"Blott de tama fåglarna har en längtan. De vilda flyger."

Elmer Diktonius

och

"Ingen fågel flyger för högt – så länge han flyger med egna vingar."

William Blake

Till minne av Inger och Algot Jansson

Oxidative damage and the DNA glycosylase MutYH

Abstract

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The DNA glycosylase MutYH is highly conserved throughout evolution, and homologs are found in most eukaryotes and prokaryotes examined. MutYH functions as a base excision repair DNA glycosylase that excises adenines misincorporated opposite 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), one of the most stable products of oxidative DNA damage. Germline mutations in *MutYH* in humans predispose to *MutYH*-associated polyposis (MAP), characterised by multiple colorectal adenomas and carcinomas. Oxidative stress and susceptibility to carcinogenesis involve additional pathways, such as the glutathione/glutathione S-transferase detoxification system. The role of the base excision repair enzyme MutYH in DNA damage repair and checkpoint control in fission yeast is here shown not to be restricted to oxidative damage.

The fission yeast gene encoding MutYH, *myh1*$^+$, displays a strong interaction with the checkpoint gene *rad1*$^+$. UV irradiation of *myh1 rad1* double mutants results in severe chromosome segregation defects and visible DNA fragmentation, and a failure to activate the checkpoint control. The *myh1 rad1* double mutants furthermore display hypersensitivity to genotoxic compounds MMS and HU. Additionally, *myh1 rad1* double mutants exhibit morphological defects in the absence of DNA damaging agents.

Fission yeast MutYH (Myh1) has a role in DNA repair after treatment with DNA crosslinking and strand-breaking chemotherapeutic agents. Myh1 contributes to survival upon genotoxic stress, particularly in a *rad1* mutant background, and relocalises to the nucleus after exposure to the DNA crosslinking agent cisplatin or following oxidative stress. An asymmetric function of the 9-1-1 checkpoint sensor complex is conceivable in MutYH-mediated base excision repair, and further extends the view of MutYH function in DNA damage checkpoint control and DNA repair.

Phylogenetic distribution and sequence analysis of the MutY, MutM, and MutT homologs, enzymes involved in repair of 8-oxodG, indicate highly conserved protein domains and evolutionary loss of individual 8-oxodG repair components, predominantly within the fungal domain of eukaryotic life. The MutM homolog is the most prevalent 8-oxodG repair enzyme among eukaryotes. This likely indicates the MutM component as the major repair enzyme in removal of 8-oxodG damage.

S. cerevisiae mutants lacking glutathione S-transferases (GSTs) display a number of phenotypic defects under different stress conditions. The phenotypes of single and multiple mutants defective in GSTs, exposed to oxidants and other toxic agents, indicate the importance of yeast GSTs in protection against oxidative stress. A complex relationship most likely exists between different GSTs in general protection against oxidative stress and in specific protein redox regulation.

Keywords: Oxidative damage, 8-oxo-7,8-dihydro-2′-deoxyguanosine, Base excision repair, DNA glycosylase, Mismatch repair, DNA damage checkpoint, 9-1-1 complex, Glutathione-S-transferases, Colon cancer.

List of publications

This thesis is based on the following papers, which are referred to by their roman numerals:

I Jansson, K., Warringer, J., Farewell, A., Park, H-O., Hoe, K-L., Kim, D-U., Hayles, J. and Sunnerhagen, P. (2008). The tumor suppressor homolog in fission yeast, *myh1*$^{+}$, displays a strong interaction with the checkpoint gene *rad1*$^{+}$. *Mutat Res* 644: 48-55.

II Jansson, K., Viktorsson, K., Warringer, J., Lewensohn, R. and Sunnerhagen, P. (2010). A novel role for Myh1 in DNA repair after treatment with DNA cross linking and strand-breaking chemotherapeutic agents. Submitted for publication.

III Jansson, K., Alm Rosenblad, M., Blomberg, A. and Sunnerhagen, P. (2010). Evolutionary loss of 8-oxo-G repair components among eukaryotes. Manuscript.

IV Barreto, L., Garcera´, A., Jansson, K., Sunnerhagen, P. and Herrero, E. (2006). A peroxisomal glutathione transferase of *Saccharomyces cerevisiae* is functionally related to sulphur amino acid metabolism. *Eukaryot Cell* 5: 1748-59.

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Introduction

DNA is prone to numerous forms of damage that can injure cells and may lead to mutations, cell death, cancer or premature aging. Living organisms therefore have evolved an array of mechanisms to repair these injuries and preserve genome integrity. DNA damage response mechanisms include pathways of DNA repair, cell cycle checkpoints and programmed cell death. These processes are part of a complex network of DNA damage recognition and processing, checkpoint signalling cascades and DNA repair.

Cell cycle checkpoints and DNA damage

Cell cycle checkpoints are control mechanisms that ensure the fidelity of cell division in eukaryotic cells. These checkpoints verify whether the processes at each phase of the cell cycle have been accurately completed before progression into the next phase. Checkpoints in the cell cycle include monitoring the state of DNA integrity, DNA replication, cell size, and the surrounding environment [1]. It is especially important for multi-cellular organisms to maintain integrity of the genome, and multiple checkpoints prior to DNA replication and mitosis monitor the state of the genome.

In mammalian cells the checkpoint response to DNA damage or replication stress regulates cellular processes such as cell-cycle progression, apoptosis, DNA repair, and DNA replication. The DNA damage checkpoint recognizes DNA damage, and is required to restrain cell cycle progression during DNA repair and to maintain chromosome stability [2]. Damaged DNA is detected by sensor proteins and is relayed to downstream effectors leading to cell cycle arrest, activation of DNA repair processes and apoptotic cell death [3]. Cells lacking functional checkpoints display genomic instability due to a failure to properly respond to DNA damage, faulty DNA replication, or aberrant chromosome segregation, resulting in an accelerated mutator phenotype [4].

The integrated checkpoint response to genome insult protects the genome from cytotoxic and mutagenic effects of chemicals and radiation in the environment. Genetic defects in the DNA damage response may lead to genome instability and predispose individuals to cancer diseases.

The DNA damage checkpoint

Cell cycle checkpoints represent integral components of DNA repair that coordinate cooperation between the machinery of the cell cycle and several biochemical pathways that respond to damage and restore DNA structure. By delaying progression through the cell cycle, checkpoints provide more time for repair before the critical phases of DNA replication, when the genome is replicated, and of mitosis, when the genome is segregated. Checkpoints operate throughout the cell cycle and respond in several ways to DNA damage. DNA strand breaks, prior to the replication phase, induce G_1 arrest [5], thereby delaying the progression of G_1 cells into the S-phase until repair is completed. Other DNA damage responsive checkpoints can be observed in S and G_2 cells. An S-phase DNA damage checkpoint in *Schizosaccharomyces pombe* discriminates between different types of damage induced by UV-irradiation and gamma-irradiation [6]. UV irradiation, which causes base modification that can be repaired during G_1 and S-phase, invokes the checkpoint, while gamma irradiation, which causes double strand breaks, does not invoke this particular S-phase checkpoint if induced before replication. The G_2 checkpoint will arrest damaged cells in G_2, delaying entry into mitosis until the damage has been repaired. Single stranded DNA, a result of stalled DNA replication or processing of chromosomal lesions, appears to be central to the activation of the G_2 damage checkpoint [7].

The DNA damage checkpoint response system involves a signal transduction pathway consisting of sensors, transducers, and effector proteins (fig. 1). The DNA damage is detected by sensors that, with the aid of mediators and transducers, relay the signal to downstream effectors. Transducer proteins activate or inactivate effector proteins that directly participate in inhibiting the G_1/S transition, S-phase progression, or the G_2/M transition. However, there is not an absolute differentiation between the various components of the checkpoint response. For instance, damage sensors may also function as signal transducers. Although the G_1/S, intra-S, and the G_2/M checkpoints are distinct, the damage sensor molecules that activate the various checkpoints appear to either be shared by all three pathways or to play a primary sensor role in one pathway and a back-up role in the others. Similarly, the signal transducing molecules, which are protein kinases and phosphatases, are shared by the different checkpoints to varying degrees [8,9]. Although it is commonly believed that sensors for DNA repair and for the checkpoint response are distinct, there are reasons to consider a potential overlap at the damage detection step [10].

