aging. Proc Natl Acad Sci USA 91:10771-10778 (1994).

18. Ames, B.N., Shigenaga, M.K., Hagen, T.M. Mitochondrial decay in aging. Biochim Biophys Acta 1271:165-70 (1995).

19. Wallace, D.C., Shoffner, J.M., Trounce, I., Brown, M.D., Ballinger, S.W., Corraldebrinski, M., Horton, T., Jun, A.S., Lott, M.T. Mitochondrial-DNA mutations in human degenerative diseases and aging. Biochim Biophys Acta 1271:141-151 (1995).

20. Heidenreich E, Wintersberger U. Replication-dependent and selection-induced mutations in respiration-competent and respiration-deficient strains of *Saccharomyces cerevisiae*. Mol Gen Genet 260:395-400 (1998).

21. Ni, T. T., Marsischky, G. T. & Kolodner, R. D. MSH2 and MSH6 are required for removal of adenine misincorporated opposite 8-oxo-guanine in *S. cerevisiae*. Mol. Cell 4, 439-444 (1999).

22. Grant CM, MacIver FH, Dawes IW. Mitochondrial function is required for resistance to oxidative stress in the yeast Saccharomyces cerevisiae. FEBS Lett 410:219-22 (1997)

23. Epstein CB, Waddle JA, Hale W 4th, Dave V, Thornton J, Macatee TL, Garner HR, Butow RA. Genome-wide responses to mitochondrial dysfunction Mol Biol Cell 12:297-308 (2001)

24. Loffler M, Jockel J, Schuster G, Becker C. Dihydroorotat-ubiquinone oxidoreductase links mitochondria in the biosynthesis of pyrimidine nucleotides. Mol Cell Biochem 174:125-129 (1997).

25. Sekito T, Thornton J, Butow RA. Mitochondria-to-nuclear signaling is regulated by the subcellular localization of the transcription factors Rtg1p and Rtg3p. Mol Biol Cell 11:2103-2115 (2000)

26. Liu, Z. & Butow, R.A. A transcriptional switch in the expression of yeast tricarboxylic acid cycle genes in response to a reduction or loss of respiratory function. Mol. Cell. Biol. 19, 6720-6728 (1999).

27. Lawrence CW, Hinkle DC. DNA polymerase zeta and the control of DNA damage induced mutagenesis in eukaryotes. Cancer Surv 28:21-31 (1996).

28. Baynton K, Bresson-Roy A, Fuchs RP. Distinct roles for Rev1p and Rev7p during translesion synthesis in *Saccharomyces cerevisiae*. Mol Microbiol 34:124-133 (1999).

29. Sherman, F., Fink, G.R.& Hicks, J.B. *Methods in Yeast Genetics: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Plainview, NY, (1994).

30. Von Borstel, R. C. Measuring spontaneous mutation frequencies in yeast. Methods Cell Biol. 20, 1-24 (1978).

31. Madeo F, Frohlich E, Ligr M, Grey M, Sigrist SJ, Wolf DH, Frohlich KU (1999) Oxygen stress: a regulator of apoptosis in yeast. J Cell Biol 145:757-767 (1999).

32. Cotton DW, Rogers S. Ageing, cancer, and mitochondrial deterioration. Lancet 341:281-282 (1993)

Table 1 • *S. cerevisiae* strains used in this study

	Strain	Genotype
Wild type	RKY3109	*MAT***a**, *ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl,hom3-10, ade8, ade2Δ1*
rho^-	YAKR145	RKY3109 rho^-
rho^0	YAKR144	RKY3109 rho^0
rho^0 *rev1*	YAKR184-188	YAKR144 *REV1Δ::URA3*
rho^0 *rev3*	YAKR189-193	YAKR144 *REV3Δ::URA3*
rho^0 *rev7*	YAKR194-198	YAKR144 *REV7Δ::URA7*

Figure Legends:

Figure 1:

Strains with mtDNA mutations (rho^-) or strains lacking mtDNA (rho^0) were generated by treatment of strains with ethidium bromide (29). The mutants were selected as cells unable to form colonies on yeast extract-peptone-glycerol (YPG) plates. Wild type and rho^- cells show mtDNA after cells were stained with DAPI (4,6-diamidino-2-phenylindole). DAPI stains both the nuclear and mtDNA. The rho^0 cells show nuclear DNA staining but they do not show punctuate staining indicating loss of mtDNA. The rho^- status was verified by genetic crossing with rho^0 cells. Fluorescence was examined using a DAPI optimized filter on Zeiss-Axiovert 135 TV inverted microscope equipped with a PXI camera (SEBSYS photometrica).

Figure 2 A and B:

Strains were tested in *CAN1*^{R} assay as described in Methods. The average median frequency in each assay is presented. $CAN1^S$ mutation assay detects frequency of *CAN1*^{S} to *CAN1*^{R} forward mutations. A: Frequency of mutation in *CAN1* gene in yeast strains defective in mitochondrial function. B: Frequency of mutation in *CAN1* gene after respiratory inhibition. Inhibition of respiration was carried out as described in material methods. The data presented are an average of at least 10 independent cultures.

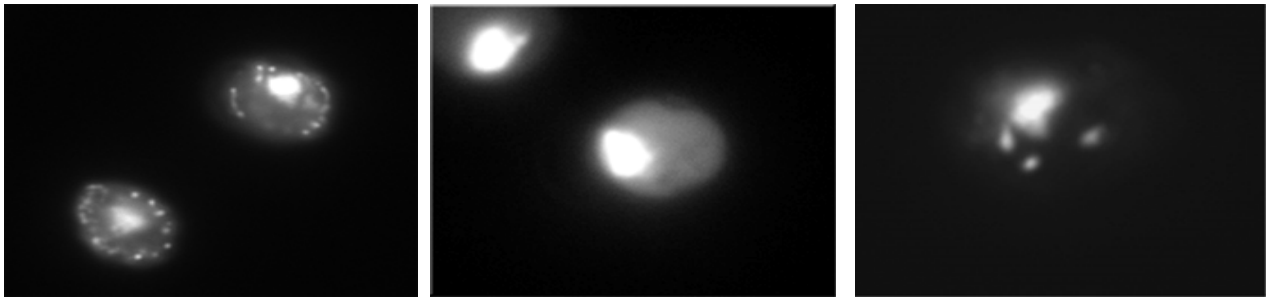
Figure 3 A and B:

Production of reactive oxygen species in yeast strains lacking mitochondrial function. A) superoxide and B) hydrogen peroxide. Free radical production was measured as described in Methods. Superoxide was measured by using dihydroethidium (DHE) and 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) was used to measure H_2O_2.

Figure 4:

Mutation frequency in yeast strain lacking *rev1*, *rev3* or *rev7* gene expression. Strains were tested in *CAN1*^{R} assay as described in Methods. The average median frequency in each assay is presented. *CAN1*^{S} mutation assay detects frequency of *CAN1*^{S} to *CAN1*^{R} forward mutations. The data presented are an average of at least 10 independent cultures.

Figure 1



Wild type rho^0 rho$^-$

Figure 2 A

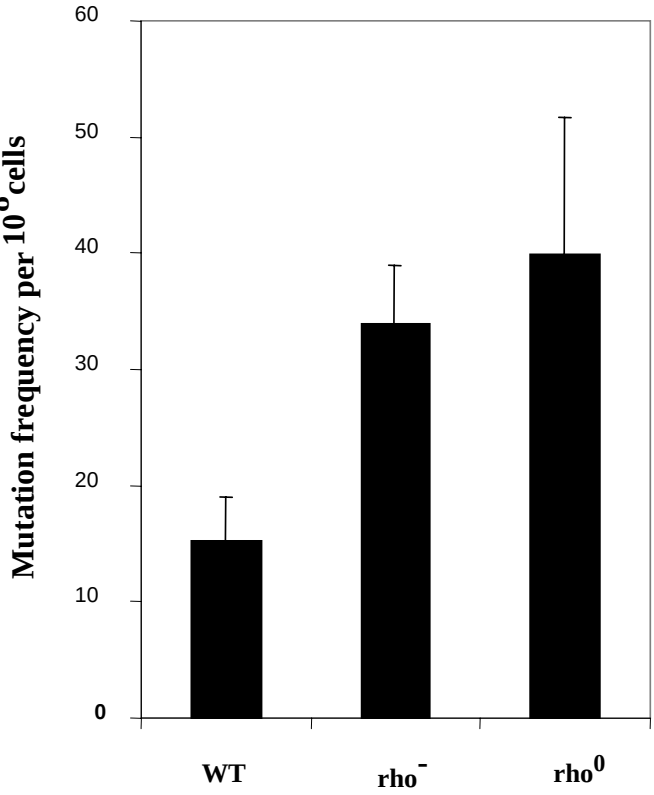


Figure 2 B

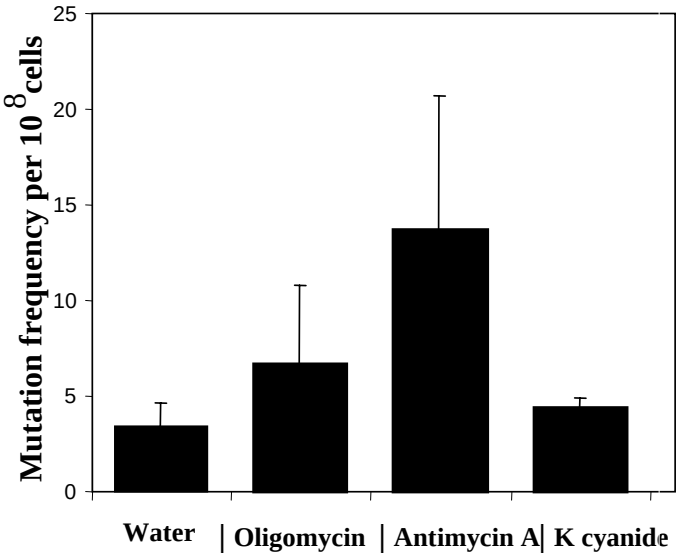


Figure 3 A

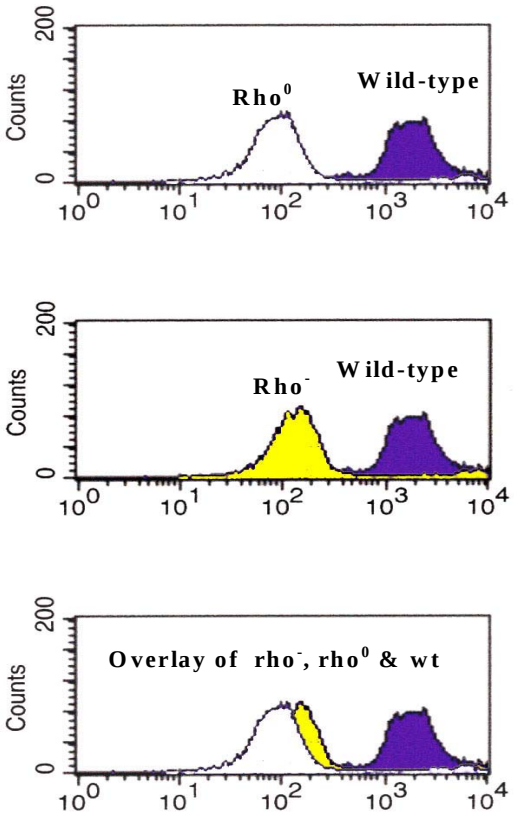


Figure 3 B

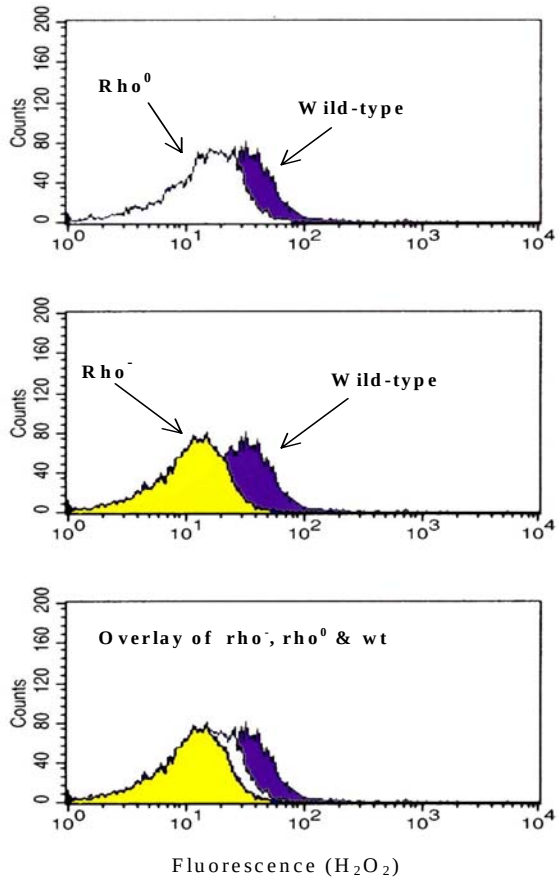
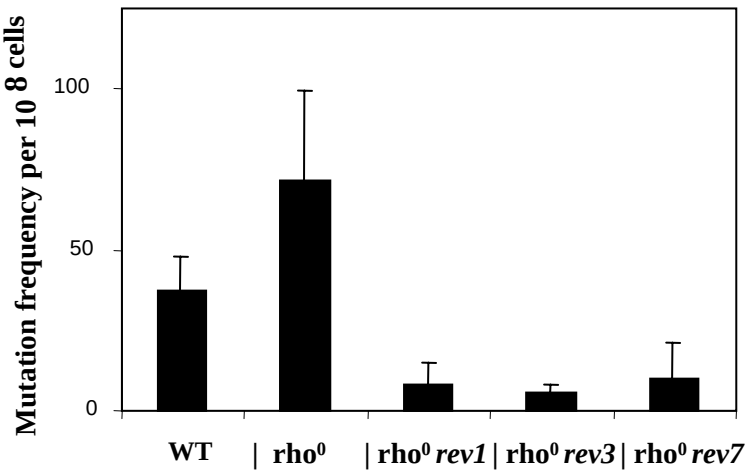


Figure 4



Manuscript IV

Anne Karin Rasmussen, Lene Juel Rasmussen, and Keshav K. Singh. Mitochondrial dysfunction suppresses the mutator phenotype of *Saccharomyces cerevisiae* superoxide dismutase deficient cells. **Manuscript in preparation.**

Mitochondrial dysfunction suppresses the mutator phenotype of *Saccharomyces cerevisiae* superoxide dismutase deficient cells

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Abstract

Oxidative stress and accumulation of mutation are associated with increased risk of cancer and aging. Alterations of oxidative phosphorylation affect intracellular levels of reactive oxygen species (ROS) which induce oxidative stress. DNA lesions introduced by ROS are potentially cytotoxic and mutagenic. We used *Saccharomyces cerevisiae* to understand the mechanisms of mutagenesis in cells deficient in superoxide dismutases (encoded by the *SOD1* and *SOD2* genes). Our analysis reveals that *SOD1* gene inactivation leads to mutations in the nuclear genome and that mitochondrial dysfunction suppress this mutator phenotype. We also demonstrate that agents which introduce oxidative damage decrease cell survival and increase mutations in cells with mitochondrial dysfunction.

Introduction

Mitochondria generate more than 80% of cellular energy in the form of ATP. In addition, these organelles carry out other cellular functions, such as respiration and heme, lipid, amino acid and nucleotide biosynthesis (Kang *et al*., 1998). Mitochondrial respiration is a major endogenous source of reactive oxygen species (ROS), including superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^\bullet$) (Cadenas and Davies, 2000). These ROS also arise from external environmental factors such as drugs, radiation, and heavy metals (Cadenas, 1989). As a result, ROS cause damage to proteins, lipids, and nucleic acids and thereby compromise cell viability. Both prokaryotic and eukaryotic cells have developed defense mechanisms against the lethal and mutagenic effect of ROS. Superoxide dismutases (SOD) are enzymes that eliminate O_2^- and thus protect cells from damage induced by free radicals (Halliwell and Gutterridge, 1985; Fridovich, 1995; Oberley and Buettner, 1979). ROS are also reduced by intracellular antioxidant enzymes such as glutathione peroxidase (GPx) and catalase (CAT) as well as some antioxidant molecules like glutathione (Raha & Robinson, 2001; Jamieson, 1998). Therefore, the balance between ROS production and antioxidant defenses determines the degree of oxidative stress.

Superoxide is the primary oxygen free radical produced by the mitochondrial respiratory chain. However, O_2^- is rapidly dismutated to H_2O_2 by either Sod2 (mitochondria) or Sod1 (nucleus, cytoplasm, and lysosomes) (Srinivasan *et al*., 2000). Hydrogen peroxide is reduced to water by CAT and GPx (Wallace, 1998). *Saccharomyces cerevisiae* cells deficient in Sod1 activity show a variety of phenotypes such as growth inhibition under aerobic conditions, sensitivity to killing by menadione or freeze-thawing, decreased viability in stationary phase, auxothropy for the amino acids methionine and lysine as well as increased spontaneous mutation rates under aerobic growth conditions. In contrast, cells deficient in Sod2 activity have a less dramatic phenotype when grown in glucose media but are oxygen-sensitive and grow poorly on carbon sources that require respiration for metabolization. The phenotype of the *sod1sod2* double mutant strain includes all phenotypes of the single mutants (Gralla and Valentine, 1991; Liu *et al*., 1992; Longo *et al*., 1996; Park *et al*., 1998).

Oxidative phosphorylation generates ROS which suggests a role for mitochondrial activity in cell survival and mutagenesis. Guidot *et al*. (1993) found that *S. cerevisiae* cells deficient in the mitochondrially-located superoxide dismutase (Sod2) failed to grow normally in hyperoxia. In contrast, strains deficient in both Sod2 and mitochondrial activity (rho^0) grew normally in hyperoxia. In addition, the viability was partially restored by mutations, which disrupt the electron transport chain. Lack of respiration also prevents viability loss of *sod1* and *sod1sod2* double mutant strains (Longo *et al*., 1996). These data imply that mitochondrial function and respiration play an important role in viability of *sod* mutant strains. Studies have shown that rho^0 strains as well as strains carrying deletions in their mitochondrial DNA (rho^-), are more sensitive to killing by O_2^- and H_2O_2 than wild type cells (Flatter-O'Brien *et al*., 1993; Jamieson, 1992; Collinson and Dawes, 1992; Grant *et al*., 1997).

Mammalian cells respond to oxidative stress by increasing antioxidant enzymes, which are capable of detoxifying reactive oxygen species (Akashi *et al*., 1995; Brambilla *et al*., 1997; Esposito *et al*., 1999; de Hann *et al*., 1998). However, there exists tissue specificity in the cellular response to oxidative stress. Skeletal muscle dramatically induced *SOD2* expression whereas heart only increased *SOD2* expression modestly upon oxidative stress. In contrast, expression of GPx1 was induced similarly in both tissues. In *S. cerevisiae*, the expression of both superoxide dismutases, *SOD1* and *SOD2*, glutathione peroxidase *GPX2*, *CTT1* encoding the cytosolic catalase T, and glutathione reductase *GLR*, which catalyses reduction of glutathione disulfide (GSSG) to glutathione (reduced form, GSH) are induced in response to oxidative stress (Galiazzo *et al*., 1987; Gasch *et al*., 2000; Grant *et al*., 1996; Inoue *et al*., 1999; Marchler *et al*., 1993).

In this paper we examined the role of Sod1 and Sod2 in mitochondria-mediated mutagenesis in *S. cerevisiae*. We show that cells deficient in Sod activity increase spontaneous mutation levels in the nuclear genome and that this mutator phenotype is suppressed by inactivation of mitochondrial activity. In order to characterize the role of mitochondrial activity in mutagenesis and cell survival we treated wild type and rho^0 cells with various drugs that affect intracellular levels of ROS. We show that both H_2O_2 and menadione increase spontaneous mutations in nuclei; but that rho^0 cells are 10-20 fold more sensitive than wild type cells to mutations introduced by these agents. Furthermore, both H_2O_2 and menadione decrease survival of rho^0 cells indicating that cells deficient in

mitochondrial activity are impaired in repair of DNA damage introduced by these agents. We used DNA microarray hybridization analysis to further characterize the cellular response to oxidative stress. We compared gene expression patterns in *sod1* and *sod1*rho^0 mutant strains. We did not detect any increase in the expression of the majority of DNA repair genes in *sod1*rho^0 cells compared to *sod1* cells. However, gene expression analysis revealed that genes involved in DNA repair are induced in *sod1* compared to wild type cells.

Materials and Methods

Media and strains. Growth media were prepared as described in Sherman *et al*., 1994. The *S. cerevisiae* strains used in this study are derived from RKY3109 (Ni *et al*., 1999) and provided by Dr. Richard Kolodner (Ludwig Institute for Cancer Research, La Jolla, CA) or strain DL1 (D273-10B) (Paul *et al*., 1989). The strains used in this study are listed in Table 1.

Generation of yeast strains. Mutations in the superoxide dismutase genes (*sod1* and *sod2*) were introduced by one-step gene deletion/replacement in RKY3109. The plasmids pUC-*SOD1Δ*::*URA3*-A and pG-*SOD2Δ*::*TRP1*-A were provided by Dr. Gralla (Department of Chemistry and Biochemistry, University of California, Los Angeles, CA). The *sod1* mutant strain YAKR129 was generated using the *SOD1*::*URA3* deletion/disruption cassette as described in (Gralla and Valentine, 1991). The *sod2* mutant strain was generated similarly using the *SOD2*::*TRP1* deletion/disruption cassette (Marres *et al*., 1985). The *sod* deletion derivatives were verified by PCR analysis (data not shown).

Generation of rho$^-$ and rho^0 strains. Strains with mitochondrial mutations (rho$^-$) or strains lacking mtDNA (rho^0) were generated by treatment of wild type, *sod1* and *sod2* strains with ethidium bromide (Sherman *et al*., 1994). The mutants were selected as cells unable to form colonies on extract-pentose-glycerol (YPG) plates. The loss of mtDNA was verified by DAPI (4,6-diamidino-2-phenylindole) staining (data not shown).

Determination of spontaneous mutation frequency. Mutation frequency was determined using fluctuation test method described in (von Borstel, 1978). For each strain, five to seven independent cultures were grown in 5 ml yeast extract-pentose-dextrose (YPD). Appropriate dilutions were plated on YPD agar plates to determine the number of viable cell in the cultures. The frequency of forward mutation at the *CAN1* gene locus was determined from the number of canavanine resistant colonies that grew on selective minimal dropout plates (SD/-Arg + 60 mg/liter canavanine). The colonies were counted after incubation at 30^{o}C for 3-5 days for wild type and rho^0 cells and for 11-14 days for *sod* mutant strains. The data were analyzed by the method of the median (Lea 1949, von Borstel, 1978). Each experiment was repeated at least twice.

Drug treatment. Overnight cultures grown in YPD medium were diluted into fresh growth medium at A_{600} of 0.15 and incubated until cell density reached A_{600} of 0.4 - 0.5. Aliquots of 5 ml were treated with various concentrations of drugs at 30^{o}C with vigorous shaking for 1 hr. The concentrations of the drugs were: H_2O_2 (0, 2 and 4 mM), 4-NQO (4-Nitroquinole N-oxide) (0, 2 and 4 μg/ml), adriamycin (0, 20 and 40 μg/ml), menadione sodium bisulfite (0, 10 and 20 mM), and diamide (0, 25 and 50 mM). Relative cell survival was determined by immediately diluting the samples into sterile H_2O and plating on YPD plates. Mutation frequency was determined from the number of canavanine resistant colonies by the method of the median (von Borstel, 1978). The yeast cells were collected by centrifugation, washed in water before plating on SD/-Arg + canavanine plates. All reagents were obtained from Sigma Chemical Co. (St Louis, MO) with the exception of adriamycin (Johns Hopkins Hospital, MD).

Gene array analysis. Total RNA was isolated from log-phase *S. cerevisiae* strains according to manufactures guide lines using RNeasy (QIAGEN). Total RNA (5 μg) was converted into double stranded cDNA by GIBCO BRL's SuperScript Choice system for cDNA synthesis (LifeTechnologies) and a T7-$(dT)_{24}$ oligomer provided by Research Genetics (Huntsville, AL). Double stranded cDNA was purified by phenol/chloroform extraction and ethanol precipitation. *In vitro* transcription was performed with T7 RNA polymerase following the instructions from BioArray high yield RNA transcript Labeling kit from Enzo (distributed by Affymetrix). The Biotin-labeled cRNA was purified on an affinity resin (RNeasy mini cleanup, QIAGEN) and the amount of labeled cRNA was determined by measuring absorbance at 260 nm and using the convention that 1 OD at 260 nm corresponds to 40 μg/ml RNA. The Yeast Genome S98 Array (Affymetrix) containing approximately 6,400 open reading frames (ORFs) of *S. cerevisiae* genome was used for gene expression analysis. Hybridization, reading and analysis were performed by Research Genetics (Huntsville, AL). The classification of genes into functional groups was done as described in MIPS database, Munich Information Center for Protein Sequences (Mewes *et al*., 1997; http://mips.gsf.de).

Results and Discussion

Mitochondrial dysfunction prevents mutations in the nuclear genome in superoxide dismutase deficient cells (Sod1 and Sod2) deficient cells.

Saccharomyces cerevisiae coq3sod1 double mutant deficient in superoxide dismutase activity and oxidative phosphorylation show increased viability compared to the superoxide deficient *sod1* mutant alone (Longo *et al*., 1996). This indicates that free radicals generated during oxidative phosphorylation in mitochondria are cytotoxic and that superoxide dismutases protect the cell from the lethal effect of mitochondrial-mediated damage. In order to characterize the interaction between mitochondrial-mediated mutagenesis and repair in more detail we measured spontaneous mutation frequencies. We applied a mutation detection assay that measures the stability of the nuclear genome. The *CAN1* gene of *S. cerevisiae* encodes a transmembrane amino acid transporter that renders the cell sensitive to the lethal arginine analogue, canavanine. Any inactivating mutation in this gene results in a canavanine resistant phenotype (CAN^R). We calculated the frequency of canavanine resistant colonies as a measure of spontaneous nuclear mutational events in *sod1* and *sod2* mutant strains (Table 2). As expected, based on earlier work (Gralla and Valentine, 1991; Longo *et al*., 1997), mutation frequencies are increased in the *sod1* mutant strain compared to the wild type parental strain (Table 2). Inactivation of the *sod2* gene increased mutation frequencies about 2-fold, which is similar to the mutator phenotype generated by inhibiting mitochondrial activity (rho^0 or rho^-) (Rasmussen *et al*., (III)). The increased mutation frequencies observed in superoxide dismutase deficient cells were suppressed by inactivation of mitochondrial activity (*sod1*rho^0 and *sod2*rho^0) (Table 2). These data suggest that the mutator phenotype observed in superoxide dismutase deficient cells is dependent on mitochondrial activity.

Differential expression of repair genes in superoxide dismutase deficient strains.

We used genome analysis by DNA microarray hybridization analysis to identify DNA repair transcripts differentially expressed as a result of mitochondrial dysfunction and superoxide dismutase deficiency (Table 3). We found that a large number of DNA repair genes acting in various DNA repair pathways are induced by the *sod1* mutation (Table 3). The differentially expressed genes included not only DNA repair genes known to repair oxidative DNA damage (*NTG2*), but also genes involved in repair of other kinds of DNA

damage such as base excision repair (*MAG1*), direct reversal repair (*MGT1*), nucleotide excision repair (*RAD10*, *RAD14*, *RAD16*, *RAD26* and *RAD28*), recombinational repair (*RAD50*, *RAD52*, *RAD59*, *SGS1*, *MUS81*, and *PSO1*), mismatch repair (*MSH3*), and meiotic recombination (*MSH4*). It has been shown that the transcript of *MAG1*, encoding a 3-methyladenine DNA glycosylase, is induced after treatment with the methylating agent methylmethane sulfonate (MMS) as well as with the oxidizing agents H_2O_2 and menadione (Jelinsky and Samson, 1999; Gasch *et al*., 2001). The induction of DNA repair genes observed in the *sod1* mutant strain suggests that one or more shared transcription factors responsible for expression of DNA repair genes are activated in response to endogenous DNA damage in superoxide dismutase deficient cells.

Further analysis of gene expression patterns revealed that several checkpoint genes were induced in the *sod1* mutant strain (Table 3). The *MEC3* gene is a checkpoint gene that together with *RAD9*, *RAD17*, *RAD24* and *DDC1* are required for detection of DNA damage and activation of the checkpoint pathways (Elledge, 1996, Zhou and Elledge, 2000). Induction of *MEC3* and *RAD9* suggest that *sod1* mutant cells generate DNA damage that is detected by the checkpoint pathways.

We show in Table 2 that the mutator phenotype of the *sod1* mutant strain is suppressed by inactivating mitochondrial activity (*sod1*rho^0). We compared gene expression patterns of the *sod1* mutant strain with the *sod1*rho^0 mutant strain in order to identify DNA repair genes involved in this suppression. Interestingly, only seven DNA repair genes were differentially expressed (Table 4). One of these is the *OGG1* gene encoding the 8-oxoguanine DNA glycosylase. Both purine and pyrimidine residues in DNA are sensitive to ROS-mediated DNA damage (Croteau and Bohr, 1997). However, the most common purine DNA lesion is the mutagenic 8-hydroxyguanine (8-oxoG) (Steenken and Jovanovic, 1997; Burrows and Muller, 1998). We have recently shown that complete loss of the mitochondrial genome (rho^0) results in lower levels of ROS (Rasmussen *et al*., (III)). Therefore, it is tempting to speculate that the *sod1* mutant strain induces Ogg1 in response to high levels of ROS.

Cells deficient in mitochondrial activity are sensitive to H_2O_2 and menadione induced DNA damage.

Studies have shown that rho^- and rho^0 cells, which are respiratorically deficient, are more sensitive to the cytotoxic effect of ROS (Flatter-O'Brien *et al*., 1993; Jamieson, 1992; Collinson and Dawes, 1992; Grant *et al*., 1997). We treated exponentially growing wild type, rho^-, and rho^0 cells with various drugs that affect intracellular levels of ROS to determine the degree of sensitivity and mutation frequencies for each drug (Figure 1). Adriamycin (Doxorubicin) is an anti-tumor drug that can inhibit DNA replication by direct intercalation between DNA base pairs (Singal *et al*., 1997). Single electron donors such as NADPH cytochrome P450 reductase can reduce the quinoid ring in adriamycin to semiquinone. In the presence of molecular oxygen the semiquinone radicals are rapidly oxidized to the parent compound in a process, which generate O_2^- (Olson and Mushlin, 1990). NADPH-cytochrome P450 reductase catalyzes the reduction of menadione leading to the formation of semiquinones that is readily autooxidized generating O_2^- (Lind *et al*., 1982). 4-nitroquinoline (4-NQO) is metabolized *in vivo* to the carcinogenic 4-hydroxyaminoquinoline 1-oxide (4-HAQO), which is oxidized generating H_2O_2 (Hozumi, 1969). However, 4-NQO also undergoes redox cycling to generate superoxide, which can be converted to H_2O_2 and form oxidative DNA damage such as 8-oxoG and DNA strand breaks (Nunoshiba and Demple, 1993; Yamamoto *et al*., 1993). In addition, acylated 4-HAQO reacts with DNA to form stable quinoline-purine monoadducts at the N^2 of guanine and N^6 of adenine and 4-NQO is therefore also known as a "UV-mimetic" (Ramotar *et al*., 1998). Finally, diamide induces oxidation of cysteine SH-residues of proteins present in mitochondrial membranes and this oxidation also induces formation of O_2^- (de Souza and Geibel, 1999).