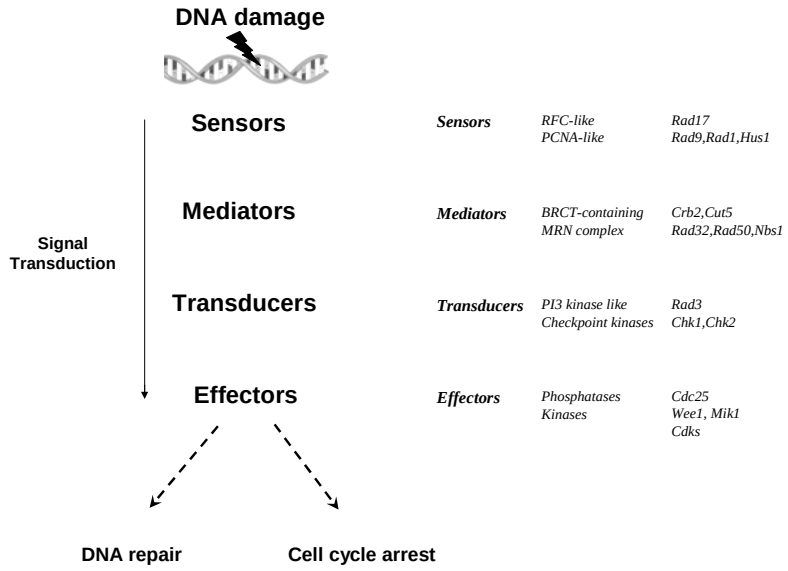

Fig 1. The DNA damage checkpoint response. DNA damage is detected by checkpoint sensor proteins and is relayed to downstream effectors, leading to cell cycle arrest and activation of DNA repair.

The *S. pombe* checkpoint response to DNA damage involves the checkpoint Rad proteins (Rad1, Rad3, Rad9, Rad17, Rad26, and Hus1). Rad3 and Rad26 exist as a complex [11], which signals to downstream effectors in response to DNA damage through the ATM- related lipid kinase motif activity of Rad3 [12]. The Rad1-Rad9-Hus1 proteins form a sensor complex that resembles the PCNA sliding clamp that may act to generate a checkpoint signal at aberrant DNA structures [13]. Rad17 belongs to a further complex which, by analogy with the *S. cerevisiae* and human homologues, contains the four small subunits of the replication factor C and is required for the association of Rad9-Rad1-Hus1 (9-1-1) complex proteins with DNA damage [14]. The checkpoint Rad family proteins are required for both the replication check checkpoint, which is activated by replication fork block, and the DNA damage checkpoint, acting to arrest cells in late S or G_2 following DNA damage.

The G2/M checkpoint control

The G_2/M checkpoint prevents DNA-damaged cells from entering mitosis and allows for the repair of DNA that was damaged in late S or G_2 phases prior to mitosis. A weakened G_2/M checkpoint control may trigger cell death via mitotic catastrophe for cells with unrepairable DNA damage. Mitotic catastrophe is a delayed mitosis-linked cell death, resulting from

premature or inappropriate entry of cells into mitosis with incomplete DNA synthesis, missegregated chromosomes, and premature chromosome condensation.

Progression of cells from G_2 phase of the cell cycle to mitosis is a tightly regulated cellular process that requires activation of the cyclin-dependent Cdc2 kinase, which determines onset of mitosis in all eukaryotic cells [15] (fig. 2). In both human and fission yeast cells, the activity of Cdc2 is regulated in part by the phosphorylation status of tyrosine 15 (Tyr15) on Cdc2, which is phosphorylated by Wee1 and Mik1 kinases during late G_2 and is rapidly dephosphorylated by the Cdc25 tyrosine phosphatase to trigger entry into mitosis [16] (fig. 2). These Cdc2 regulators are the downstream targets of two well characterized G_2/M pathways which prevent cells from entering mitosis when cellular DNA is damaged or when DNA replication is inhibited.

The DNA damage checkpoint is activated by ionizing radiation or ultraviolet light, and activation of this checkpoint leads to inhibitory Tyr15 phosphorylation of Cdc2 by a multistep pathway. The early genes in the pathway, which include Rad1, Rad3, Rad9, Rad17, Rad26 and Hus1, are thought to sense the DNA damage and lead to phosphorylation of the Chk1 protein [17]. In response to double strand DNA breaks (DSBs) induced by ionizing radiation, for example, Rad17 acts as a checkpoint specific loading factor, which responds to the DNA damage by loading the Rad9-Rad1-Hus1 (9-1-1) complex onto the sites where DNA is damaged [18]. In addition, the Rad3-Rad26 protein complex also binds to sites of DNA damage independently of the 9-1-1 complex [19]. Activation of Chk1 is mediated by Crb2, which may bridge Rad3 and Chk1. The activated Chk1 kinase then directly phosphorylates the Cdc25 phosphatase [20]. The phosphorylated Cdc25 binds Rad24/25 protein, and this complex is transported out of the nucleus to render Cdc25 inactive [21]. The activated Chk1 also regulates the Mik1 kinase to inhibit Cdc2 [22]. DNA damage thus initiates a Chk1 mediated protein phosphorylation cascade ending in the inactivation of the protein phosphatase Cdc25 and activation of the protein kinase Mik1 to increase inhibitory phosphorylation of Tyr15 on Cdc2.

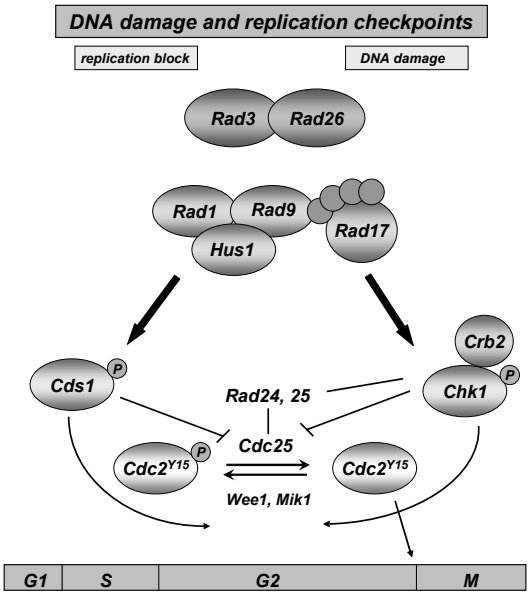

Fig 2. The DNA damage and replication checkpoint controls. The G2/M DNA damage checkpoint prevents damaged cells from entering mitosis. Progression of G2 phase cells into mitosis requires activation of the cyclin-dependent Cdc2 kinase, which is partly regulated by the phosphorylation status of Tyr15. DNA damage triggers phosphorylation of Chk1, which inactivates Cdc25 and activates Mik1 to increase inhibitory phosphorylation of Tyr15 on Cdc2.
The DNA replication checkpoint also controls the G2 to M transition through inhibitory phosphorylation of Cdc2. Phosphorylation of Cds1 inactivates Cdc25 and activate Mik1, which phosphorylates Tyr15 of Cdc2 and prevents entry into mitosis.

The DNA replication checkpoint is activated by treatment with hydroxyurea, which inhibits DNA replication, and this checkpoint also controls the G_2 to M transition through inhibitory phosphorylation of Cdc2 [23] (fig. 2). Parts of this DNA replication checkpoint are shared with the DNA damage checkpoint as Rad1, Rad3, Rad9, Rad17, Rad26 and Hus1, required for both checkpoints in fission yeast. The same 9-1-1 and Rad3-Rad26 checkpoint protein complexes may associate with the DNA replication complex [24]. However, the DNA replication checkpoint acts primarily through phosphorylation of the protein kinase Cds1. Activated Cds1 inactivates Cdc25 through a similar mechanism as Chk1 [25]. Cds1 also activates the kinase Mik1, which phosphorylates Tyr15 of Cdc2 [26].

The link between DNA damage checkpoint activation and recruitment of repair machineries to DNA lesions has been demonstrated through interactions and colocalization of checkpoint

sensors with proteins involved in various DNA repair processes upon DNA damage [27,28]. Activation of the first cascade checkpoint kinase is probably not due to the lesion itself, but it requires recognition and initial processing of the damage by a specific repair mechanism. Repair enzymes likely convert a variety of physically and chemically different lesions to a unique common structure, which is the checkpoint triggering signal. It has been suggested that the checkpoint proteins may detect a common intermediate, such as single stranded DNA coated by replication protein A (RPA) [29]. Recently, several reports support a hypothesis that checkpoint proteins may require a series of "adaptors" to recognize DNA damage [27,30]. Such adaptor proteins may be DNA damage recognition proteins involved in mismatch repair, nucleotide excision repair, base excision repair, and double-strand break repair.

DNA damage

DNA in living cells is subjected to many chemical alterations. If the genetic information encoded in the DNA is to remain uncorrupted, any chemical changes must be corrected. A failure to repair DNA before it is replicated produces a mutation. Damage to DNA is induced by certain wavelengths of radiation, ionizing radiation and ultraviolet rays, highly-reactive oxygen radicals, chemicals in the environment, and chemicals used in chemotherapy of cancers. Various forms of DNA damage arise from exposure to endogenous and exogenous DNA damaging agents. All four of the different bases in DNA (A, T, C, G) can be covalently modified at various positions. One of the most frequent base modifications is a deamination, i.e. a loss of an amino group. Mismatches of DNA bases can arise because of a failure of proofreading during DNA replication. DNA strand breaks in the backbone, from ionizing radiation or chemicals, can be limited to one of the two strands giving rise to a single strand break (SSB), or on both strands causing a double strand break (DSB). Crosslinks or covalent linkages, induced by several chemotherapeutic drugs, can be formed between bases on the same DNA strand (intrastrand breaks) or on the opposite strand (interstrand breaks).

Repair of damaged DNA base residues

Damaged or inappropriate base residues can be repaired by several DNA repair mechanisms. Excision of damaged bases is carried out by repair enzymes, called glycosylases. Specific DNA glycosylases remove the mismatched base residue, thereby restoring the correct DNA base. This is done without the need to break the DNA backbone. The enormous amount of different types of chemical DNA base modifications requires its own repair mechanism to

correct. A more general mechanism capable of correcting all sorts of chemical damage with a limited array of repair enzymes is the mechanism of excision repair.

Base excision repair (BER) mainly repairs non-bulky lesions produced by alkylation, oxidation or deamination of DNA base residues. The damaged base is identified and removed by a damage specific DNA glycosylase. DNA glycosylases cleave the N-glycosidic bond between the target base and the deoxyribose moiety. Excision of the damaged base leaves behind an apurinic/apyrimidinic (AP) site in the DNA. The resulting single strand gap is filled by a DNA polymerase and repair is completed by a DNA ligase that seals the remaining nick (fig. 3). While the base excision repair machinery can recognize specific lesions in the DNA it can correct only damaged bases that can be removed by a specific glycosylase, the nucleotide excision repair (NER) enzymes recognize bulky distortions in the DNA double helix, such as inter- and intra-strand cross-links. Damage recognition leads to the removal of a short single stranded DNA segment that includes the lesion, creating a single strand gap in the DNA, which is subsequently filled in by DNA polymerase, using the undamaged strand as a template. Nucleotide excision repair can be divided into two subpathways, global genomic NER and transcription coupled NER. Global genomic NER refers to the excision of altered, transcriptionally silent, DNA nucleotides. Mismatch repair (MMR) is responsible for the recognition and repair of mispaired nucleotides. The repair system removes errors that escape proofreading and targets mispaired bases that arise through replication errors during homologues recombination and as a result of DNA damage.

There is considerable overlapping activity between the different pathways. Mismatch repair (MMR) and BER appear to have partially overlapping functions [31]. Although oxidative DNA damage is mainly processed by the base excision repair (BER) pathway [32], all three pathways have been implicated in the repair of oxidative damage [33,34]. There is additionally mounting evidence to implicate cooperation of DNA glycosylases with components of repair pathways other than BER [35]. A single lesion may thus be processed by BER, NER and MMR proteins until properly repaired. This suggests a mechanism for crosstalk between different repair and checkpoint signaling pathways in cellular DNA damage response [36-38].

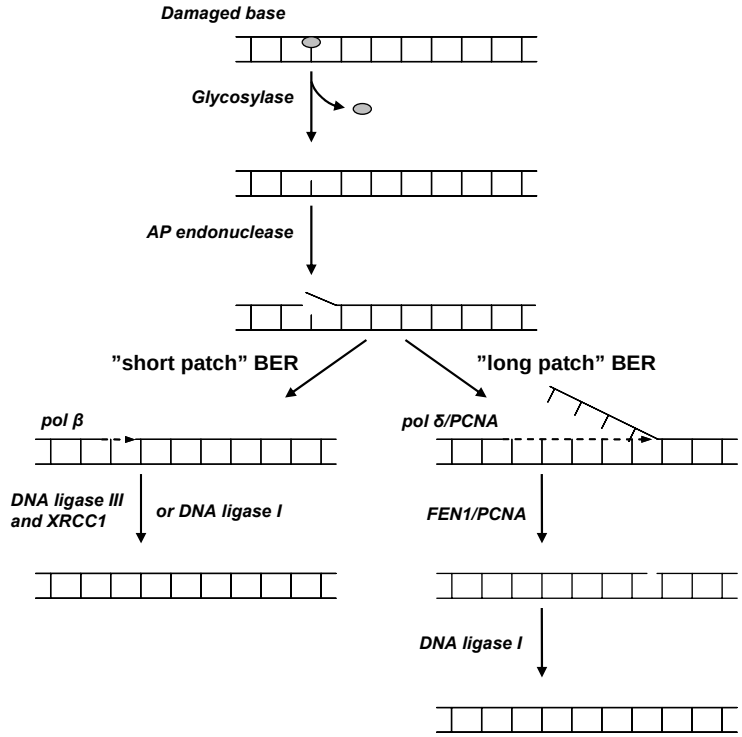

Fig 3. DNA damage processed by base excision repair (BER). The damaged base is identified and removed by a DNA glycosylase. Excision of the damaged base leaves an apurinic/apyrimidinic (AP) site. The repair of the resulting AP site involves the replacement of either a single nucleotide (short patch BER) or of several nucleotides (long patch BER). The resulting single strand gap is filled by a DNA polymerase and repair is completed by a DNA ligase that seals the remaining nick.

Base excision repair and DNA glycosylases

The base excision repair (BER) of modified nucleotides is initiated by damage-specific DNA glycosylases. The repair of the resulting apurinic/apyrimidinic site (AP site) involves the replacement of either a single nucleotide (short patch BER) or of several nucleotides (long patch BER) (fig. 3). The mechanism that controls the selection of either BER pathway is unknown. DNA glycosylases cleave the N-glycosidic bond between the target base and the deoxyribose, releasing a free base and an AP site. In the short patch model, an AP endonuclease cleaves the phosphodiester bond immediately 5′ to the AP site, generating 5′-sugar phosphate and 3′-OH ends as it nicks the DNA (fig. 3). Removal of the 5′- sugar phosphate moiety by a deoxyribophosphodiesterase (dRpase) results in a single nucleotide gap that is then filled by a DNA polymerase and sealed by DNA ligase [39]. As in short patch BER, AP sites in long patch BER are processed by an AP endonuclease which cleaves

immediately 5′to the AP site, generating 5′- sugar phosphate and 3′-OH ends. However, in this process, the 5′-sugar phosphate residue is not removed by a dRpase, rather a DNA polymerase adds several nucleotides to the 3′ end of the nick displacing the 5′- sugar phosphate as part of a single stranded flap structure. This flap structure is recognized and excised by flap endonuclease (FEN1) and DNA is finally ligated by DNA ligase. This pathway is dependent on proliferating cell nuclear antigen (PCNA), which presumably plays a role in loading DNA polymerase onto the DNA as well as stimulating the activity of FEN1 [40].