As expected, based on earlier work (Flatter-O'Brien *et al*., 1993; Jamieson, 1992; Collinson and Dawes, 1992; Grant *et al*., 1997), rho^0 cells are more sensitive to killing by H_2O_2 and menadione compared to wild type. We showed that these agents at the same time increased spontaneous mutations in rho^0 cells (Figure 1A and 1B, Table 5). In contrast, mitochondrial dysfunction mediated resistance to killing by 4-NQO and adriamycin as well as increased 4-NQO-induced mutations (Figure 1D and 1E, Table 5). A phenotype not only observed for yeast as human HeLa rho^0 cells have been shown to be more resistant to adriamycin than HeLa rho^+ cells (Singh *et al*., 1999). We did neither detect any difference in cell survival nor any difference in spontaneous mutations in rho^0 cells after treatment with diamide (Figure 1C, Table 5). These results suggest that antioxidant and/or repair

defense against damage generated by H_2O_2 and menadione is impaired in rho^0 cells. However, the data also indicate that the mutagenic and cytotoxic effect of 4-NQO and adriamycin can be separated in rho^0 cells.

The transcript of *REV1* is induced in rho^0 cells compared to wild type cells (Rasmussen *et al*., III). The Rev1 protein is involved in error-prone translesion synthesis and possesses a transferase activity, which incorporate dCMP opposite abasic site in the DNA template (Lawrence and Maher, 2001). The Rev1 protein is also involved in bypass of a T-T (6-4) UV photoproduct (Nelson *et al*., 2000). Thus, the enzymatic nature of this Rev1p function is not yet fully understood. We are currently testing a Rev1-model to see if Rev1 can bypass DNA lesions introduced by 4-NQO (UV-mimetic) but not H_2O_2 and menadione.

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References

Akashi M, Hachiya M, Paquette RL, Osawa Y, Shimizu S, Suzuki G. Irradiation increases manganese superoxide dismutase mRNA levels in human fibroblasts. Possible mechanisms for its accumulation. J Biol Chem. 1995;270(26):15864-9.

Brambilla L, Cairo G, Sestili P, O'Donnel V, Azzi A, Cantoni O. Mitochondrial respiratory chain deficiency leads to overexpression of antioxidant enzymes. FEBS Lett. 1997;418(3):247-50.

Cadenas E. Biochemistry of oxygen toxicity. Annu Rev Biochem. 1989;58:79-110.

Cadenas E, Davies KJ. Mitochondrial free radical generation, oxidative stress, and aging. Free Radic Biol Med. 2000;29(3-4):222-30.

Collinson LP, Dawes IW. Inducibility of the response of yeast cells to peroxide stress. J Gen Microbiol. 1992;138 (Pt 2):329-335.

Croteau DL, Bohr VA. Repair of oxidative damage to nuclear and mitochondrial DNA in mammalian cells. J Biol Chem. 1997;272(41):25409-12.

de Haan JB, Bladier C, Griffiths P, Kelner M, O'Shea RD, Cheung NS, Bronson RT, Silvestro MJ, Wild S, Zheng SS, Beart PM, Hertzog PJ, Kola I. Mice with a homozygous null mutation for the most abundant glutathione peroxidase, Gpx1, show increased susceptibility to the oxidative stress-inducing agents paraquat and hydrogen peroxide. J Biol Chem. 1998;273(35):22528-36.

de Souza Pereira R, Geibel J. Direct observation of oxidative stress on the cell wall of Saccharomyces cerevisiae strains with atomic force microscopy. Mol Cell Biochem. 1999;201(1-2):17-24.

Elledge SJ. Cell cycle checkpoints: preventing an identity crisis. Science. 1996;274(5293):1664-72.

Esposito LA, Melov S, Panov A, Cottrell BA, Wallace DC. Mitochondrial disease in mouse results in increased oxidative stress. Proc Natl Acad Sci U S A. 1999;96(9):4820-5.

Flattery-O'Brien J, Collinson LP, Dawes IW. Saccharomyces cerevisiae has an inducible response to menadione which differs from that to hydrogen peroxide. J Gen Microbiol. 1993;139:501-7.

Galiazzo F, Schiesser A, Rotilio G. Glutathione peroxidase in yeast. Presence of the enzyme and induction by oxidative conditions. Biochem Biophys Res Commun. 1987;147(3):1200-5.

Gasch AP, Spellman PT, Kao CM, Carmel-Harel O, Eisen MB, Storz G, Botstein D, Brown PO. Genomic expression programs in the response of yeast cells to environmental changes. Mol Biol Cell. 2000;11(12):4241-57.

Gasch AP, Huang M, Metzner S, Botstein D, Elledge SJ, Brown PO. Genomic expression responses to dna-damaging agents and the regulatory role of the yeast atr homolog mec1p. Mol Biol Cell. 2001;12(10):2987-3003.

Gralla EB, Valentine JS. Null mutants of Saccharomyces cerevisiae Cu,Zn superoxide dismutase: characterization and spontaneous mutation rates. J Bacteriol. 1991;173(18):5918-20.

Grant CM, MacIver FH, Dawes IW. Glutathione is an essential metabolite required for resistance to oxidative stress in the yeast Saccharomyces cerevisiae. Curr Genet. 1996;29(6):511-5.

Grant CM, MacIver FH, Dawes IW. Mitochondrial function is required for resistance to oxidative stress in the yeast Saccharomyces cerevisiae. FEBS Lett. 1997;410(2-3):219-22.

Guidot DM, McCord JM, Wright RM, Repine JE. Absence of electron transport (Rho 0 state) restores growth of a manganese-superoxide dismutase deficient Saccharomyces cerevisiae in hyperoxia. Evidence for electron transport as a major source of superoxide generation in vivo. J Biol Chem. 1993;268(35):26699-703.

Halliwell B, Gutterridge JMC. Free Radicals in Biology and Medicine. Oxford University Press, New York, 1985.

Hozumi M. Production of hydrogen peroxide by 4 hydroxyaminoquinoline 1-oxide. Gann. 1969;60(1):83-90.

Inoue Y, Matsuda T, Sugiyama K, Izawa S, Kimura A. Genetic analysis of glutathione peroxidase in oxidative stress response of Saccharomyces cerevisiae. J Biol Chem. 1999;274(38):27002-9.

Jamieson DJ. Saccharomyces cerevisiae has distinct adaptive responses to both hydrogen peroxide and menadione. J Bacteriol. 1992;174(20):6678-81.

Jamieson DJ. Oxidative stress responses of the yeast Saccharomyces cerevisiae. Yeast. 1998;14(16):1511-27.

Jelinsky SA, Samson LD. Global response of Saccharomyces cerevisiae to an alkylating agent. Proc Natl Acad Sci U S A. 1999;96(4):1486-91.

Kang D, Takeshige K, Sekiguchi M, Singh KK. Mitochondrial DNA mutations in aging, disease and cancer. Springer-Verlag and R.G. Landes Company (ed. Keshav K. Singh) 1998;1-15

Lawrence CW, Maher VM. Mutagenesis in eukaryotes dependent on DNA polymerase zeta and Rev1p. Philos Trans R Soc Lond B Biol Sci. 2001;356(1405):41-6.

Lea DE, Coulson CA. The distribution of numbers of mutants in bacterial populations. J Genet. 1949; 49:264-85.

Lind C, Hochstein P, Ernster L. DT-diaphorase as a quinone reductase: a cellular control device against semiquinone and superoxide radical formation. Arch Biochem Biophys. 1982;216(1):178-85.

Liu XF, Elashvili I, Gralla EB, Valentine JS, Lapinskas P, Culotta VC. Yeast lacking superoxide dismutase. Isolation of genetic suppressors. J Biol Chem. 1992;267(26):18298-302.

Longo VD, Gralla EB, Valentine JS. Superoxide dismutase activity is essential for stationary phase survival in Saccharomyces cerevisiae. Mitochondrial production of toxic oxygen species in vivo.
J Biol Chem. 1996;271(21):12275-80.

Longo VD, Ellerby LM, Bredesen DE, Valentine JS, Gralla EB. Human Bcl-2 reverses survival defects in yeast lacking superoxide dismutase and delays death of wild-type yeast. J Cell Biol. 1997;137(7):1581-8.

Marchler G, Schuller C, Adam G, Ruis H. A Saccharomyces cerevisiae UAS element controlled by protein kinase A activates transcription in response to a variety of stress conditions. EMBO J. 1993;12(5):1997-2003.

Marres CA, Van Loon AP, Oudshoorn P, Van Steeg H, Grivell LA, Slater EC. Nucleotide sequence analysis of the nuclear gene coding for manganese superoxide dismutase of yeast mitochondria, a gene previously assumed to code for the Rieske iron-sulphur protein. Eur J Biochem. 1985;147(1):153-61.

Nelson JR, Gibbs PE, Nowicka AM, Hinkle DC, Lawrence CW. Evidence for a second function for Saccharomyces cerevisiae Rev1p. Mol Microbiol. 2000;37(3):549-54.

Mewes HW, Albermann K, Heumann K, Liebl S, Pfeiffer F. MIPS: a database for protein sequences, homology data and yeast genome information. Nucleic Acids Res. 1997;25(1):28-30

Ni TT, Marsischky GT, Kolodner RD. MSH2 and MSH6 are required for removal of adenine misincorporated opposite 8-oxo-guanine in S. cerevisiae. Mol Cell. 1999;4(3):439-44.

Nunoshiba T, Demple B. Potent intracellular oxidative stress exerted by the carcinogen 4-nitroquinoline-N-oxide. Cancer Res. 1993;53(14):3250-2.

Oberley LW, Buettner GR. Role of superoxide dismutase in cancer: a review. Cancer Res. 1979;39(4):1141-9.

Olson RD, Mushlin PS. Doxorubicin cardiotoxicity: analysis of prevailing hypotheses. FASEB J. 1990;4(13):3076-86.

Park JI, Grant CM, Davies MJ, Dawes IW. The cytoplasmic Cu,Zn superoxide dismutase of saccharomyces cerevisiae is required for resistance to freeze-thaw stress. Generation of free radicals during freezing and thawing. J Biol Chem. 1998;273(36):22921-8.

Paul MF, Velours J, Arselin de Chateaubodeau G, Aigle M, Guerin B. The role of subunit 4, a nuclear-encoded protein of the F0 sector of yeast mitochondrial ATP synthase, in the assembly of the whole complex. Eur J Biochem 1989;185(1):163-71

Raha S, Robinson BH. Mitochondria, oxygen free radicals, and apoptosis. Am J Med Genet. 2001;106(1):62-70.

Ramotar D, Belanger E, Brodeur I, Masson JY, Drobetsky EA. A yeast homologue of the human phosphotyrosyl phosphatase activator PTPA is implicated in protection against oxidative DNA damage induced by the model carcinogen 4-nitroquinoline 1-oxide. J Biol Chem. 1998;273(34):21489-96.

Sherman F, Fink GR, Hicks JB. Methods in Yeast Genetics: A Laboratory Manual
Cold Spring Harbor Laboratory Press, Plainview, NY. 1994.

Singal PK, Iliskovic N, Li T, Kumar D. Adriamycin cardiomyopathy: pathophysiology and prevention. FASEB J. 1997;11(12):931-6.

Srinivasan C, Liba A, Imlay JA, Valentine JS, Gralla EB. Yeast lacking superoxide dismutase(s) show elevated levels of "free iron" as measured by whole cell electron paramagnetic resonance. J Biol Chem. 2000;275(38):29187-92.

Von Borstel, R. C. Measuring spontaneous mutation frequencies in yeast. Methods Cell Biol. 1978 (20):1-24

Wallace DC, Melov S. Radicals r'aging. Nat Genet. 1998;19(2):105-6.

Yamamoto K, Inoue S, Kawanishi S. Site-specific DNA damage and 8-hydroxydeoxyguanosine formation by hydroxylamine and 4-hydroxyaminoquinoline 1-oxide in the presence of Cu(II): role of active oxygen species. Carcinogenesis. 1993;14(7):1397-401.

Zhou BB, Elledge SJ. The DNA damage response: putting checkpoints in perspective. Nature. 2000;408(6811):433-9.

Table 1. *Saccharomyces cerevisiae* strains used in this study

	Strain	Genotype
wild type	RKY3109	*MAT***a**, *ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade8, ade2Δ1*
rho$^-$	YAKR145	RKY3109 rho$^-$
rho^0	YAKR144	RKY3109 rho^0
sod1	YAKR129	RKY3109 *sod1*::*URA3*
sod1 rho^0	YAKR163-166	RKY3109 rho^0
sod2	YAKR133	RKY3109 *sod2*::*TRP1*
sod2 rho^0	YAKR135	RKY3109 rho^0
sod1 sod2	YAKR137	RKY3109, *sod1*::*URA3, sod2*::*TRP1*
wild type	DL1	*MATα, met*
rho^0	DL1 rho^0	DL1 rho^0

Table 2. Spontaneous mutation frequencies in superoxide deficient strains

Strain	***can1^r* (x10^{-8})**	**Fold increase**
Wild type	15.3 ± 3.7	1
rho$^-$	34.0 ± 5.0	2.2
rho^0	39.9 ± 11.7	2.6
sod2	30.5± 5.6	2.0
sod2 rho^0	6.4 ± 2.4	0.4
	***can1^r* (x10^{-6})**	
wild type	1.3 ± 0.3	1
sod1	165.5 ± 5.1	127.3
sod1 rho^0	8.9 ± 1.8	6.8

Spontaneous mutation frequency in rho$^-$, rho^0, *sod2*, *sod2 rho^0*, *sod1* and *sod1 rho^0* is significantly different ($P < 0.01$) from wild type, as determined by t-Test: Two-Sample Assuming Equal Variances.

Table 3. Functional classification of DNA repair genes induced by the *SOD1* mutation

Gene	Function	Human homolog	Fold induction in *sod1*
MEC3	Checkpoint control and DNA repair		2.8
RAD9	Checkpoint control and DNA repair	hRAD9	3.4
MSH3	MMR	hMSH3	2.9
MSH4	Meiotic recombination	hMSH4	5.1
NTG2	BER		2
MAG1	BER	AAG	3.2
MGT1	Direct reversal repair	MGMT	6.3
RAD14	NER	XPA	2.4
RAD16	NER		4.2
RAD10	NER	ERCC1	3.1
RAD26	NER	CSB/ERCC6	2.3
RAD28	NER	CSA	2.6
RAD50	DSBR	hRAD50	3.8
RAD52	DSBR	hRAD52	3.1
RAD59	DSBR		2.8
SGS1	DSBR	BLM	2.2
MUS81	DSBR		3.8
PSO2	DNA repair/recombination		2.2
RNC1	DNA repair/recombination		2.8
THI4	Unknown DNA repair function		8.6
LIF1	DSBR	XRCC4	2.6
ECM32	Unknown DNA repair function		2.5
TFB3	NER		2.3

DNA mismatch repair (MMR), Base excision repair (BER), Nucleotide excision repair (NER) and Double strand break repair (DSBR)

Table 4. Functional classification of DNA repair genes induced by the *SOD1* mutation (*sod1* vs *sod1*rho^0)

Gene	Function	Human homolog	Fold induction in *sod1*
RAD9	Checkpoint control and DNA repair	hRAD9	2.2
OGG1	BER	hOGG1	2.5
RAD26	NER	CSB/ERCC6	2.1
RAD28	NER	CSA	2.2
MUS81	DSBR		2.7
CDC2 (POL3)	BER, NER		2.1
FUN30	Unknown DNA repair function		2