Many DNA glycosylases have been discovered and isolated, and their reaction mechanisms and substrate specificities have been elucidated. Most of the known products of oxidative damage to DNA are substrates of DNA glycosylases with broad or narrow substrate specificities. Some possess cross-activity and remove both pyrimidine- and purine-derived lesions. Overlapping activities between enzymes also exist. DNA glycosylases with overlapping substrate ranges provide back-up or redundant functions, so that the absence of one will be covered by another for the repair of a critical mutagenic lesion. The stability and correct function of the DNA is necessary for normal cellular functions and damage to the DNA can lead to cellular dysfunction, cancer and other diseases, or to cell death. Deficiencies and polymorphisms in DNA glycosylase-encoding genes have been shown to be related to human disease susceptibility [41].

8-Oxoguanine DNA glycosylases

A major product of DNA oxidation is the miscoding base 7,8-dihydro-8-oxoguanine (8-oxodG). It is found in DNA following oxidative damage mediated by reactive oxygen species (ROS), and is the most stable and deleterious product known caused by oxidative damage to DNA. It frequently mispairs with the incoming dAMP during DNA replication, leading to G:C→T:A transversions [42].

In prokaryotes, several DNA repair enzymes known as the “GO system” prevent mutagenesis via 8-oxodG [43]. This system consists of MutT, a 8-oxodGTPase that prevents incorporation of 8-oxodG into DNA from the triphosphate pool [44]; Fpg (MutM), an 8-oxoguanine-DNA glycosylase that preferentially excises 8-oxodG paired with C [45]; and MutY, an adenine-DNA glycosylase that preferentially excises A paired with 8-oxodG [46], initiating a round of base excision repair that restores the 8-oxoG:C pair, a substrate for Fpg. The mechanism to defend against the mutagenic effects of 8-oxodG lesions is conserved among organisms.

Human cells possess functional homologs of MutT (hMTH1), MutM (hOGG1, hOGG2) and MutY (hMYH). The MutY homolog (MYH or MutYH) and the functional MutM/Fpg homolog (OGG) are not present in *S. cerevisiae and S. pombe*, respectively.

The repair of 8-oxodG has received considerable research interest. An impaired or defective repair system may result in elevated levels of lesions and an increased risk of disease. Lesions such as 8-oxodG are potentially mutagenic in mammalian cells, and 8-oxodG derived G:C→T:A transversions are commonly observed in tumor suppressor genes in cancer. Hence 8-oxoG DNA repair capacity has been seen as a potential marker of cancer susceptibility [47].

The MutYH glycosylase

MutYH (MYH) is an adenine DNA glycosylase and initiates post-replication BER by recognizing and removing adenine residues from DNA paired to 8-oxodG or dG [48-50]. Apurinic (AP) sites generated by MYH glycosylase is cleaved by AP endonuclease (APE), generating 3′-OH and 5′-deoxyribose phospate (dRP). The 3′-OH residue serves as a primer terminus for repair synthesis. Unlike regular BER, the DNA glycosylase MYH removes the undamaged base adenine, and DNA polymerase inserts a nucleotide opposite the lesion 8-oxodG. The reactions must ensure the formation of 8-oxoG:C pair, which is then repaired to G:C by regular BER initiated by 8-oxoguanine DNA glycosylase (OGG1). MYH is located in replication foci [51] and interacts with the proliferating cell nuclear antigen (PCNA), APE1, MSH6 and RPA [50,52]. The post-replication repair is coupled with replication [53] and is suggested to follow long-patch BER. The human MutYH is targeted to both the mitochondria and the nuclei. The subcellular localization of MUTYH in human cells indicates that mitochondrial DNA is an important target for BER initiated by MUTYH as well as the MutMH (hOGG1), probably because of the increased level of oxidative stress in mitochondria [54,55].

MutYH can remove adenine from A/GO, A/G, A/C and guanine from G/GO mismatches. The adenine specificity to A/G and A/GO is consistent with the mutation phenotype of MutY mutants for G:C→T:A transversions. In *E. coli*, the frequency of adenine misincorporation opposite 8-oxodG is approximately 30% when measured in MutM- and MutY-deficient cells, and more than 90% of these misincorporated residues are removed by MutY initiated post-replicative repair. Although a misincorporation frequency of adenine residues has not yet been determined in human cells, a similar repair process is expected to operate since MYH defective cells are mutation prone [56]. The *S. pombe* MYH knockout strain displays a 36-

fold higher mutation frequency than the wildtype strain [57]. Because *S. pombe* does not contain any MutM or OGG1 homolog, a single SpMYH mutant may behave like a MutY/MutM double mutant of *E. coli* whose mutation rate is three orders of magnitude higher than wild type cells [46].

MutY has a high affinity to its DNA product containing an AP site [58]. The purpose of the tight binding of MutY to its product is to prevent the toxic effect of AP site until other components are recruited to carry out the next repair step. Another biological significance of MutY binding to AP/GO mismatches following its glycosylase activity may be to prevent removal of GO or cleavage at the AP site by MutM, and thus to avoid the formation of double-strand breaks.

MutYH deficiency and colorectal cancer

MAP (MutYH-associated polyposis) is a recently described colorectal adenoma and carcinoma predisposition syndrome that is associated with biallelic inherited mutations of the human MutY homolog gene hMYH. MAP tumours display a mutational signature of somatic G:C→T:A transversions in the adenomatous polyposis coli and K-ras genes, reflecting the role of MutYH in the base excision repair of adenines misincorporated opposite 7,8-dihydro-8-oxoguanine. The pattern of somatic mutation observed in adenomas and colorectal cancers from MAP patients supports a causal relationship between MutYH associated deficiency in BER and colorectal tumorigenesis in MAP [59,60].

The adenomatous polyposis syndromes are the most common inherited syndromes that increase the risk for colorectal cancer and, in some cases, for other cancers. The adenomatous polyposis syndromes include familial adenomatous polyposis (FAP), attenuated FAP (AFAP), and MYH -associated polyposis (MAP). The most distinct difference between the inherited colorectal cancer syndromes is the number of polyps that develop in the colon and rectum. FAP is caused by inherited mutations in the APC (adenomatous polyposis coli) gene. AFAP is less well characterized than FAP.

Mutation analysis of MutYH has been undertaken in affected siblings with FAP-like and AFAP-like phenotypes in whom no inherited APC mutation could be identified [61,62]. Biallelic MutYH mutations have been identified in approximately 25% of such cases, and more than 20 truncating mutations, missense mutations, and splice site mutations, that are likely to be pathogenic, have been identified to date. Non-truncating mutations appear to

cluster in known functional domains of MutYH, while truncating mutations have been identified throughout the coding region.

Few MutYH mutations have so far been subjected to functional analysis, although the two most frequent mutations (Y165C, G382D) have been investigated [60]. Both mutations severely diminished the substrate recognition ability and the capability to discriminate between guanine and 8-oxodG. Also the glycosylase activity of both mutants was noted to be less efficient. Two further missense mutations (R227W, V232F), that lie close to, or within, the putative MSH6 binding domain of MutYH, have been investigated [63]. Neither mutation affected MSH6 binding, but both mutant proteins were compromised in A:8-oxoG binding and in their glycosylase activities.

MutYH and mismatch repair

OGG1 and MYH have a central role in preventing a build-up of DNA 8-oxodG damage and tumour formation. However, alternative pathways that act independently to exclude, or remove, 8-oxodG from DNA exist. DNA mismatch repair (MMR) is one of the alternative pathways for controlling DNA 8-oxodG levels, and the oxidized base accumulates extensively in the DNA of MMR-deficient human and mouse cells treated with oxidizing agents or low dose rate ionizing radiation [64,65]. *Saccharomyces cerevisiae* MMR appears to compensate for the apparent absence of an MYH function and removes misincorporated A from 8-oxoG:A base pairs (Ni, 1999). It has been shown that the human MMR complex MSH2/MSH6 is activated upon recognition of 8-oxodG [66]. Furthermore, it has been demonstrated that MYH interacts with the MSH2/MSH6 heterodimer via MSH6, and MSH2/MSH6 stimulates the DNA binding and glycosylase activities of MYH with an 8-oxoG:A mismatch [52]. Because both MYH and MSH6 interact with PCNA and colocalize to the replication foci, PCNA may act as a coordinator of both repair pathways [50,67]. Therefore, MYH-mediated BER may cooperate with MMR to prevent 8-oxodG-mediated mutagenesis.

MutYH interactions with cell cycle checkpoint proteins

DNA repair is coordinated with cell cycle progression and DNA damage checkpoints. In response to specific forms of genotoxic stress, unique sensor proteins detect the damage and trigger cell cycle arrest. The ataxiatelangiectasia mutant (ATM) and the ATM and Rad3-related (ATR) gene products are critical proteins necessary for maintaining the fidelity of these checkpoint pathways. The Rad9-Rad1-Hus1 (9-1-1) complex has been characterized as a sensor of DNA damage and targeted to the nucleus and damaged DNA following genotoxic

stress [68]. The 9-1-1 complex is believed to act as an integral part of the DNA damage sensing system in collaboration with ATM and ATR to promote efficient cell cycle arrest and genotoxin resistance.

Recent data suggest that the 9-1-1 complex and its Rad9 component serve different and multiple functions in cells by sensing DNA damage, stimulating apoptosis, DNA repair, and regulating gene transcription [69-71]. It is shown that 9-1-1 interacts with and/or stimulates components of the BER pathway including the human polymerase β (Polβ), flap endonuclease 1 (FEN1), RPA, DNA ligase 1, and *S. pombe* MutY homolog (SpMYH). All three domains of the 9-1-1 complex associate with SpMYH DNA glycosylase. Furthermore, the SpMYH and 9-1-1 interaction has been shown to significantly increase following hydrogen peroxide treatment and is dependent on SpMYH expression [69]. This association is correlated with genotoxin-induced phosphorylation of the Hus1 protein, providing further evidence of a damage specific interaction.

It is shown that more than one subunit of the 9-1-1 complex can be associated with the same protein. The hRad17 protein interacts predominately with hRad9 and to a much lesser degree with hRad1 but not with hHus1 [72]. It is possible that one of the three 9-1-1 subunits is involved in major interactions with other proteins. Whether the specific type of genotoxic stress is correlated with specific interactions between the 9-1-1 individual subunits and their corresponding interactions partners remains unclear. The SpMYH has been shown to interact with each subunit of the 9-1-1 complex and with individual subunits in the absence of the other two subunits [69]. This indicates that SpMYH may undergo an asymmetrical interaction with the 9-1-1 complex.

The observation of the 9-1-1 sensor complex as a component in base excision DNA repair indicates a wider role for the 9-1-1 complex, beyond as a damage sensor to activate checkpoint control. Studies in yeast have revealed other direct roles of 9-1-1 in translesion synthesis repair [73] and in responses to DNA double strand breaks [74]. It is thought to have a role in recruitment and stimulation of both damage response mediators and DNA repair proteins, and may act as a recruitment platform for different factors involved in BER. This supports a model where checkpoint proteins require a series of “adaptors” to recognize DNA damage [69]. A DNA glycosylase would initially recognize specific DNA lesions, and then recruit the 9-1-1 complex to initiate the checkpoint control signaling and activation of cell cycle arrest and DNA repair.

Evolution of DNA repair genes

All known forms of life need efficient systems to maintain the integrity of their genetic material. As DNA is under constant attack by different environmental agents and metabolic by-products, evolution has provided organisms with several DNA repair pathways to remove lesions in their genetic material. These pathways have important contrasting roles in evolution, safeguarding the genome, and allowing for a certain level of mutations in the course of evolution. The critical balance of these two activities is probably the best reason for the high levels of conservation observed in DNA repair related proteins, even across the three kingdoms; bacteria, archaea, and eukaryotes [75]. Selection for different mutation rates and exposure to fluctuating levels of DNA-damaging agents in different organisms and in different environments are likely to have shaped the complex and highly redundant DNA repair pathways in modern organisms [76]. Duplications of DNA repair-related genes may have resulted in redundancy and furthermore improved the organisms' responses to environmental challenges. Duplicated genes and divergent evolution of ancestral enzymes have resulted in superfamilies of modern-day DNA repair enzymes that differ in their substrate specificity and reaction specificity [77]. Most such gene duplications seem to have arisen very early in evolution and played a central role in the diversification of enzymatic function.

Other evolutionary patterns, such as domain fusion events, also contribute to the diversification of repair proteins. The mechanism of domain recruitment most likely provides a more favourable pathway in creation of novel enzymatic activities than single point mutations, because of the use of pre-existing binding and catalytic motifs. Throughout biology, and particularly in DNA repair, cellular processes are carried out by multiprotein complexes [78]. The evolution of these protein-protein interactions has probably been a critical component of evolving DNA repair pathways. Moreover, regulation of these complexes via posttranslational modification provides an additional level of complexity [79] in evolutionary aspects.

The vast diversity of DNA repair enzymes and their tremendous catalytic diversity suggest that DNA repair pathways themselves are under selective pressure to be evolvable [76]. The ability to recognize and repair new types of DNA damage provides a powerful evolutionary force in response to changing environments.

Evolution of base excision repair DNA glycosylases

DNA glycosylases constitute one of the largest classes of repair enzymes. The helix-hairpin-helix (HhH) superfamily of base excision repair DNA glycosylases is composed of multiple phylogenetically diverse enzymes, that are capable of excising varying spectra of oxidatively and alkyl-damaged bases. Several DNA glycosylases are able to efficiently catalyse the excision of both purine- and pyrimidine derived base lesions [80]. Traditionally, DNA glycosylases have been distinguished as having either narrow or broad substrate specificity. The HhH superfamily members have diverged to accept a wide variety of very different substrates, and enzymes with narrow substrate specificity seem to be less common. Presumably the broad substrate specificity of these enzymes is advantageous because of the structural diversity of DNA damage, and allows a single enzyme to protect against multiple types of damage. Additionally, a narrow specificity would likely require a specific enzyme for each specific lesion. More importantly, this strategy could provide capacity to repair new types of DNA damage and from an evolutionary point of view contribute to new repair activities [76]. However, most DNA glycosylases appear to have a single preferred substrate and exhibit substantially lower turnover rates for other substrates.

Both substrate specificity and reaction specificity has varied in the HhH superfamily of DNA glycosylases, and the amino acid sequences have diverged considerably among these enzymes. Yet, the overall three-dimensional protein fold, the active site location, and in many cases key catalytic residues have been conserved. Residues critical for DNA binding, nucleotide flipping, and N-glycosidic bond cleavage are highly conserved throughout the superfamily. Nevertheless, convergent evolution can occur, and the existence of several different structural families of DNA glycosylases demonstrates that these enzymes have evolved independently at different times in the past [76]. It is not yet known whether extensive structural differences between HhH glycosylases may reflect some additional functional differences, such as protein-protein interactions.

Six major helix-hairpin-helix gene families are identified, and sequences from all three domains of life are represented in four of the families [81]. Each of the six HhH gene families is named according to functionally characterized glycosylases included in the respective families (Nth, Ogg1, MutY/Mig, AlkA, MpgII, and OggII). Three gene families (MutY/Mig, Ogg1, and OggII) contain glycosylases that either directly excise 8-oxoguanines or correct base pairing mismatches associated with this type of damage. All of the helix-hairpin-helix

DNA glycosylases share the basic HhH DNA binding domain, whose amino acid sequences are thought to impart specificity in damage recognition [82]. The HhH superfamily includes monofunctional and bifunctional DNA glycosylases, with MutY sharing both monofunctional and bifunctional features.

The evolutionary relationships of different HhH homologs and the extent to which they are conserved across phylogeny still remain unclear. The HhH superfamily is thought to have diverged very early in evolution, prior to the divergence of the three domains of life, and phylogeny provides evidence for multiple lineage-specific gene duplication events in some eukaryotic homologs. Also the number of HhH glycosylase genes show extensive variation between different organisms, possibly reflecting major differences among species in DNA repair pathways and mechanisms [81].