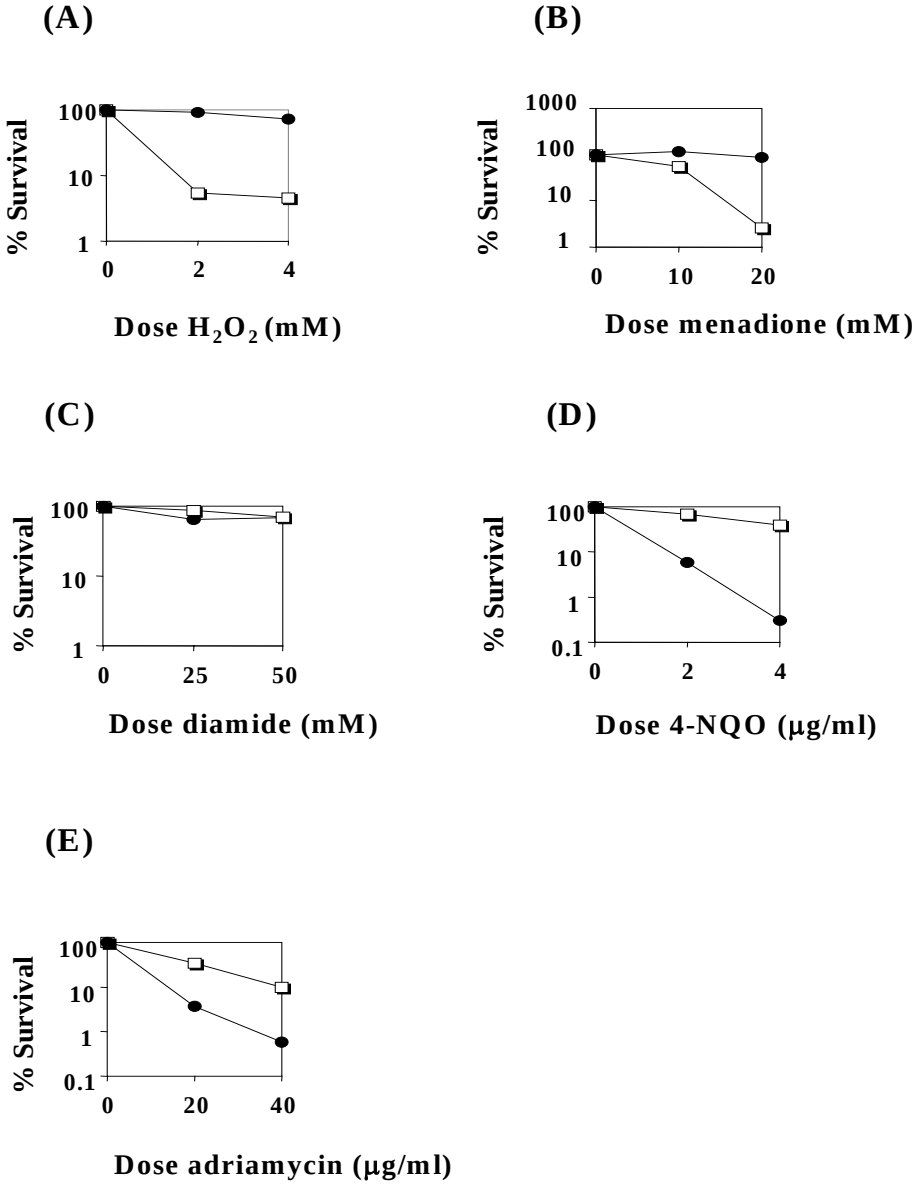
Base excision repair (BER), Nucleotide excision repair (NER) and and Double strand break repair (DSBR)

Table 5. Spontaneous mutation frequency (x10^{-8})										**Spontaneous mutation frequency (x10^{-7})**					
	H_2O_2 (mM)			**Menadione (mM)**			**Diamide (mM)**			**4-NQO (μg/ml)**			**Adriamycin (μg/ml)**		
Conc.	***0***	***2***	***4***	***0***	***10***	***20***	***0***	***25***	***50***	***0***	***2***	***4***	***0***	***20***	***40***
wt	2.5	6.8	4.0	3.6	1.7	2.4	1.6	4.2	2.2	5.6	34.8	39.2	5.0	6.4	9.7
rho^0	3.8	87.6	85.4	6.1	11.6		4.2	5.9	5.8	27.5	158.1	214.8	71.3	115.1	95.3

Figure Legends

Figure 1. Cell survival of wild type and rho^0 cells after drug treatment. Percent survival is expressed relative to the untreated control culture (100%). Cells were treated with various concentrations of (A) H_2O_2, (B) menadione, (C) diamide, (D) 4-NQO, and (E) adriamycin. Each data point represents an average of duplicate measurements from one representative experiment. Closed symbols: wild type. Open symbols: rho^0.

Figure 1.



Supplemental materials

Table • Genes repressed in DL1 vs *sod1*Δ cells

Probe Set Name	ORF	Gene name	AC	Fold Change in DL1	Descriptions
DNA Damage Checkpoint					
10048_at	YLR288C	*MEC3*	P	2.8	Involved in checkpoint control and DNA repair.
6274_at	YDR217C	**RAD9**	P	3.4	Cell cycle arrest protein.
DNA Mismatch Repair					
6787_at	YCR092C	**MSH3**	P	2.9	MutS homolog, forms a complex with Msh2p to repair insertion-deletion mispairs.
Meiotic recombination					
5385_at	YFL003C	**MSH4**	A	5.1	MutS homolog, localizes to discrete sites on meiotic chromosomes, meiosis specific protein.
Base Excision Repair					
Oxidative DNA Repair					
8609_at	YOL043C	**NTG2**	P	2	Endonuclease III-like glycosylase 2.
DNA Alkylation Repair					
5582_at	YER142C	**MAG1**	A	3.2	3-methyladenine DNA glycosylase.
Direct Repair					
6663_at	YDL200C	**MGT1**	A	6.3	6-O-methylguanine-DNA methylase.
Nucleotide Excision Repair					
9428_at	YMR201C	**RAD14**	P	2.4	Human xeroderma pigmentosum group A DNA repair gene homolog.
7250_at	YBR114W	**RAD16**	P	4.2	Radiation repair protein, putative DNA helicase.
9749_at	YML095C	**RAD10**	A	3.1	Endonuclease (with Rad1p) that degrades single-stranded DNA for repair.
9750_at	YML095C	**RAD10**	A	3.2	Questionable ORF
11009_at	YJR035W	**RAD26**	P	2.3	DNA-dependent ATPase, homologous to human Cockayne syndrome B gene ERCC6, that is a putative helicase.
6444_at	YDR030C	**RAD28**	P	2.6	Protein involved in the same pathway as Rad26p, has beta-transducin (WD-40) repeats.
Recombination Repair					
9099_at	YNL250W	**RAD50**	P	3.8	Protein contains a purine-binding domain, two heptad repeats and a hydrophobic tail, Rad50p interacts with Mre11p and Xrs2p in two-hybrid and immunoprecipitation analyses\; it co-localizes to spots with Mre11p and Xrs2p in a rad50s background.
9678_at	YML032C	**RAD52**	A	3.1	Questionable ORF.
6539_at	YDL059C	**RAD59**	P	2.8	A mutation in this gene results in RADiation sensitivity and recombination defects, which are general properties of the RAD52 epistasis group mutants. rad59 is epistatic to rad52 for its repair and recombination defects. The RAD59 gene product has homology to the Rad52 protein.
9461_at	YMR190C	**SGS1**	P	2.2	Has DNA helicase signature motifs.
6083_at	YDR386W	**MUS81**	A	3.8	MMS and UV Sensitive\; Mus81p and Rad54p are found together in a complex from whole-cell extracts.
9491_at	YMR137C	**PSO2**	P	2.2	Interstrand crosslink repair protein.
Other/unknown DNA Repair Function					
10528_at	YKR056W	**RNC1**	P	2.8	Endo-exonuclease yNucR.
4844_at	YGR144W	**THI4**	A	8.6	Component of the biosynthetic pathway producing the thiazole precursor of thiamine.
5107_at	YGL090W	**LIF1**	P	2.6	Ligase Interacting Factor 1\; physically interacts with DNA ligase 4 protein.
5524_at	YER176W	**ECM32**	P	2.5	DNA Helicase I.
6019_at	YDR460W	**TFB3**	P	2.3	TFIIH subunit Tfb3 \; similar to mammalian CAK subunit.

AC = the Absolute Call; The transcript is present (P) or absent (A) for DL1. When the transcript is absent is the fold change an approximation and calculated using the noise level.

4. Concluding Remarks

Increased spontaneous mutation frequency is associated with higher risk of cancer. However, the relative contribution of spontaneous endogenous mutagenesis to carcinogenesis is not yet known. Among the normal cellular metabolic processes that lead to elevated spontaneous mutation rates are (Jackson *et al*., 1998; Rossman & Goncharova, 1998; Rasmussen *et al*., III):

- DNA polymerase errors,
- Depurination, which gives rise to abasic sites,
- Deamination of deoxycytidine residues (C → U and 5-methyl-C → T),
- Methylation of DNA (O^6-methylguanine),
- Damage by oxygen free radicals,
- and *mitochondrial dysfunction*.

Thus, both a main repair pathway and one or more backup pathways can generally remove these lesions. For example the main repair pathway for eliminating mutations, caused by the deoxyguanosine oxidation product 8-oxoG, is BER (hOGG1, hMYH and hMTH). However, studies in *S. cerevisiae* have shown that MSH2-MSH6-dependent MMR is involved in repairing 8-oxoG:A mispairs and 8-oxoG:C base pairs. These results suggest that MMR is functioning as a backup pathway under high oxidative stress (Ni *et al*., 1999). The MMR pathway is also involved in repair of O^6-MeG and O^6-MeG-triggered cell death when MGMT repair capacity is saturated (see 2.3 Direct Repair: O^6-methylguanine-DNA methyl-transferase). Furthermore, it has been suggested that the hMLH1 protein, from the MMR pathway, has an impact on the apoptosis pathway after oxidative stress. The mitochondria were suggested to be involved in this apoptosis pathway because increased mitochondrial permeability and cytochrome *c* release after H_2O_2 treatment were observed preferentially in hMLH proficient cell lines (Hardman *et al*., 2001).

Therefore, it seems like inactivation or dysregulation of the DNA protection system are important for generating a mutator phenotype. Its importance in human disease is supported by the discovery that defects in several kinds of DNA repair are known to raise spontaneous mutation frequency i.e. mutations in MMR genes cause hereditary non-polyposis colon cancer, HNPCC (Hoeijmakers, 2001).

In an attempt to identify new MMR genes, we employed the yeast two-hybrid system, using the human mismatch repair protein hMSH2 (Rasmussen *et al*., 2000) or hMLH1 as bait and a fetal liver cDNA library as prey. We demonstrated that hMSH2 interacts with exonuclease hEXO1 suggesting that hEXO1 plays a role in MMR. Futhermore, we showed that hMSH2 and EXO1 are co-expressed at high levels in fetal liver but not in adult liver where cell turnover is normally very slow. The hEXO1 protein was highly expressed in several liver cancer cell lines as well as in colon and pancreas adenocarcinomas, but not in the corresponding non-neoplastic tissue. These results support a role for hEXO1 in cellular processes such as MMR, recombination repair or replication, which are expected to be active in proliferating cells (Rasmussen *et al*., 2000).

The mismatch repair protein hMLH1 is known to interact with hPMS1. However, the exact biochemical role for the hMLH1-hPMS1 complex has not yet been determined. We showed that hMLH1 interacts with hPMS1 in the yeast two-hybrid system using hMLH1 as bait and fetal liver cDNA library as prey. Dot-blot analysis of multiple tissue RNA revealed that hPMS1 is predominantly expressed in fetal liver, adult liver, pancreas, kidney, testis and appendix. Unfortunately, we do not have the corresponding dot-blot analysis for hPMS2. However, it is tempting to speculate that hPMS1 is tissue specific and that the hMLH1-hPMS1 complex plays an important role in DNA repair in liver. Furthermore, it could be interesting to compare hPMS1 and hPMS2 expression levels in different cancer cell lines with non-neoplastic tissue to investigate if any up or down regulation will occur in the cancer cells.

Mutations in MMR genes are known to cause spontaneous mutations. Defects in translocation of repair proteins could be a way to inactivate the DNA protection system. We present preliminary data showing that hMSH2 and hMLH1 interact with an importin-α homolog and importin-α, respectively. Importin-α is part of the translocation system to the nucleus. Protein-protein interaction and nuclear localization studies with HNPCC-hMSH2 mutants or HNPCC-hMLH1 mutants together with importin-α and the importin-α homolog could be a way to determine if mutations in MMR mutant genes found in HNPCC patients interfere with the translocation system.

Another model to inactivate the DNA repair system is oxidative damage. The mitochondrial theory of aging postulates that accumulation of oxidative damage in the mtDNA leads to mitochondrial dysfunction that results in energy deficiencies in old cells. Declined mitochondrial energy capacity could in turn lead to lack in repair (Ames, 1989).

Our results in *S. cerevisiae* showed that mitochondrial dysfunction caused significantly higher spontaneous mutation frequencies in nDNA compared to wild type and this was not due to increased levels of ROS in the cells. However, we demonstrated that disruption of the respiratory chain function using various mitochondrial inhibitors caused an increase in spontaneous mutation frequencies, indicating that increased mitochondrial ROS production can diffuse to the nucleus where it causes DNA damage. Furthermore, in rho^0 cells we found an up-regulation of Rev1 compared to wild type and we showed that mitochondrial-mediated mutator phenotype of rho^0 cells is suppressed by preventing Rev1/Rev3/Rev7-dependent translesion synthesis (Rasmussen *et al.*, III). Even though we do not know today how mitochondrial dysfunction leads to spontaneous nuclear DNA damage, mitochondrial dysfunction elevates the spontaneous mutation frequencies.

The nuclear (Sod1) and mitochondrial (Sod2) superoxide dismutases eliminate the superoxide anion and thus protect cells from damage induced by free radicals. We find that strains deficient in *SOD* activity increase spontaneous mutations and that this mutator phenotype is suppressed both in *sod1Δ*rho^0 and *sod2Δ*rho^0 mutant strains. Our results suggest that mitochondrial dysfunction either reduces oxidative damage to DNA by preventing formation of ROS and/or increases repair of oxidative DNA damage. We compared gene expression patterns of the *sod1* mutant strain with the *sod1*rho^0 mutant strain in order to identify DNA repair genes involved in this suppression. This analysis suggested that *sod1*rho^0 cells did not increase repair response compared to *sod1* cells. This infers that removal of mitochondrial function reduces intracellular levels of ROS and consequently decreases mutagenesis caused by oxidative damage to DNA (Rasmussen *et al.*, IV).

We found that both H_2O_2 and menadione treatments reduced cell survival of rho^0 cells but at the same time increased spontaneous mutations in these cells compared to wild type. Gene expression analysis showed that the induced antioxidant enzymes in rho^0 cells were different from the ones induced in the wild type. Implying that defense against oxidative

damage generated by H_2O_2 and Menadione is different in rho^0 cells. These results emphasize the significance of mitochondrial activity in controlling cell death as well as mutational events following DNA damage. Proteins involved in such control mechanisms have been identified and include checkpoint proteins such as the human tumor suppressor protein p53. It is therefore tempting to consider mitochondria as a new guardian of genomic stability.

Given the fact that the integrity of the mtDNA is important it could be beneficial to understand the molecular processes and components responsible for mtDNA repair. Most DNA glycosylases that have been identified in the nucleus have also been identified in mitochondria, which indicates that BER is operating in mitochondria.
It has not yet been determined if mitochondrial DNA is repaired by MMR. Our two-hybrid screenings with hMSH2 or hMLH1 as baits and a fetal liver cDNA library as prey gave no answers to this question. However, we found that full-length MGMT, when expressed as a GFP fusion protein in human MCF12A (breast) epithelial cells, were localized to the nuclei but not to the mitochondria (Rasmussen *et al*., II). These data suggest that the alkyltransferase mechanism, which seems to be operating within the mitochondrion in mammalian species, is not MGMT in human breast epithelial cells.

Expression of the mitochondrial genes are required to maintain proper function of the organelle, suggesting that even a slight alteration of mitochondrial function may have profound effects. Interestingly, one of the profound features of cancer cells is their defective mitochondrial function (Warburg, 1956; Polyak *et al*., 1998; Fliss *et al*., 2000).

References

Acharya S, Wilson T, Gradia S, Kane MF, Guerrette S, Marsischky GT, Kolodner R, Fishel R. hMSH2 forms specific mispair-binding complexes with hMSH3 and hMSH6. Proc Natl Acad Sci U S A. 1996;93(24):13629-34.

Allen DJ, Makhov A, Grilley M, Taylor J, Thresher R, Modrich P, Griffith JD. MutS mediates heteroduplex loop formation by a translocation mechanism. EMBO J. 1997;16(14):4467-76.