On average, archaeal and eukaryotic genomes contain more HhH glycosylase genes than bacterial genomes [81]. Species with fewer glycosylase genes still have to deal with the diverse spectrum of DNA base damage in their genome. It is possible that a given glycosylase in these organisms is able to recognize a broader spectrum of damage than the orthologous glycosylases found in species with genomes encoding multiple HhH homologs. Damaged DNA bases could also be repaired by BER glycosylases outside the HhH superfamily, and/or by completely different DNA repair pathways. Separate DNA repair pathways may play a role in the repair of oxidatively damaged purines and methyl-damaged bases. Overlap between BER and NER, in the recognition of 8-oxoguanine, is supported by studies in yeast [38]. Mismatch repair (MMR) is another likely candidate for overlap with BER, as many base-pairing mismatches are repaired by the mismatch repair system and BER glycosylases such as MutY [52].

Glutathione S-trasferases and oxidative DNA damage

The extent of oxidative DNA damage is dependent on detoxifying enzymes such as glutathione S-transferases (GSTs), antioxidant defense systems, macromolecules that scavenge electrophilic intermediates, and DNA repair systems. Glutathione S-transferase is a member of the GST family of proteins, which help protect the cell against DNA damage, and are believed to exert a critical role in cellular protection against ROS. The quantitative presence of this enzyme system is important for defining the sensitivity of cells toward genotoxic factors. Certain polymorphisms in GSTs are associated with changes in enzyme activity and sensitivity to chemotherapeutic agents [83].

GST enzymes attach reduced glutathione to electrophilic groups in a wide variety of toxic compounds, including chemotherapeutic agents and ROS. The function of GSTs is to bring the substrate into close proximity with GSH by binding both the electrophilic substrate and GSH to activate the sulfhydryl group of GSH, thereby allowing nucleophilic attack of glutathione on the substrate. Many of the compounds that induce GST are themselves substrates for these enzymes, or are metabolized (by cytochrome P-450 monooxygenases) to compounds that can serve as GST substrates. The metabolism of foreign compounds usually involves two distinct stages, commonly referred to as phase I and II metabolism. Phases I and II enzymes catalyze the conversion of a lipophilic, non-polar xenobiotic into a more water soluble and therefore less toxic metabolite, which can then be eliminated more easily from the cell. Phase I metabolism involves an initial oxidation of the xenobiotic by cytochrome P450 (CYP) monooxygenases. This step is followed by phase II metabolism, which frequently involves conjugation reactions catalyzed by glutathione S-transferases (GST). The biochemical basis for protection by GST includes not only conjugation reactions, but also drug sequestration. Different GSTs may exhibit different activities for either a specific compound or metabolites formed from the particular compound.

A key determinant of the cellular response to oxidative stress relates to the level and form of glutathione. Glutathione itself is a critical factor in maintaining the cellular redox balance and has been demonstrated to be involved in regulation of cell signalling and repair pathways [84]. A major factor that affects glutathione homeostasis is its utilization by conjugation, primarily via GST. The ability of GST to alter levels of cellular glutathione in response to production of ROS has been implicated in protection of cells from ROS-inducing agents [85]. The level of intracellular glutathione is thought to be a key regulator for the induction of stress-activated signal transduction pathways [86], and GSTs are known to have additional roles in the cell as regulatory molecules in the mitogen-activated protein kinase (MAPK) pathways [87].

Reactive oxygen species are critical in the regulation of stress responses, and acts as transduction signals that modulate the activity of the MAPK signal transduction pathways [88]. It also appears probable that GSTs are regulated *in vivo* by ROS, because hydrogen peroxide is shown to induce GST expression in mammalian cells [89]. GST induction represents part of an adaptive response mechanism to chemical stress caused by electrophilic substances.

Oxidative stress is a known mediator of cancer decease, and reactive oxygen species can play a role at different levels. Oxidative damage to DNA could lead to the mutational events of tumor initiation and progression. Another important consequence of oxidative insult is interruption of reactive oxygen-dependent cell signalling pathways controlling gene expression that contribute to all stages of cancer, in particular tumor promotion.

Glutathione S-trasferases in colorectal cancers

The colon may be especially susceptible to oxidants by the route of the gut lumen. This contains bacteria that can generate free radicals, hydrogen peroxide, and genotoxins. Colon carcinogenesis is a multifactorial process influenced by hereditary as well as environmental factors. The glutathione/glutathione S-transferase detoxification system in human colon cells is important for protection against carcinogens. A low glutathione detoxification capacity might therefore contribute to increased colon cancer risk. An association of low colonic glutathione/glutathione S-transferase activity levels and high clinical risk for the development of colorectal cancer is recently observed [90]. In addition, certain GST polymorphisms are also reported to be associated with changes in enzyme activity and sensitivity to chemotherapy in colorectal cancer [91].

The critical role of the glutathione S-transferase (GST) multigene family in cellular protection in combination with the large individual variability in the expression has made them useful as valuable biomarker proteins in many cancers, including colorectal cancers. They are considered to be important in cancer prevention and susceptibility, and high levels are linked to drug resistance [92].

Yeast glutathione S-transferases in oxidative stress

Glutathione S-transferases were first discovered in mammalian tissues, and they are still best studied in higher eukaryotes, especially in rat, human and mouse. The characterization of mammalian GSTs has been facilitated by their activity with the model substrates, as 1-chloro-2,4-dinitrobenzene (CDNB) and their ability to bind to bind to glutathione. These GSTs were initially divided into three classes, alpha, mu, and pi, on the basis of their distinct but broad and overlapping specificity for model substrates. Soluble or cytosolic GSTs, membrane associated GSTs, and mitochondral GSTs are generally recognized as distinct multigene subfamilies. Cytosolic mammalian and non-mammalian GSTs have been divided into a number of classes on the basis of primary sequence and substrate specificity.

In unicellular organisms the picture is less clear. In contrast to higher eukaryotes, both bacteria and fungi have a relatively small number of known GSTs with very diverse and often unusual functions. The *S. cerevisiae* Gtt1 and Gtt2, and *S. pombe* Gst3 are the best characterized GSTs in fungi. Recombinant Gtt1 and Gtt2 exhibit GST activity with CDNB [93]. They are likely to participate, via their GST activity, in the elimination of toxic metabolites that accumulate during oxidative stress conditions from hydrogen peroxide [94].

The *S. cerevisiae* glutathione S-transferases, Gto1, Gto2, and Gto3 display similarities with human omega class GSTs. Omega class GSTs deviate from other GST classes because they display no activity against standard GST substrates, whereas they are active thiol transferases (glutaredoxins) [95]. Thus they likely have a function in response to oxidative stress as GSH-dependent redox regulators of thiol groups.

Present study

The tumor suppressor homolog in fission yeast, *myh1*$^+$, displays a strong interaction with the checkpoint gene *rad1*$^+$ (Paper I)

The 8-oxodG repair system is crucial for the prevention of mutation related diseases, such as cancer. The 8-oxodG repair enzymes exist in a wide variety of bacteria, yeast, mammals, and plants. Inherited defects in the human MutYH (MYH) component of the 8-oxodG system are associated with multiple colorectal tumors and somatic G:C→T:A mutations in the adenomatous polyposis coli (APC) gene [59]. Furthermore, MYH mouse knockouts resulted in spontaneous cancer [96] and a mutator phenotype in embryonic stem cells and *Schizosaccharomyces pombe* [56,57]. Despite these pieces of evidence emphasizing the importance of MYH-initiated base excision repair in mutation/cancer avoidance, very little is known regarding DNA glycosylases overlapping functions in BER and other DNA repair pathways. Repair of AP sites has turned out to be unexpectedly complex and involves BER, NER, recombination repair and TLS. Genetic evidence has moreover demonstrated overlapping specificities of the BER, NER, recombination repair, and the TLS pathway for oxidative base damage in *S. cerevisiae* [38]. Several lines of evidence indicate overlapping pathways for repair of damage from ultraviolet light, and repair of alkylation damage is also considered to involve more than one pathway. This raises the question whether the MYH may have role in DNA repair of damage other than from oxidants.