Altshuler KB, Hodes CS, Essigmann JM. Intrachromosomal probes for mutagenesis by alkylated DNA bases replicated in mammalian cells: a comparison of the mutagenicities of O^4-methylthymine and O^6-methylguanine in cells with different DNA repair backgrounds. Chem Res Toxicol. 1996;9(6):980-7.

Ames BN. Endogenous oxidative DNA damage, aging, and cancer. Free Radic Res Commun. 1989;7(3-6):121-8.

Anderson CT, Friedberg EC. The presence of nuclear and mitochondrial uracil-DNA glycosylase in extracts of human KB cells. Nucleic Acids Res. 1980;8(4):875-88.

Anderson S, Bankier AT, Barrell BG, de Bruijn MH, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJ, Staden R, Young IG. Sequence and organization of the human mitochondrial genome. Nature. 1981;290(5806):457-65.

Ayi TC, Loh KC, Ali RB, Li BF. Intracellular localization of human DNA repair enzyme methylguanine-DNA methyltransferase by antibodies and its importance. Cancer Res. 1992;52(23):6423-30.

Bai C, Elledge SJ. Searching for interaction proteins with the Two-Hybrid system I (The yeast Two-Hybrid system ed. Bartel PL, Fields S) Oxford University Press, Inc.,New York, NY. 1997:11-28

Bai J, Rodriguez AM, Melendez JA, Cederbaum AI. Overexpression of catalase in cytosolic or mitochondrial compartment protects HepG2 cells against oxidative injury. J Biol Chem. 1999;274(37):26217-24.

Bai J, Cederbaum AI. Mitochondrial catalase and oxidative injury. Biol Signals Recept. 2001;10(3-4):189-99.

Bailly V, Verly WG, O'Connor T, Laval J. Mechanism of DNA strand nicking at apurinic/apyrimidinic sites by *Escherichia coli* [formamidopyrimidine]DNA glycosylase. Biochem J. 1989;262(2):581-9.

Bambara RA, Murante RS, Henricksen LA. Enzymes and reactions at the eukaryotic DNA replication fork. J Biol Chem. 1997;272(8):4647-50.

Belanich M, Randall T, Pastor MA, Kibitel JT, Alas LG, Dolan ME, Schold SC Jr, Gander M, Lejeune FJ, Li BF, White AB, Wasserman P, Citron ML, Yarosh DB. Intracellular Localization and intercellular heterogeneity of the human DNA repair protein *O^6*-methylguanine-DNA methyltransferase. Cancer Chemother Pharmacol. 1996;37(6):547-55.

Bestwick RK, Moffett GL, Mathews CK. Selective expansion of mitochondrial nucleoside triphosphate pools in antimetabolite-treated HeLa cells. J Biol Chem. 1982;257(16):9300-4.

Bhattacharyya NP, Skandalis A, Ganesh A, Groden J, Meuth M. Mutator phenotypes in human colorectal carcinoma cell lines. Proc Natl Acad Sci U S A. 1994;91(14):6319-23.

Bianchi NO, Bianchi MS, Richard SM. Mitochondrial genome instability in human cancers. Mutat Res. 2001;488(1):9-23.

Bogdanov MB, Ramos LE, Xu Z, Beal MF. Elevated "hydroxyl radical" generation in vivo in an animal model of amyotrophic lateral sclerosis. J Neurochem. 1998;71(3):1321-4.

Boldogh I, Milligan D, Lee MS, Bassett H, Lloyd RS, McCullough AK. hMYH cell cycle-dependent expression, subcellular localization and association with replication foci: evidence suggesting replication-coupled repair of adenine:8-oxoguanine mispairs. Nucleic Acids Res. 2001;29(13):2802-9.

Boulikas T. Nuclear import of DNA repair proteins. Anticancer Res. 1997;17(2A):843-63.

Bouziane M, Miao F, Bates SE, Somsouk L, Sang BC, Denissenko M, O'Connor TR. Promoter structure and cell cycle dependent expression of the human methylpurine-DNA glycosylase gene.
Mutat Res. 2000;461(1):15-29.

Boveris A. Mitochondrial production of superoxide radical and hydrogen peroxide.
Adv Exp Med Biol. 1977;78:67-82.

Boveris A, Chance B. The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. Biochem J. 1973;134(3):707-16.

Bowers J, Tran PT, Joshi A, Liskay RM, Alani E.
MSH-MLH complexes formed at a DNA mismatch are disrupted by the PCNA sliding clamp.
J Mol Biol. 2001 Mar 9;306(5):957-68.

Bronstein SM, Cochrane JE, Craft TR, Swenberg JA, Skopek TR. Toxicity, mutagenicity, and mutational spectra of N-ethyl-N-nitrosourea in human cell lines with different DNA repair phenotypes.
Cancer Res. 1991;51(19):5188-97.

Bronstein SM, Skopek TR, Swenberg JA. Efficient repair of O^6-ethylguanine, but not O^4-ethylthymine or O2-ethylthymine, is dependent upon O^6-alkylguanine-DNA alkyltransferase and nucleotide excision repair activities in human cells. Cancer Res. 1992;52(7):2008-11.

Bruijn LI, Becher MW, Lee MK, Anderson KL, Jenkins NA, Copeland NG, Sisodia SS, Rothstein JD, Borchelt DR, Price DL, Cleveland DW. ALS-linked SOD1 mutant G85R mediates damage to astrocytes and promotes rapidly progressive disease with SOD1-containing inclusions. Neuron. 1997;18(2):327-38.

Buermeyer AB, Deschenes SM, Baker SM, Liskay RM. Mammalian DNA mismatch repair.
Annu Rev Genet. 1999;33:533-64.

Burdett V, Baitinger C, Viswanathan M, Lovett ST, Modrich P. In vivo requirement for RecJ, ExoVII, ExoI, and ExoX in methyl-directed mismatch repair. Proc Natl Acad Sci U S A. 2001;98(12):6765-70.

Burrows CJ, Muller JG. Oxidative nucleobase modifications leading to strand scission.
Chem. Rev. 1998;98:1109-51.

Cadenas E, Davies KJ. Mitochondrial free radical generation, oxidative stress, and aging.
Free Radic Biol Med. 2000;29(3-4):222-30.

Carethers JM, Hawn MT, Chauhan DP, Luce MC, Marra G, Koi M, Boland CR. Competency in mismatch repair prohibits clonal expansion of cancer cells treated with N-methyl-N'-nitro-N-nitrosoguanidine.
J Clin Invest. 1996;98(1):199-206.

Ceccotti S, Aquilina G, Macpherson P, Yamada M, Karran P, Bignami M. Processing of O^6-methylguanine by mismatch correction in human cell extracts. Curr Biol. 1996;6(11):1528-31.

Ceska TA, Sayers JR. Structure-specific DNA cleavage by 5' nucleases.
Trends Biochem Sci. 1998;23(9):331-6.

Chance B, Sies H, Boveris A. Hydroperoxide metabolism in mammalian organs.
Physiol Rev. 1979;59(3):527-605.

Cheng KC, Cahill DS, Kasai H, Nishimura S, Loeb LA. 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G→T and A→C substitutions. J Biol Chem. 1992;267(1):166-72.

Chen C, Merrill BJ, Lau PJ, Holm C, Kolodner RD. Saccharomyces cerevisiae pol30 (proliferating cell nuclear antigen) mutations impair replication fidelity and mismatch repair. Mol Cell Biol. 1999;19(11):7801-15.

Chien CT, Bartel PL, Sternglanz R, Fields S. The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest. Proc Natl Acad Sci U S A. 1991;88(21):9578-82.

Christmann M, Kaina B. Nuclear translocation of mismatch repair proteins MSH2 and MSH6 as a response of cells to alkylating agents. J Biol Chem. 2000;275(46):36256-62.

Clayton DA, Doda JN, Friedberg EC. The absence of a pyrimidine dimer repair mechanism in mammalian mitochondria. Proc Natl Acad Sci U S A. 1974;71(7):2777-81.

Crapo JD, Oury T, Rabouille C, Slot JW, Chang LY. Copper,zinc superoxide dismutase is primarily a cytosolic protein in human cells. Proc Natl Acad Sci U S A. 1992;89(21):10405-9.

Crone TM, Pegg AE. A single amino acid change in human O^6-alkylguanine-DNA alkyltransferase decreasing sensitivity to inactivation by O^6-benzylguanine. Cancer Res. 1993;53(20):4750-3.

Crone TM, Goodtzova K, Pegg AE. Amino acid residues affecting the activity and stability of human O^6-alkylguanine-DNA alkyltransferase. Mutat Res. 1996;363(1):15-25.

Croteau DL, Bohr VA. Repair of oxidative damage to nuclear and mitochondrial DNA in mammalian cells. J Biol Chem. 1997;272(41):25409-12.

Croteau DL, Stierum RH, Bohr VA. Mitochondrial DNA repair pathways. Mutat Res. 1999;434(3):137-48.

de Haan JB, Bladier C, Griffiths P, Kelner M, O'Shea RD, Cheung NS, Bronson RT, Silvestro MJ, Wild S, Zheng SS, Beart PM, Hertzog PJ, Kola I. Mice with a homozygous null mutation for the most abundant glutathione peroxidase, Gpx1, show increased susceptibility to the oxidative stress-inducing agents paraquat and hydrogen peroxide. J Biol Chem. 1998;273(35):22528-36.

de Laat WL, Jaspers NG, Hoeijmakers JH. Molecular mechanism of nucleotide excision repair. Genes Dev. 1999;13(7):768-85.

de Souza-Pinto NC, Hogue BA, Bohr VA. DNA repair and aging in mouse liver: 8-oxodG glycosylase activity increase in mitochondrial but not in nuclear extracts. Free Radic Biol Med. 2001;30(8):916-23.

de Vries S, Marres CA. The mitochondrial respiratory chain of yeast. Structure and biosynthesis and the role in cellular metabolism. Biochim Biophys Acta. 1987;895(3):205-39.

de Vries S, Grivell LA. Purification and characterization of a rotenone-insensitive NADH:Q6 oxidoreductase from mitochondria of Saccharomyces cerevisiae. Eur J Biochem. 1988;176(2):377-84.

De Vries S, Van Witzenburg R, Grivell LA, Marres CA. Primary structure and import pathway of the rotenone-insensitive NADH-ubiquinone oxidoreductase of mitochondria from Saccharomyces cerevisiae. Eur J Biochem. 1992;203(3):587-92.

DiMauro S, Schon EA. Mitochondrial DNA mutations in human disease. Am J Med Genet. 2001;106(1):18-26.

Dobson AW, Xu Y, Kelley MR, LeDoux SP, Wilson GL. Enhanced mitochondrial DNA repair and cellular survival after oxidative stress by targeting the human 8-oxoguanine glycosylase repair enzyme to mitochondria. J Biol Chem. 2000;275(48):37518-23.

Dosanjh MK, Galeros G, Goodman MF, Singer B. Kinetics of extension of O^6-methylguanine paired with cytosine or thymine in defined oligonucleotide sequences. Biochemistry. 1991;30(49):11595-9.

Dosanjh MK, Menichini P, Eritja R, Singer B. Both O^4-methylthymine and O^4-ethylthymine preferentially form alkyl T.G pairs that do not block in vitro replication in a defined sequence. Carcinogenesis. 1993;14(9):1915-9.

Dosch J, Christmann M, Kaina B. Mismatch G-T binding activity and MSH2 expression is quantitatively related to sensitivity of cells to methylating agents. Carcinogenesis. 1998;19(4):567-73.

Drummond JT, Genschel J, Wolf E, Modrich P. DHFR/MSH3 amplification in methotrexate-resistant cells alters the hMutSalpha/hMutSbeta ratio and reduces the efficiency of base-base mismatch repair. Proc Natl Acad Sci U S A. 1997;94(19):10144-9.

Duckett DR, Drummond JT, Murchie AI, Reardon JT, Sancar A, Lilley DM, Modrich P. Human MutSalpha recognizes damaged DNA base pairs containing O6-methylguanine, O4-methylthymine, or the cisplatin-d(GpG) adduct. Proc Natl Acad Sci U S A. 1996;93(13):6443-7.

Edara S, Kanugula S, Pegg AE. Expression of the inactive C145A mutant human O6-alkylguanine-DNA alkyltransferase in E.coli increases cell killing and mutations by N-methyl-N'-nitro-N-nitrosoguanidine. Carcinogenesis. 1999;20(1):103-8.

Encell LP, Loeb LA. Enhanced in vivo repair of O(4)-methylthymine by a mutant human DNA alkyltransferase. Carcinogenesis. 2000;21(7):1397-402.

Eshleman JR, Lang EZ, Bowerfind GK, Parsons R, Vogelstein B, Willson JK, Veigl ML, Sedwick WD, Markowitz SD. Increased mutation rate at the hprt locus accompanies microsatellite instability in colon cancer. Oncogene. 1995;10(1):33-7.

Esposito LA, Melov S, Panov A, Cottrell BA, Wallace DC. Mitochondrial disease in mouse results in increased oxidative stress. Proc Natl Acad Sci U S A. 1999;96(9):4820-5.

Evans AR, Limp-Foster M, Kelley MR. Going APE over ref-1. Mutat Res. 2000;461(2):83-108.

Fang WH, Modrich P. Human strand-specific mismatch repair occurs by a bidirectional mechanism similar to that of the bacterial reaction. J Biol Chem. 1993;268(16):11838-44.

Fields S, Song O. A novel genetic system to detect protein-protein interactions.
Nature. 1989;340(6230):245-6.

Fink D, Aebi S, Howell SB. The role of DNA mismatch repair in drug resistance. Clin Cancer Res. 1998;4(1):1-6.

Finkel T, Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. Nature. 2000;408(6809):239-47.

Flint DH, Tuminello JF, Emptage MH. The inactivation of Fe-S cluster containing hydro-lyases by superoxide. J Biol Chem. 1993;268(30):22369-76.

Fliss MS, Usadel H, Caballero OL, Wu L, Buta MR, Eleff SM, Jen J, Sidransky D. Facile detection of mitochondrial DNA mutations in tumors and bodily fluids. Science. 2000;287(5460):2017-9.

Flores-Rozas H, Kolodner RD. The Saccharomyces cerevisiae MLH3 gene functions in MSH3-dependent suppression of frameshift mutations. Proc Natl Acad Sci U S A. 1998;95(21):12404-9.

Flores-Rozas H, Clark D, Kolodner RD. Proliferating cell nuclear antigen and Msh2p-Msh6p interact to form an active mispair recognition complex. Nat Genet. 2000;26(3):375-8.

Foury F, Roganti T, Lecrenier N, Purnelle B. The complete sequence of the mitochondrial genome of Saccharomyces cerevisiae. FEBS Lett. 1998;440(3):325-31.

Frey TG, Mannella CA. The internal structure of mitochondria. Trends Biochem Sci. 2000;25(7):319-24.

Fung H, Kow YW, Van Houten B, Taatjes DJ, Hatahet Z, Janssen YM, Vacek P, Faux SP, Mossman BT.

Asbestos increases mammalian AP-endonuclease gene expression, protein levels, and enzyme activity in mesothelial cells. Cancer Res. 1998;58(2):189-94.

Galloway SM, Greenwood SK, Hill RB, Bradt CI, Bean CL. A role for mismatch repair in production of chromosome aberrations by methylating agents in human cells. Mutat Res. 1995;346(4):231-45.

Gardner PR, Raineri I, Epstein LB, White CW. Superoxide radical and iron modulate aconitase activity in mammalian cells. J Biol Chem. 1995;270(22):13399-405.

Gary R, Ludwig DL, Cornelius HL, MacInnes MA, Park MS. The DNA repair endonuclease XPG binds to proliferating cell nuclear antigen (PCNA) and shares sequence elements with the PCNA-binding regions of FEN-1 and cyclin-dependent kinase inhibitor p21. J Biol Chem. 1997;272(39):24522-9.

Gary R, Park MS, Nolan JP, Cornelius HL, Kozyreva OG, Tran HT, Lobachev KS, Resnick MA, Gordenin DA. A novel role in DNA metabolism for the binding of Fen1/Rad27 to PCNA and implications for genetic risk. Mol Cell Biol. 1999;19(8):5373-82.