The MYH is directly linked to cell cycle checkpoint control via Hus1/Rad1/Rad9 (9-1-1 sensor complex). The MYH glycosylase activity is thereby stimulated by the 9-1-1 complex. The interactions of the 9-1-1 complex with DNA repair enzymes support a model where damage recognition proteins are adaptors for checkpoint sensor proteins to activate damage response [69]. A few DNA damage recognition proteins involved in mismatch repair (MMR), nucleotide excision repair (NER), and double-strand break repair have been shown to interact with checkpoint proteins [27,97,98]. It was previously believed that an intact 9-1-1 complex may be critical for association with its binding partners. However, the possibility of asymmetrical interactions with individual subunits of the 9-1-1 sensor complex is plausible.

The fission yeast gene encoding MYH (Myh1) displays a strong interaction with the gene encoding the 9-1-1 checkpoint complex Rad1 subunit specifically. The *myh1 rad1* double mutant shows hypersensitivity to UV irradiation, HU, and MMS. This is not observed for

wildtype cells, or the *myh1* single mutant, which displays wildtype resistance to replication stress and alkylation damage. The *rad1* single mutant shows sensitivity when exposed to UV irradiation, a DNA replication inhibitor, or to DNA damage, as expected for checkpoint mutants. The highly DNA damage sensitive *myh1 rad1* double mutant, exposed to UV irradiation, undergoes a pronounced "mitotic burst" that can be measured by a septation index calculation. The number of cells passing through mitosis is considerable higher than for the *rad1* single mutants, even at half the UV dose. The wildtype and the *myh1* single mutant cells exhibit a reduced fraction of cells passing through mitosis in response to UV-induced DNA damage, indicating proper activation of the checkpoint response. The *myh1* single mutant triggers the checkpoint response to UV earlier than the wildtype, and exhibits a lower fraction of cells passing through mitosis. Also the *myh1* mutant checkpoint response is accompanied by a marked cell elongation, in average 15% more than the wildtype, possibly indicating higher levels of unrepaired DNA damage. The pronounced mitotic burst observed in the *myh1 rad1* double mutant is consistent with a severely defective DNA damage response, and UV irradiation of mutant cells results in severe chromosome segregation defects and visible DNA fragmentation. The *myh1 rad1* double mutant moreover displays morphological aberrations, even in the absence of DNA damaging agents. This is not seen in any other of the strains tested.

Besides the very strong DNA damage sensitive phenotype seen for the *myh1 rad1* double mutant, a less marked synthetic phenotype is also seen with the 9-1-1 checkpoint protein Rad9 and Myh1 for replication stress and alkylating DNA damage. Neither the *myh1 rad1* nor the *myh1 rad9* double mutants were sensitive to any of the non-genotoxic compounds tested. Strong interactions between *myh1* and *rad1* on one hand, and *myh1* and *rad9* on the other, were observed, but not with other checkpoint mutations. In addition, the checkpoint 9-1-1 single mutants show distinct differences in their sensitivity to HU and MMS, suggesting an underlying complexity in the responses of individual checkpoint genes to genotoxic stress.

Fission yeast *Schizosaccharomyces pombe* possesses two types of excision repair systems for UV-induced DNA damage, nucleotide excision repair (NER) and UV-damaged DNA endonuclease (UVDE)-dependent excision repair (UVER). The endonuclease Rad13 is a key component in the NER pathway that is considered to be the major repair system of UV-induced damage. The endonuclease Rad2 has a function in the alternative UVER excision repair pathway. Deficiency in any of these excision repair genes is associated with a UV-sensitive phenotype. A combined deletion of *myh1* in a *rad13* or a *rad2* mutant background

resulted in a slightly more or equally sensitive phenotype compared to the corresponding single mutants. Although the repair of UV-induced damage is typically processed by the NER system, mismatch repair are likely to some extent functionally overlap with base excision repair in *S. pombe*. A combined deletion of *myh1*$^+$ and *msh6*$^+$ generates a somewhat more UV-sensitive phenotype compared to the corresponding single mutants. Interestingly, the *S. pombe rhp51* homologous recombination repair single mutant is more UV-sensitive than the *myh1 rhp51* double mutant. This indicates a phenotypic suppression of the *rhp51* mutation by *myh1*.

While a role for DNA glycosylase activity in base excision repair (BER) is well understood, the mechanisms by which DNA glycosylases interact with non-BER pathways are, in many cases, poorly understood. Interplay between DNA glycosylases and MMR in the repair of oxidative lesions and in recognition and repair of non-oxidative lesions are becoming clear. The binding of MMR protein Msh6 to DNA glycosylase MutYH and the slightly observed UV-sensitivity of the fission yeast *myh1 msh6* double mutant further support the accumulating evidence of coordination of MMR and BER in processing of complex patterns of DNA damage [31]. Several DNA glycosylases are found to interact with proteins involved in the NER pathway. Base excision repair of oxidative DNA damage has been shown to be activated by the human orthologue to fission yeast Rad13 [99]. It is therefore likely that *S. pombe* Myh1 contributes to some extent in repair of UV-induced DNA damage in cooperation with the NER Rad13 protein. This is implied by the somewhat more UV-sensitive *rad13 myh1* double mutant compared to the *rad13* mutant alone. The observed genetic interaction of fission yeast *myh1* with *rhp51* moreover indicates the Myh1 involvement in recombinative repair of UV-induced DNA damage. Abasic (AP) sites as a result of DNA glycosylase removal of base lesions from alkylating DNA damage are substrates for nucleotide excision repair, long- and short-patch BER and recombination repair in *S. pombe* [100]. The deletion of an alkylation product BER glycosylase (Mag1) in the *rhp51* mutant background partially relieves the MMS sensitivity of the *rhp51* single mutant [101]. This somewhat surprisingly complex genetic interaction is the same as seen for the *myh1 rhp51* double mutant with UV-induced DNA damage, indicating a partial overlap of the BER and recombination repair pathways in processing of alkylating and UV-induced DNA damage. A crosstalk between BER, MMR, NER, and recombination repair in damage recognition and repair is conceivable and the different repair pathways are likely to compete, complement or cooperate with each other. Mechanistic explanations remain elusive, but cell cycle-dependent regulation of protein

expression, post-translational modifications, and protein degradation may favour one specific repair pathway over another.

DNA repair and/or signalling for cell cycle arrest may be a result of collective actions by several repair proteins. Several reports support the hypothesis of DNA damage recognition proteins in the BER, MMR, and the NER pathway as "adaptors" to recruit checkpoint sensor proteins to the site of damage. Recent findings support a model that MutYH is one of the adaptors for checkpoint proteins in recognition of DNA lesions [69]. In this model MutYH functions upstream of the 9-1-1 sensor complex in the damage signalling pathway. The *myh1* mutation displays a very strong interaction with the checkpoint gene *rad1* and less marked DNA damage phenotypes seen in combination with different DNA repair deficiencies in the MMR, NER, postreplication repair, and recombination repair pathways. This suggests a major role of Myh1 in activating the DNA damage checkpoint response via the 9-1-1 sensor complex. No synthetic interactions were seen with other checkpoint mutations than the 9-1-1 complex. The Myh1 is therefore likely to operate early in the initiation of the DNA damage response. The more subtle phenotypes observed, from UV-induced damage, in *myh1* double mutants with other repair pathways also implies that Myh1 is involved in recognition and repair of a wider range of DNA damage than previously thought.

The very strong genetic interaction between *rad1* and *myh1*, and to a lesser extent between *rad9* and *myh1*, is an interesting observation. It is possible that individual subunits of the 9-1-1 complex may be partly substituted by Myh1, and some data indicate that Myh1 may also undergo an asymmetrical interaction with the 9-1-1 complex upon oxidative DNA damage [69]. It would be interesting to find out more about the *myh1* interaction with individual checkpoint sensor genes depending on the type of induced DNA damage.