Genschel J, Littman SJ, Drummond JT, Modrich P. Isolation of MutSbeta from human cells and comparison of the mismatch repair specificities of MutSbeta and MutSalpha. J Biol Chem. 1998;273(31):19895-901.

Glassner BJ, Rasmussen LJ, Najarian MT, Posnick LM, Samson LD. Generation of a strong mutator phenotype in yeast by imbalanced base excision repair. Proc Natl Acad Sci U S A. 1998;95(17):9997-10002.

Gomes XV, Burgers PM. Two modes of FEN1 binding to PCNA regulated by DNA. EMBO J. 2000;19(14):3811-21.

Goodtzova K, Kanugula S, Edara S, Pegg AE. Investigation of the role of tyrosine-114 in the activity of human O6-alkylguanine-DNA alkyltranferase. Biochemistry. 1998;37(36):12489-95.

Gralla EB, Valentine JS. Null mutants of Saccharomyces cerevisiae Cu,Zn superoxide dismutase: characterization and spontaneous mutation rates. J Bacteriol. 1991;173(18):5918-20.

Gu L, Hong Y, McCulloch S, Watanabe H, Li GM. ATP-dependent interaction of human mismatch repair proteins and dual role of PCNA in mismatch repair. Nucleic Acids Res. 1998;26(5):1173-8.

Guerrette S, Wilson T, Gradia S, Fishel R. Interactions of human hMSH2 with hMSH3 and hMSH2 with hMSH6: examination of mutations found in hereditary nonpolyposis colorectal cancer. Mol Cell Biol. 1998;18(11):6616-23.

Guerrette S, Acharya S, Fishel R. The interaction of the human MutL homologues in hereditary nonpolyposis colon cancer. J Biol Chem. 1999;274(10):6336-41.

Guidot DM, McCord JM, Wright RM, Repine JE. Absence of electron transport (Rho 0 state) restores growth of a manganese-superoxide dismutase-deficient Saccharomyces cerevisiae in hyperoxia. Evidence for electron transport as a major source of superoxide generation in vivo. J Biol Chem. 1993;268(35):26699-703.

Gurney ME, Pu H, Chiu AY, Dal Canto MC, Polchow CY, Alexander DD, Caliendo J, Hentati A, Kwon YW, Deng HX, et al. Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. Science. 1994;264(5166):1772-5.

Görlich D. Transport into and out of the cell nucleus. EMBO J. 1998;17(10):2721-7.

Habraken Y, Sung P, Prakash L, Prakash S. A conserved 5' to 3' exonuclease activity in the yeast and human nucleotide excision repair proteins RAD2 and XPG. J Biol Chem. 1994;269(50):31342-5.

Hall MC, Jordan JR, Matson SW. Evidence for a physical interaction between the *Escherichia coli* methyl-directed mismatch repair proteins MutL and UvrD. EMBO J. 1998;17(5):1535-41.

Hall MC, Matson SW. The *Escherichia coli* MutL protein physically interacts with MutH and stimulates the MutH-associated endonuclease activity. J Biol Chem. 1999;274(3):1306-12.

Hampson R, Humbert O, Macpherson P, Aquilina G, Karran P. Mismatch repair defects and O^6-methylguanine-DNA methyltransferase expression in acquired resistance to methylating agents in human cells. J Biol Chem. 1997;272(45):28596-606.

Hardman RA, Afshari CA, Barrett JC. Involvement of mammalian MLH1 in the apoptotic response to peroxide-induced oxidative stress. Cancer Res. 2001;61(4):1392-7.

Harfe BD, Jinks-Robertson S. DNA mismatch repair and genetic instability. Annu Rev Genet. 2000;34:359-399.

A - Harrington JJ, Lieber MR. The characterization of a mammalian DNA structure-specific endonuclease. EMBO J. 1994;13(5):1235-46.

B - Harrington JJ, Lieber MR. Functional domains within FEN-1 and RAD2 define a family of structure-specific endonucleases: implications for nucleotide excision repair. Genes Dev. 1994;8(11):1344-55.

Harris LC, Potter PM, Margison GP. Site directed mutagenesis of two cysteine residues in the E. coli ogt O^6-alkylguanine DNA alkyltransferase protein. Biochem Biophys Res Commun. 1992;187(1):425-31.

Hayakawa H, Koike G, Sekiguchi M. Expression and cloning of complementary DNA for a human enzyme that repairs O^6-methylguanine in DNA. J Mol Biol. 1990;213(4):739-47.

Hazra TK, Roy R, Biswas T, Grabowski DT, Pegg AE, Mitra S. Specific recognition of O6-methylguanine in DNA by active site mutants of human O^6-methylguanine-DNA methyltransferase. Biochemistry. 1997;36(19):5769-76.

Hazra TK, Izumi T, Maidt L, Floyd RA, Mitra S. The presence of two distinct 8-oxoguanine repair enzymes in human cells: their potential complementary roles in preventing mutation. Nucleic Acids Res. 1998;26(22):5116-22.

Hickman MJ, Samson LD. Role of DNA mismatch repair and p53 in signaling induction of apoptosis by alkylating agents. Proc Natl Acad Sci U S A. 1999;96(19):10764-9.

Hoeijmakers JH. Genome maintenance mechanisms for preventing cancer. Nature. 2001;411(6835):366-74.

Holmes J Jr, Clark S, Modrich P. Strand-specific mismatch correction in nuclear extracts of human and Drosophila melanogaster cell lines. Proc Natl Acad Sci U S A. 1990;87(15):5837-41.

Huang JC, Hsu DS, Kazantsev A, Sancar A. Substrate spectrum of human excinuclease: repair of abasic sites, methylated bases, mismatches, and bulky adducts. Proc Natl Acad Sci U S A. 1994;91(25):12213-7.

Huang LC, Clarkin KC, Wahl GM. Sensitivity and selectivity of the DNA damage sensor responsible for activating p53-dependent G1 arrest. Proc Natl Acad Sci U S A. 1996;93(10):4827-32.

Humbert O, Fiumicino S, Aquilina G, Branch P, Oda S, Zijno A, Karran P, Bignami M. Mismatch repair and differential sensitivity of mouse and human cells to methylating agents. Carcinogenesis. 1999;20(2):205-14.

Ikeda S, Biswas T, Roy R, Izumi T, Boldogh I, Kurosky A, Sarker AH, Seki S, Mitra S. Purification and characterization of human NTH1, a homolog of *Escherichia coli* endonuclease III. Direct identification of Lys-212 as the active nucleophilic residue. J Biol Chem. 1998;273(34):21585-93.

Imlay JA, Linn S. DNA damage and oxygen radical toxicity. Science. 1988;240(4857):1302-9.

A - Ishibashi T, Nakabeppu Y, Sekiguchi M. Artificial control of nuclear translocation of DNA repair methyltransferase. J Biol Chem. 1994;269(10):7645-50.

B - Ishibashi T, Nakabeppu Y, Kawate H, Sakumi K, Hayakawa H, Sekiguchi M. Intracellular localization and function of DNA repair methyltransferase in human cells. Mutat Res. 1994;315(3):199-212.

Ishii N, Fujii M, Hartman PS, Tsuda M, Yasuda K, Senoo-Matsuda N, Yanase S, Ayusawa D, Suzuki K. A mutation in succinate dehydrogenase cytochrome b causes oxidative stress and ageing in nematodes. Nature. 1998;394(6694):694-7.

Izumi T, Hazra TK, Boldogh I, Tomkinson AE, Park MS, Ikeda S, Mitra S. Requirement for human AP endonuclease 1 for repair of 3'-blocking damage at DNA single-strand breaks induced by reactive oxygen species. Carcinogenesis. 2000;21(7):1329-34.

Jackson AL, Newcomb TG, Loeb LA. Origin of multiple mutations in human cancers. Drug Metab Rev. 1998;30(2):285-304.

Jans DA, Chan CK, Huebner S. Signals mediating nuclear targeting and their regulation: application in drug delivery. Med Res Rev. 1998;18(4):189-223.

Jans DA, Xiao CY, Lam MH. Nuclear targeting signal recognition: a key control point in nuclear transport? Bioessays. 2000;22(6):532-44.

Jiricny J, Nystrom-Lahti M. Mismatch repair defects in cancer. Curr Opin Genet Dev. 2000 Apr;10(2):157-61.

Johnson RE, Kovvali GK, Prakash L, Prakash S. Requirement of the yeast RTH1 5' to 3' exonuclease for the stability of simple repetitive DNA. Science. 1995;269(5221):238-40.

Johnson RE, Kovvali GK, Guzder SN, Amin NS, Holm C, Habraken Y, Sung P, Prakash L, Prakash S. Evidence for involvement of yeast proliferating cell nuclear antigen in DNA mismatch repair. J Biol Chem. 1996;271(45):27987-90.

Jonassen T, Clarke CF. Isolation and functional expression of human COQ3, a gene encoding a methyltransferase required for ubiquinone biosynthesis. J Biol Chem. 2000;275(17):12381-7.

Jones JB, Song JJ, Hempen PM, Parmigiani G, Hruban RH, Kern SE. Detection of mitochondrial DNA mutations in pancreatic cancer offers a "mass"-ive advantage over detection of nuclear DNA mutations. Cancer Res. 2001;61(4):1299-304.

Joseph-Horne T, Hollomon DW, Wood PM. Fungal respiration: a fusion of standard and alternative components. Biochim Biophys Acta. 2001;1504(2-3):179-95.

Jäger AC, Rasmussen M, Bisgaard HC, Singh KK, Nielsen FC, Rasmussen LJ. HNPCC mutations in the human DNA mismatch repair gene hMLH1 influence assembly of hMutLalpha and hMLH1-hEXO1 complexes. Oncogene. 2001;20(27):3590-5.

Kaina B, Ziouta A, Ochs K, Coquerelle T. Chromosomal instability, reproductive cell death and apoptosis induced by O^6-methylguanine in Mex-, Mex+ and methylation-tolerant mismatch repair compromised cells: facts and models. Mutat Res. 1997;381(2):227-41.

Kang D, Nishida J, Iyama A, Nakabeppu Y, Furuichi M, Fujiwara T, Sekiguchi M, Takeshige K. Intracellular localization of 8-oxo-dGTPase in human cells, with special reference to the role of the enzyme in mitochondria. J Biol Chem. 1995;270(24):14659-65.

Kang D, Takeshige K, Sekiguchi M, Singh KK. Mitochondrial DNA mutations in aging, disease and cancer. Springer-Verlag and R.G. Landes Company (ed. Keshav K. Singh) 1998:1-15

Kanugula S, Goodtzova K, Edara S, Pegg AE. Alteration of arginine-128 to alanine abolishes the ability of human O^6-alkylguanine-DNA alkyltransferase to repair methylated DNA but has no effect on its reaction with O6-benzylguanine. Biochemistry. 1995;34(21):7113-9.

Karran P, Bignami M. DNA damage tolerance, mismatch repair and genome instability. Bioessays. 1994;16(11):833-9.

Karran P, Hampson R. Genomic instability and tolerance to alkylating agents. Cancer Surv. 1996;28:69-85.

Kasai H, Tanooka H, Nishimura S. Formation of 8-hydroxyguanine residues in DNA by X-irradiation. Gann. 1984;75(12):1037-9.

Kawate H, Sakumi K, Tsuzuki T, Nakatsuru Y, Ishikawa T, Takahashi S, Takano H, Noda T, Sekiguchi M. Separation of killing and tumorigenic effects of an alkylating agent in mice defective in two of the DNA repair genes. Proc Natl Acad Sci U S A. 1998;95(9):5116-20.

Kleczkowska HE, Marra G, Lettieri T, Jiricny J. hMSH3 and hMSH6 interact with PCNA and colocalize with it to replication foci. Genes Dev. 2001;15(6):724-36.

Klein JC, Bleeker MJ, Roelen HC, Rafferty JA, Margison GP, Brugghe HF, van den Elst H, van der Marel GA, van Boom JH, Kriek E, et al. Role of nucleotide excision repair in processing of O4-alkylthymines in human cells. J Biol Chem. 1994;269(41):25521-8.

Khrapko K, Coller HA, Andre PC, Li XC, Hanekamp JS, Thilly WG. Mitochondrial mutational spectra in human cells and tissues. Proc Natl Acad Sci U S A. 1997;94(25):13798-803.

Kim K, Biade S, Matsumoto Y. Involvement of flap endonuclease 1 in base excision DNA repair. J Biol Chem. 1998;273(15):8842-8.

Kleibl K, Margison GP. Increasing DNA repair capacity in bone marrow by gene transfer as a prospective tool in cancer therapy. Neoplasma. 1998;45(4):181-6.

Kogelnik AM, Lott MT, Brown MD, Navathe SB, Wallace DC. MITOMAP: a human mitochondrial genome database--1998 update. Nucleic Acids Res. 1998;26(1):112-5.

Köhler M, Speck C, Christiansen M, Bischoff FR, Prehn S, Haller H, Gorlich D, Hartmann E. Evidence for distinct substrate specificities of importin alpha family members in nuclear protein import. Mol Cell Biol. 1999;19(11):7782-91.

Kokoska RJ, Stefanovic L, Buermeyer AB, Liskay RM, Petes TD. A mutation of the yeast gene encoding PCNA destabilizes both microsatellite and minisatellite DNA sequences. Genetics. 1999;151(2):511-9.

Kokoszka JE, Coskun P, Esposito LA, Wallace DC. Increased mitochondrial oxidative stress in the Sod2 (+/-) mouse results in the age-related decline of mitochondrial function culminating in increased apoptosis. Proc Natl Acad Sci U S A. 2001;98(5):2278-83.

Kolodner RD, Marsischky GT. Eukaryotic DNA mismatch repair. Curr Opin Genet Dev. 1999;9(1):89-96.

Kondo E, Horii A, Fukushige S. The interacting domains of three MutL heterodimers in man: hMLH1 interacts with 36 homologous amino acid residues within hMLH3, hPMS1 and hPMS2. Nucleic Acids Res. 2001;29(8):1695-702.

Kooistra R, Zonneveld JB, Watson AJ, Margison GP, Lohman PH, Pastink A. Identification and characterisation of the Drosophila melanogaster O^6-alkylguanine-DNA alkyltransferase cDNA. Nucleic Acids Res. 1999;27(8):1795-801.

Kreklau EL, Kurpad C, Williams DA, Erickson LC. Prolonged inhibition of O^6-methylguanine DNA methyltransferase in human tumor cells by O^6-benzylguanine in vitro and in vivo. J Pharmacol Exp Ther. 1999;291(3):1269-75.

Kuchino Y, Mori F, Kasai H, Inoue H, Iwai S, Miura K, Ohtsuka E, Nishimura S. Misreading of DNA templates containing 8-hydroxydeoxyguanosine at the modified base and at adjacent residues. Nature. 1987;327(6117):77-9.

Lahue RS, Au KG, Modrich P. DNA mismatch correction in a defined system. Science. 1989;245(4914):160-4.

Lakshmipathy U, Campbell C. The human DNA ligase III gene encodes nuclear and mitochondrial proteins. Mol Cell Biol. 1999;19(5):3869-76.

Lakshmipathy U, Campbell C. Antisense-mediated decrease in DNA ligase III expression results in reduced mitochondrial DNA integrity. Nucleic Acids Res. 2001;29(3):668-76.

Larson K, Sahm J, Shenkar R, Strauss B. Methylation-induced blocks to in vitro DNA replication. Mutat Res. 1985;150(1-2):77-84.

Lawrence CW, Maher VM. Mutagenesis in eukaryotes dependent on DNA polymerase zeta and Rev1p. Philos Trans R Soc Lond B Biol Sci. 2001;356(1405):41-6.

Lebovitz RM, Zhang H, Vogel H, Cartwright J Jr, Dionne L, Lu N, Huang S, Matzuk MM. Neurodegeneration, myocardial injury, and perinatal death in mitochondrial superoxide dismutase-deficient mice. Proc Natl Acad Sci U S A. 1996;93(18):9782-7.

LeDoux SP, Wilson GL, Beecham EJ, Stevnsner T, Wassermann K, Bohr VA. Repair of mitochondrial DNA after various types of DNA damage in Chinese hamster ovary cells. Carcinogenesis. 1992;13(11):1967-73.

LeDoux SP, Driggers WJ, Hollensworth BS, Wilson GL. Repair of alkylation and oxidative damage in mitochondrial DNA. Mutat Res. 1999;434(3):149-59.

Lee BI, Wilson DM 3rd. The RAD2 domain of human exonuclease 1 exhibits 5' to 3' exonuclease and flap structure-specific endonuclease activities. J Biol Chem. 1999;274(53):37763-9.

Li GM, Modrich P. Restoration of mismatch repair to nuclear extracts of H6 colorectal tumor cells by a heterodimer of human MutL homologs. Proc Natl Acad Sci U S A. 1995;92(6):1950-4.

Li Y, Huang TT, Carlson EJ, Melov S, Ursell PC, Olson JL, Noble LJ, Yoshimura MP, Berger C, Chan PH, et al. Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. Nat Genet. 1995;11(4):376-81.

Lieber MR. The FEN-1 family of structure-specific nucleases in eukaryotic DNA replication, recombination and repair. Bioessays. 1997;19(3):233-40.

Liem LK, Lim A, Li BF. Specificities of human, rat and E. coli O6-methylguanine-DNA methyltransferases towards the repair of O6-methyl and O6-ethylguanine in DNA. Nucleic Acids Res. 1994;22(9):1613-9.

Lim A, Li BF. The nuclear targeting and nuclear retention properties of a human DNA repair protein O6-methylguanine-DNA methyltransferase are both required for its nuclear localization: the possible implications. EMBO J. 1996;15(15):4050-60.

Lindahl T, Sedgwick B, Sekiguchi M, Nakabeppu Y. Regulation and expression of the adaptive response to alkylating agents. Annu Rev Biochem. 1988;57:133-57.

Lindahl T, Wood RD. Quality control by DNA repair. Science. 1999;286(5446):1897-905.

Lindahl T. Suppression of spontaneous mutagenesis in human cells by DNA base excision-repair. Mutat Res. 2000;462(2-3):129-35.

Lindenau J, Noack H, Possel H, Asayama K, Wolf G. Cellular distribution of superoxide dismutases in the rat CNS. Glia. 2000;29(1):25-34.

Lipkin SM, Wang V, Jacoby R, Banerjee-Basu S, Baxevanis AD, Lynch HT, Elliott RM, Collins FS.
MLH3: a DNA mismatch repair gene associated with mammalian microsatellite instability. Nat Genet. 2000;24(1):27-35.

Liu XF, Elashvili I, Gralla EB, Valentine JS, Lapinskas P, Culotta VC. Yeast lacking superoxide dismutase. Isolation of genetic suppressors. J Biol Chem. 1992;267(26):18298-302.

Liu D, Wen J, Liu J, Li L. The roles of free radicals in amyotrophic lateral sclerosis: reactive oxygen species and elevated oxidation of protein, DNA, and membrane phospholipids. FASEB J. 1999;13(15):2318-28.

Loft S, Poulsen HE. Cancer risk and oxidative DNA damage in man. J Mol Med. 1996;74(6):297-312.

Longley MJ, Prasad R, Srivastava DK, Wilson SH, Copeland WC. Identification of 5'-deoxyribose phosphate lyase activity in human DNA polymerase gamma and its role in mitochondrial base excision repair in vitro. Proc Natl Acad Sci U S A. 1998;95(21):12244-8.

Longo VD, Gralla EB, Valentine JS. Superoxide dismutase activity is essential for stationary phase survival in Saccharomyces cerevisiae. Mitochondrial production of toxic oxygen species in vivo. J Biol Chem. 1996;271(21):12275-80.

Longo VD, Gralla EB, Valentine JS. Superoxide dismutase activity is essential for stationary phase survival in Saccharomyces cerevisiae. Mitochondrial production of toxic oxygen species in vivo. J Biol Chem. 1996;271(21):12275-80.

Lynch HT, de la Chapelle A. Genetic susceptibility to non-polyposis colorectal cancer. J Med Genet. 1999;36(11):801-18.

Malins DC, Haimanot R. Major alterations in the nucleotide structure of DNA in cancer of the female breast. Cancer Res. 1991;51(19):5430-2.

Marcelino LA, Andre PC, Khrapko K, Coller HA, Griffith J, Thilly WG. Chemically induced mutations in mitochondrial DNA of human cells: mutational spectrum of N-methyl-N'-nitro-N-nitrosoguanidine. Cancer Res. 1998;58(13):2857-62.

Marcelino LA, Thilly WG. Mitochondrial mutagenesis in human cells and tissues. Mutat Res. 1999;434(3):177-203.

Marra G, Iaccarino I, Lettieri T, Roscilli G, Delmastro P, Jiricny J. Mismatch repair deficiency associated with overexpression of the MSH3 gene. Proc Natl Acad Sci U S A. 1998;95(15):8568-73.

Matton N, Simonetti J, Williams K. Identification of mismatch repair protein complexes in HeLa nuclear extracts and their interaction with heteroduplex DNA. J Biol Chem. 2000;275(23):17808-13.

Melov S, Coskun P, Patel M, Tuinstra R, Cottrell B, Jun AS, Zastawny TH, Dizdaroglu M, Goodman SI, Huang TT, Miziorko H, Epstein CJ, Wallace DC. Mitochondrial disease in superoxide dismutase 2 mutant mice. Proc Natl Acad Sci U S A. 1999;96(3):846-51.

Melov S, Ravenscroft J, Malik S, Gill MS, Walker DW, Clayton PE, Wallace DC, Malfroy B, Doctrow SR, Lithgow GJ. Extension of life-span with superoxide dismutase/catalase mimetics. Science. 2000;289(5484):1567-9.

Michaels ML, Cruz C, Grollman AP, Miller JH. Evidence that MutY and MutM combine to prevent mutations by an oxidatively damaged form of guanine in DNA. Proc Natl Acad Sci U S A. 1992;89(15):7022-5.

Mita S, Monnat RJ Jr, Loeb LA. Resistance of HeLa cell mitochondrial DNA to mutagenesis by chemical carcinogens. Cancer Res. 1988;48(16):4578-83.

Miyako K, Takamatsu C, Umeda S, Tajiri T, Furuichi M, Nakabeppu Y, Sekiguchi M, Hamasaki N, Takeshige K, Kang D. Accumulation of adenine DNA glycosylase-sensitive sites in human mitochondrial DNA. J Biol Chem. 2000;275(16):12326-30.

Modrich P. Strand-specific mismatch repair in mammalian cells. J Biol Chem. 1997;272(40):24727-30.

Mol CD, Parikh SS, Putnam CD, Lo TP, Tainer JA. DNA repair mechanisms for the recognition and removal of damaged DNA bases. Annu Rev Biophys Biomol Struct. 1999;28:101-28.

Mu D, Tursun M, Duckett DR, Drummond JT, Modrich P, Sancar A. Recognition and repair of compound DNA lesions (base damage and mismatch) by human mismatch repair and excision repair systems. Mol Cell Biol. 1997;17(2):760-9.

Myers KA, Saffhill R, O'Connor PJ. Repair of alkylated purines in the hepatic DNA of mitochondria and nuclei in the rat. Carcinogenesis. 1988;9(2):285-92.

Myrnes B, Giercksky KE, Krokan H. Interindividual variation in the activity of O6-methyl guanine-DNA methyltransferase and uracil-DNA glycosylase in human organs. Carcinogenesis. 1983;4(12):1565-8.

Nachury MV, Ryder UW, Lamond AI, Weis K. Cloning and characterization of hSRP1 gamma, a tissue-specific nuclear transport factor. Proc Natl Acad Sci U S A. 1998;95(2):582-7.

Nakabeppu Y. Molecular genetics and structural biology of human MutT homolog, MTH1. Mutat Res. 2001;477(1-2):59-70.

Nakai K, Kanehisa M. A knowledge base for predicting protein localization sites in eukaryotic cells, Genomics. 1992; 14, 897-911.

Nelson WG, Kastan MB. DNA strand breaks: the DNA template alterations that trigger p53-dependent DNA damage response pathways. Mol Cell Biol. 1994;14(3):1815-23.

Nelson JR, Lawrence CW, Hinkle DC. Deoxycytidyl transferase activity of yeast REV1 protein. Nature. 1996;382(6593):729-31.

Ni TT, Marsischky GT, Kolodner RD. MSH2 and MSH6 are required for removal of adenine misincorporated opposite 8-oxo-guanine in S. cerevisiae. Mol Cell. 1999;4(3):439-44.

Nicolaides NC, Papadopoulos N, Liu B, Wei YF, Carter KC, Ruben SM, Rosen CA, Haseltine WA, Fleischmann RD, Fraser CM, et al. Mutations of two PMS homologues in hereditary nonpolyposis colon cancer. Nature. 1994;371(6492):75-80.

Nicolaides NC, Carter KC, Shell BK, Papadopoulos N, Vogelstein B, Kinzler KW. Genomic organization of the human PMS2 gene family. Genomics. 1995;30(2):195-206.

Nilsen H, Otterlei M, Haug T, Solum K, Nagelhus TA, Skorpen F, Krokan HE. Nuclear and mitochondrial uracil-DNA glycosylases are generated by alternative splicing and transcription from different positions in the UNG gene. Nucleic Acids Res. 1997;25(4):750-5.

Nishioka K, Ohtsubo T, Oda H, Fujiwara T, Kang D, Sugimachi K, Nakabeppu Y. Expression and differential intracellular localization of two major forms of human 8-oxoguanine DNA glycosylase encoded by alternatively spliced OGG1 mRNAs. Mol Biol Cell. 1999;10(5):1637-52.

Nohl K, Jordan W. The mitochondrial site of superoxide foremation. Biochem Biophys Res Commun. 1986;138(2):533-39.

Ohtsubo T, Nishioka K, Imaiso Y, Iwai S, Shimokawa H, Oda H, Fujiwara T, Nakabeppu Y. Identification of human MutY homolog (hMYH) as a repair enzyme for 2-hydroxyadenine in DNA and detection of multiple forms of hMYH located in nuclei and mitochondria. Nucleic Acids Res. 2000;28(6):1355-64.

Olinski R, Zastawny T, Budzbon J, Skokowski J, Zegarski W, Dizdaroglu M. DNA base modifications in chromatin of human cancerous tissues. FEBS Lett. 1992;309(2):193-8.

Otterlei M, Haug T, Nagelhus TA, Slupphaug G, Lindmo T, Krokan HE. Nuclear and mitochondrial splice forms of human uracil-DNA glycosylase contain a complex nuclear localisation signal and a strong classical mitochondrial localisation signal, respectively. Nucleic Acids Res. 1998;26(20):4611-7.

Papa S. Mitochondrial oxidative phosphorylation changes in the life span. Molecular aspects and physiopathological implications. Biochim Biophys Acta. 1996;1276(2):87-105.

Papadopoulos N, Nicolaides NC, Wei YF, Ruben SM, Carter KC, Rosen CA, Haseltine WA, Fleischmann RD, Fraser CM, Adams MD, et al. Mutation of a mutL homolog in hereditary colon cancer. Science. 1994;263(5153):1625-9.

Papadopoulos N, Lindblom A.
Molecular basis of HNPCC: mutations of MMR genes. Hum Mutat. 1997;10(2):89-99.

Parkes TL, Elia AJ, Dickinson D, Hilliker AJ, Phillips JP, Boulianne GL. Extension of Drosophila lifespan by overexpression of human SOD1 in motorneurons. Nat Genet. 1998;19(2):171-4.

Pascucci B, Versteegh A, van Hoffen A, van Zeeland AA, Mullenders LH, Dogliotti E. DNA repair of UV photoproducts and mutagenesis in human mitochondrial DNA. J Mol Biol. 1997;273(2):417-27.

Pegg AE. Properties of mammalian O6-alkylguanine-DNA transferases. Mutat Res. 1990;233(1-2):165-75.

Pegg AE, Byers TL. Repair of DNA containing O6-alkylguanine. FASEB J. 1992;6(6):2302-10.

Pegg AE. Repair of O(6)-alkylguanine by alkyltransferases. Mutat Res. 2000;462(2-3):83-100.

Peltomäki P, Vasen HF. Mutations predisposing to hereditary nonpolyposis colorectal cancer: database and results of a collaborative study. The International Collaborative Group on Hereditary Nonpolyposis Colorectal Cancer. Gastroenterology. 1997;113(4):1146-58.

Peltomäki P. DNA mismatch repair and cancer. Mutat Res. 2001;488(1):77-85.

Perkins GA, Frey TG. Recent structural insight into mitochondria gained by microscopy. Micron. 2000;31(1):97-111.

Pendlebury A, Frayling IM, Santibanez Koref MF, Margison GP, Rafferty JA. Evidence for the simultaneous expression of alternatively spliced alkylpurine N-glycosylase transcripts in human tissues and cells. Carcinogenesis. 1994;15(12):2957-60.

Polyak K, Li Y, Zhu H, Lengauer C, Willson JK, Markowitz SD, Trush MA, Kinzler KW, Vogelstein B.
Somatic mutations of the mitochondrial genome in human colorectal tumours. Nat Genet. 1998;20(3):291-3.

Potter PM, Wilkinson MC, Fitton J, Carr FJ, Brennand J, Cooper DP, Margison GP. Characterisation and nucleotide sequence of ogt, the O^6-alkylguanine-DNA-alkyltransferase gene of E. coli. Nucleic Acids Res. 1987;15(22):9177-93.

Prakash L. Repair of pyrimidine dimers in nuclear and mitochondrial DNA of yeast irradiated
with low doses of ultraviolet light. J Mol Biol. 1975;98(4):781-95.

Prieto-Alamo MJ, Laval F. Overexpression of the human HAP1 protein sensitizes cells to the lethal effect of bioreductive drugs. Carcinogenesis. 1999;20(3):415-9.

Pryor WA. Oxy-radicals and related species: their formation, lifetimes, and reactions. Annu Rev Physiol. 1986;48:657-67.

Pulkes T, Hanna MG. Human mitochondrial DNA diseases. Adv Drug Deliv Rev. 2001;49(1-2):27-43.

Qiu J, Qian Y, Chen V, Guan MX, Shen B. Human exonuclease 1 functionally complements its yeast homologues in DNA recombination, RNA primer removal, and mutation avoidance. J Biol Chem. 1999;274(25):17893-900.

Radi R, Turrens JF, Chang LY, Bush KM, Crapo JD, Freeman BA. Detection of catalase in rat heart mitochondria. J Biol Chem. 1991;266(32):22028-34.

Raha S, McEachern GE, Myint AT, Robinson BH. Superoxides from mitochondrial complex III: the role of manganese superoxide dismutase. Free Radic Biol Med. 2000;29(2):170-80.

Rahden-Staron I, Laval F. cDNA cloning of the rat O6-methylguanine-DNA-methyltransferase. Biochem Biophys Res Commun. 1991;177(2):597-602.

Räschle M, Marra G, Nystrom-Lahti M, Schar P, Jiricny J. Identification of hMutLbeta, a heterodimer of hMLH1 and hPMS1. J Biol Chem. 1999;274(45):32368-75.

Rasmussen LJ, Samson L, Marinus MG. Dam-directed DNA mismatch repair (DNA damage and repair, Vol. 1: DNA repair in prokaryotes and lower eukaryotes, ed. Nickoloff JA, Hoekstra MF). Humana Press Inc., Totowa, NJ. 1998: 205-28

Rasmussen LJ, Rasmussen M, Lee B, Rasmussen AK, Wilson DM, Nielsen FC, Bisgaard HC.
Identification of factors interacting with hMSH2 in the fetal liver utilizing the yeast two-hybrid system. In vivo interaction through the C-terminal domains of hEXO1 and hMSH2 and comparative expression analysis. Mutat Res. 2000;460(1):41-52.

Reagan MS, Pittenger C, Siede W, Friedberg EC. Characterization of a mutant strain of Saccharomyces cerevisiae with a deletion of the RAD27 gene, a structural homolog of the RAD2 nucleotide excision repair gene. J Bacteriol. 1995;177(2):364-71.

Reaume AG, Elliott JL, Hoffman EK, Kowall NW, Ferrante RJ, Siwek DF, Wilcox HM, Flood DG, Beal MF, Brown RH Jr, Scott RW, Snider WD. Motor neurons in Cu/Zn superoxide dismutase-deficient mice develop normally but exhibit enhanced cell death after axonal injury. Nat Genet. 1996;13(1):43-7.

Reenan RA, Kolodner RD. Characterization of insertion mutations in the Saccharomyces cerevisiae MSH1 and MSH2 genes: evidence for separate mitochondrial and nuclear functions. Genetics. 1992;132(4):975-85.

Robinson BH. Human complex I deficiency: clinical spectrum and involvement of oxygen free radicals in the pathogenicity of the defect. Biochim Biophys Acta. 1998;1364(2):271-86.

Rossman TG, Goncharova EI. Spontaneous mutagenesis in mammalian cells is caused mainly by oxidative events and can be blocked by antioxidants and metallothionein. Mutat Res. 1998;402(1-2):103-10.

Rydberg B, Spurr N, Karran P. cDNA cloning and chromosomal assignment of the human O6-methylguanine-DNA methyltransferase. cDNA expression in Escherichia coli and gene expression in human cells. J Biol Chem. 1990;265(16):9563-9.

Samson L, Han S, Marquis JC, Rasmussen LJ. Mammalian DNA repair methyltransferases shield O^4MeT from nucleotide excision repair. Carcinogenesis. 1997;18(5):919-24.

Saraste M. Oxidative phosphorylation at the fin de siecle. Science. 1999;283(5407):1488-93.

Sassanfar M, Dosanjh MK, Essigmann JM, Samson L. Relative efficiencies of the bacterial, yeast, and human DNA methyltransferases for the repair of O6-methylguanine and O4-methylthymine. Suggestive evidence for O^4-methylthymine repair by eukaryotic methyltransferases. J Biol Chem. 1991;266(5):2767-71.

Satoh MS, Huh N, Rajewsky MF, Kuroki T. Enzymatic removal of O^6-ethylguanine from mitochondrial DNA in rat tissues exposed to N-ethyl-N-nitrosourea in vivo. J Biol Chem. 1988;263(14):6854-6.

Scheffler IE. Mitochondria. Wiley-Liss, Inc., New York. 1999, pp.1-367

Schmitt ME, Clayton DA. Conserved features of yeast and mammalian mitochondrial DNA replication. Curr Opin Genet Dev. 1993;3(5):769-74.

Schmutte C, Marinescu RC, Sadoff MM, Guerrette S, Overhauser J, Fishel R. Human exonuclease I interacts with the mismatch repair protein hMSH2. Cancer Res. 1998;58(20):4537-42.

Schmutte C, Sadoff MM, Shim KS, Acharya S, Fishel R. The interaction of DNA mismatch repair proteins with human exonuclease I. J Biol Chem. 2001;276(35):33011-8.

Sedgwick B. Molecular cloning of a gene which regulates the adaptive response to alkylating agents in Escherichia coli. Mol Gen Genet. 1983;191(3):466-72.

Seo BB, Kitajima-Ihara T, Chan EK, Scheffler IE, Matsuno-Yagi A, Yagi T. Molecular remedy of complex I defects: rotenone-insensitive internal NADH-quinone oxidoreductase of Saccharomyces cerevisiae mitochondria restores the NADH oxidase activity of complex I-deficient mammalian cells. Proc Natl Acad Sci U S A. 1998;95(16):9167-71.

Seo BB, Matsuno-Yagi A, Yagi T. Modulation of oxidative phosphorylation of human kidney 293 cells by transfection with the internal rotenone-insensitive NADH-quinone oxidoreductase (NDI1) gene of Saccharomyces cerevisiae. Biochim Biophys Acta. 1999;1412(1):56-65.

Shibutani S, Takeshita M, Grollman AP. Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxodG. Nature. 1991;349(6308):431-4.

Shiota S, von Wronski MA, Tano K, Bigner DD, Brent TP, Mitra S. Characterization of cDNA encoding mouse DNA repair protein O6-methylguanine-DNA methyltransferase and high-level expression of the wild-type and mutant proteins in Escherichia coli. Biochemistry. 1992;31(7):1897-903.

Singer B, Dosanjh MK. Site-directed mutagenesis for quantitation of base-base interactions at defined sites. Mutat Res. 1990;233(1-2):45-51.

Sklar R, Strauss B. Removal of O6-methylguanine from DNA of normal and xeroderma pigmentosum-derived lymphoblastoid lines. Nature. 1981;289(5796):417-20.

Slupphaug G, Markussen FH, Olsen LC, Aasland R, Aarsaether N, Bakke O, Krokan HE, Helland DE. Nuclear and mitochondrial forms of human uracil-DNA glycosylase are encoded by the same gene. Nucleic Acids Res. 1993;21(11):2579-84.

Slupphaug G, Eftedal I, Kavli B, Bharati S, Helle NM, Haug T, Levine DW, Krokan HE. Properties of a recombinant human uracil-DNA glycosylase from the UNG gene and evidence that UNG encodes the major uracil-DNA glycosylase. Biochemistry. 1995;34(1):128-38.

Srinivasan C, Liba A, Imlay JA, Valentine JS, Gralla EB. Yeast lacking superoxide dismutase(s) show elevated levels of "free iron" as measured by whole cell electron paramagnetic resonance. J Biol Chem. 2000;275(38):29187-92.

Steenken S, Jovanovic SV. How easily oxidizable is DNA? One-electron reduction potentials of adenosine and guanosine radicals in aqueous solution. J Am Chem Soc. 1997;119:617-18

Stierum RH, Croteau DL, Bohr VA. Purification and characterization of a mitochondrial thymine glycol endonuclease from rat liver. J Biol Chem. 1999;274(11):7128-36.

Szankasi P, Smith GR. A role for exonuclease I from S. pombe in mutation avoidance and mismatch correction. Science. 1995;267(5201):1166-9.

Takao M, Aburatani H, Kobayashi K, Yasui A. Mitochondrial targeting of human DNA glycosylases for repair of oxidative DNA damage. Nucleic Acids Res. 1998;26(12):2917-22.

Takao M, Zhang QM, Yonei S, Yasui A. Differential subcellular localization of human MutY homolog (hMYH) and the functional activity of adenine:8-oxoguanine DNA glycosylase. Nucleic Acids Res. 1999;27(18):3638-44.

Tano K, Shiota S, Collier J, Foote RS, Mitra S. Isolation and structural characterization of a cDNA clone encoding the human DNA repair protein for O^6-alkylguanine. Proc Natl Acad Sci U S A. 1990;87(2):686-90.

Tchou J, Kasai H, Shibutani S, Chung MH, Laval J, Grollman AP, Nishimura S. 8-oxoguanine (8-hydroxyguanine) DNA glycosylase and its substrate specificity. Proc Natl Acad Sci U S A. 1991;88(11):4690-4.

Tell G, Crivellato E, Pines A, Paron I, Pucillo C, Manzini G, Bandiera A, Kelley MR, Di Loreto C, Damante G. Mitochondrial localization of APE/Ref-1 in thyroid cells. Mutat Res. 2001;485(2):143-52.

Thomas DC, Roberts JD, Kunkel TA. Heteroduplex repair in extracts of human HeLa cells. J Biol Chem. 1991;266(6):3744-51.

Tishkoff DX, Boerger AL, Bertrand P, Filosi N, Gaida GM, Kane MF, Kolodner RD. Identification and characterization of Saccharomyces cerevisiae EXO1, a gene encoding an exonuclease that interacts with MSH2. Proc Natl Acad Sci U S A. 1997;94(14):7487-92.

Tishkoff DX, Amin NS, Viars CS, Arden KC, Kolodner RD. Identification of a human gene encoding a homologue of Saccharomyces cerevisiae EXO1, an exonuclease implicated in mismatch repair and recombination. Cancer Res. 1998;58(22):5027-31.

Tom S, Henricksen LA, Bambara RA. Mechanism whereby proliferating cell nuclear antigen stimulates flap endonuclease 1. J Biol Chem. 2000;275(14):10498-505.

Tomkinson AE, Bonk RT, Kim J, Bartfeld N, Linn S. Mammalian mitochondrial endonuclease activities specific for ultraviolet-irradiated DNA. Nucleic Acids Res. 1990;18(4):929-35.

Tran HT, Gordenin DA, Resnick MA. The 3'→5' exonucleases of DNA polymerases delta and epsilon and the 5'→3' exonuclease Exo1 have major roles in postreplication mutation avoidance in Saccharomyces cerevisiae. Mol Cell Biol. 1999;19(3):2000-7.

Tsujimura T, Zhang YP, Fujio C, Chang HR, Watatani M, Ishizaki K, Kitamura H, Ikenaga M.
O^6-methylguanine methyltransferase activity and sensitivity of Japanese tumor cell strains to 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea hydrochloride.
Jpn J Cancer Res. 1987;78(11):1207-15.

Tsurimoto T. PCNA binding proteins. Front Biosci. 1999;4:D849-58.

Turrens JF, Alexandre A, Lehninger AL. Ubisemiquinone is the electron donor for superoxide formation by complex III of heart mitochondria. Arch Biochem Biophys. 1985;237(2):408-14.

Umar A, Buermeyer AB, Simon JA, Thomas DC, Clark AB, Liskay RM, Kunkel TA. Requirement for PCNA in DNA mismatch repair at a step preceding DNA resynthesis. Cell. 1996;87(1):65-73.

Umar A, Koi M, Risinger JI, Glaab WE, Tindall KR, Kolodner RD, Boland CR, Barrett JC, Kunkel TA. Correction of hypermutability, N-methyl-N'-nitro-N-nitrosoguanidine resistance, and defective DNA mismatch repair by introducing chromosome 2 into human tumor cells with mutations in MSH2 and MSH6. Cancer Res. 1997;57(18):3949-55.

Vallen EA, Cross FR. Mutations in RAD27 define a potential link between G1 cyclins and DNA replication. Mol Cell Biol. 1995;15(8):4291-302.

van Steeg H, Kraemer KH. Xeroderma pigmentosum and the role of UV-induced DNA damage in skin cancer. Mol Med Today. 1999;5(2):86-94.

Viswanathan M, Lovett ST. Single-strand DNA-specific exonucleases in Escherichia coli. Roles in repair and mutation avoidance. Genetics. 1998;149(1):7-16.

Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. Nature. 2000;408(6810):307-10.

Vongsamphanh R, Fortier PK, Ramotar D. Pir1p mediates translocation of the yeast Apn1p endonuclease into the mitochondria to maintain genomic stability. Mol Cell Biol. 2001;21(5):1647-55.

Wallace DC, Brown MD, Melov S, Graham B, Lott M. Mitochondrial biology, degenerative diseases and aging. Biofactors. 1998;7(3):187-90.

Wallace DC. Mitochondrial diseases in man and mouse. Science. 1999;283(5407):1482-8.

Warbrick E, Lane DP, Glover DM, Cox LS. Homologous regions of Fen1 and p21Cip1 compete for binding to the same site on PCNA: a potential mechanism to co-ordinate DNA replication and repair. Oncogene. 1997;14(19):2313-21.

Warbrick E. PCNA binding through a conserved motif. Bioessays. 1998;20(3):195-9.

Warburg O. On the origin of cancer cells. Science. 1956; 123:309-314.

Wilson DM 3rd, Carney JP, Coleman MA, Adamson AW, Christensen M, Lamerdin JE. Hex1: a new human Rad2 nuclease family member with homology to yeast exonuclease 1. Nucleic Acids Res. 1998;26(16):3762-8.

Wood RD, Mitchell M, Sgouros J, Lindahl T. Human DNA repair genes. Science. 2001;291(5507):1284-9.

Wu Y, Berends MJ, Post JG, Mensink RG, Verlind E, Van Der Sluis T, Kempinga C, Sijmons RH, van der Zee AG, Hollema H, Kleibeuker JH, Buys CH, Hofstra RM. Germline mutations of EXO1 gene in patients with hereditary nonpolyposis colorectal cancer (HNPCC) and atypical HNPCC forms. Gastroenterology. 2001;120(7):1580-7.

Wyatt MD, Allan JM, Lau AY, Ellenberger TE, Samson LD. 3-methyladenine DNA glycosylases: structure, function, and biological importance. Bioessays. 1999;21(8):668-76.

Xiao W, Derfler B, Chen J, Samson L. Primary sequence and biological functions of a Saccharomyces cerevisiae O^6-methylguanine/O^4-methylthymine DNA repair methyltransferase gene. EMBO J. 1991;10(8):2179-86.

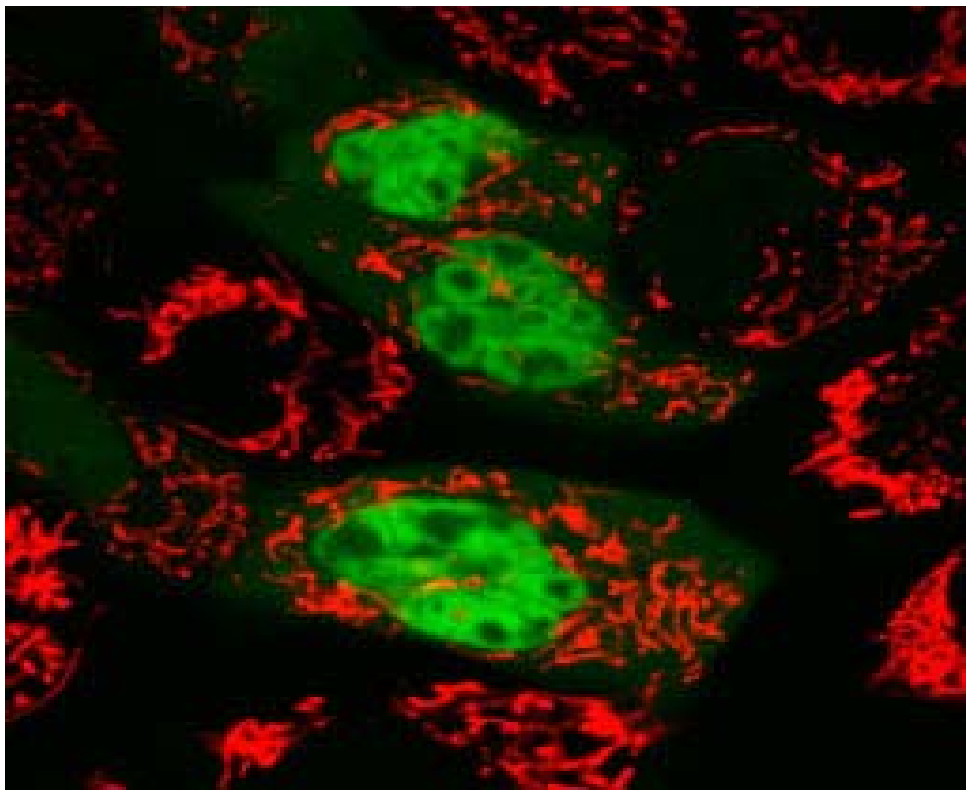
Xu-Welliver M, Kanugula S, Loktionova NA, Crone TM, Pegg AE. Conserved residue lysine165 is essential for the ability of O6-alkylguanine-DNA alkyltransferase to react with O6-benzylguanine. Biochem J. 2000;347(Pt 2):527-34.

Yarosh DB. The role of O6-methylguanine-DNA methyltransferase in cell survival, mutagenesis and carcinogenesis. Mutat Res. 1985;145(1-2):1-16.

Yim MB, Kang JH, Yim HS, Kwak HS, Chock PB, Stadtman ER. A gain-of-function of an amyotrophic lateral sclerosis-associated Cu,Zn-superoxide dismutase mutant: An enhancement of free radical formation due to a decrease in Km for hydrogen peroxide. Proc Natl Acad Sci U S A. 1996;93(12):5709-14.

Yu Z, Chen J, Ford BN, Brackley ME, Glickman BW. Human DNA repair systems: an overview. Environ Mol Mutagen. 1999;33(1):3-20.

Zak P, Kleibl K, Laval F. Repair of O6-methylguanine and O4-methylthymine by the human and rat O^6-methylguanine-DNA methyltransferases. J Biol Chem. 1994;269(1):730-3.



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