A novel role for Myh1 in DNA repair after treatment with DNA cross linking and strand-breaking chemotherapeutic agents (Paper II)

The highly conserved DNA glycosylase MutYH physically and functionally interacts with the checkpoint sensor complex 9-1-1 (paper I) [69,102].

The 9-1-1 checkpoint proteins Rad9, Rad1 and Hus1 play important roles in both cell cycle checkpoint control and DNA repair. The deletion of either of the three genes for these proteins in fission yeast *Schizosaccharomyces pombe* inactivates the S/M, intra-S and G2/M

checkpoint controls, and sensitizes fission yeast cells to killing by UV light, gamma rays and the replication inhibitor hydroxyurea [103-105]. The combined deletion of fission yeast *myh1*$^{+}$ and *rad1*$^{+}$ yields an extremely sensitive phenotype to UV-induced damage, alkylating DNA damage, and replication inhibitor hydroxyurea. The question whether the MutYH glycosylase activity is stimulated by Rad1, Rad9, or Hus1 separately, or by the entire heterotrimer 9-1-1 complex, is not clear. It is believable that an intact 9-1-1 complex may function more efficiently in stimulating BER-specific repair proteins than individual subunits. Because DNA glycosylases have distinct substrate specificities, the 9-1-1 complex likely needs to be "channelled" to different BER pathways in response to different DNA damage signals. Asymmetrical interactions between the 9-1-1 complex subunits and other proteins involved in the DNA damage checkpoint response have been reported [69,72,102]. A study of fission yeast MutYH (Myh1) interactions with the 9-1-1 components in response to an extended range of DNA damaging substances was therefore considered to be of interest in providing more data about the seemingly dynamic interplay between BER DNA glycosylases and the 9-1-1 checkpoint complex, depending on the type of DNA damage induced.

As the *rad1 myh1* double mutant is hypersensitive to UV-induced DNA damage (paper I), causing mainly pyrimidine dimmers and oxidative damage, the sensitivity to intrastrand crosslinking platinum compounds was examined. Similarly to what is observed for UV, MMS, hydroxyurea (paper I), the *rad1 myh1* mutant is hypersensitive also to platinum (II) compounds and oxaliplatin. The same observation is made for the DNA single- and double-strand break inducers bleomycin and phleomycin. Long term exposure to cisplatin is moreover consistent with a slight growth inhibition for the *rad1* single mutant, while the *myh1* single mutant displays wildtype resistance. A similar growth inhibition pattern is observed with oxaliplatin exposure, except that the *rad1* mutant exhibits more pronounced drug sensitivity. Notably, the difference between single 9-1-1 checkpoint mutants and *myh1* double mutant sensitivities is not seen for *rad9* or *hus1* mutants. The outcomes of treatment with bleomycin and phleomycin differ from that of platinum compound exposure, in the way of showing growth curves with a marked biphasic character, for all mutants deficient in a 9-1-1 component. In addition, the individual 9-1-1 checkpoint complex mutants display equal sensitivity and only the *rad1 myh1* double mutant shows drug hypersensitivity. These growth curve data strongly indicate a requirement for Myh1 in DNA damage response to DNA-crosslinking and DNA-double strand breaking substances, and asymmetric interactions between Myh1 and the 9-1-1 subunits.

Cellular response to DNA damage from cisplatin and hydrogen peroxide causes decreasing levels of soluble Myh1. A dose- and time dependent decrease is observed following short time treatment with cisplatin and hydrogen peroxide respectively. This could be consistent with a protein relocalisation, downregulation or degradation. Extraction of both soluble and chromatin binding proteins, under similar conditions of exposure, alters the Myh1 level. Following treatment with cisplatin or hydrogen peroxide, a slight increase in the total cellular amount of Myh1 is observed. This likely indicates Myh1 chromatin binding following cisplatin-induced DNA damage, and further support the growth data of the Myh1 requirement in DNA damage response to cisplatin-induced intrastrand-crosslinks.

The observed increased Myh1 chromatin binding upon cisplatin and hydrogen peroxide treatment suggests a protein relocalisation and a possible protein upregulation. A Myh1 relocalisation to the cell nuclei upon DNA damage is confirmed using GFP-tagged Myh1 examined with fluorescence microscopy. A strong nuclear GFP signal is demonstrated in treated cells, while no relocalisation signal is observed in untreated cells. This clearly shows an activation of Myh1 in response to DNA damage induced by cisplatin and hydrogen peroxide.

The role of Myh1 in repair of cisplatin-induced damage is however not clear and not earlier investigated. More recent data imply multiple DNA repair pathways in cooperation with checkpoint signalling in order to organize the cellular DNA damage response to chemotherapeutic drugs [106,107]. Cross-talk and functional overlap between primarily the BER and MMR pathways, in processing complex spectrum of DNA damages, are becoming evident [31]. The molecular interactions and mechanisms however are unknown. The Myh1 base excision repair protein may have a role in initial damage recognition of DNA-Pt adducts, by competing with or assisting mismatch repair. The Myh1 protein most likely has a critical function in activating the DNA damage checkpoint response and mediating DNA repair by the BER pathway upon DNA damage induced by hydrogen peroxide and cisplatin. It is plausible that the MutYH glycosylase has an integrated role in DNA damage detection, checkpoint control activation, and processing of diverse DNA damage spectrum.

The checkpoint complex 9-1-1 components are known to be associated with damaged DNA induced by genotoxic stress [68,108]. Because of the highly sensitive DNA damage phenotype of the *rad1 myh1* mutant and the observed cisplatin and hydrogen peroxide-induced nuclear relocalization of Myh1, a functional link between Myh1 and Rad1 in DNA

damage recognition and repair is conceivable. The demonstrated unequal function of the 9-1-1 complex subunits with Myh1 in response to hydrogen peroxide, UV-irradiation, MMS and hydroxyurea, (paper I) [69], is further supported by data on cross-linking and DNA-dsb inducing agents in this work. The hypersensitivity seen for the *rad1 myh1* double mutant upon bleomycin and phleomycin exposure is not observed for a combined deletion of *myh1* with *rad9* or *hus1* respectively.

Human MutYH (hMYH) is reported to physically interact with hHus1 and hRad1, but not with hRad9. Also human MYH can interact with hHus1 even in the absence of hRad1 and hRad9 [102]. It is not yet clear whether these reported asymmetric interactions between MutYH and the 9-1-1 complex are dependent of the nature of the DNA damage. *In vitro*, the interaction of Myh1-Hus1 is demonstrated to be selectively enhanced by hydrogen peroxide treatment [69]. One may speculate that Myh1 could compensate for a Rad1 deficiency at the site of damage, and thereby stabilising a larger repair complex of checkpoint sensor proteins and proteins required for regulating DNA replication and repair. This raises questions about the nature and function of the 9-1-1 checkpoint sensor complex itself. The 9-1-1 complex is thought to be a damage specific substitute for PCNA, in acting as a platform and coordination factor for DNA repair proteins [109]. Other results point to a direct role of 9-1-1 as a component of DNA repair systems. The 9-1-1 complex stimulates or associates with enzymes involved in nearly every step of the long-patch base excision repair pathway, among them the MutYH glycosylase [110]. Recent findings moreover indicate a 9-1-1 stimulation of the long-patch BER protein DNA ligase I, independently whether the checkpoint 9-1-1 trimeric complex is able to load onto the DNA or not [111]. It is therefore possible that 9-1-1 stimulation of BER enzymes may derive from protein-protein interactions that do not involve DNA encirclement at all. A stimulation of the MutYH glycosylase activity by either of the Rad9, Rad1 and Hus1 subunits separately, from protein-protein interactions, can not be ruled out. The highly sensitive DNA damage profile seen for the lack of Myh1 upon genotoxic stress, other than oxidative, mainly in the *rad1* mutant background may further support the hypothesis of specific protein-protein interactions between MutYH and the 9-1-1 checkpoint complex in response to DNA damage. In line with this it would be interesting to perform additional biochemical analyses in DNA damage dependent phosphorylation of the 9-1-1 complex components and their subsequent interactions with Myh1.

Evolutionary loss of 8-oxo-G repair components among eukaryotes (Paper III)

The basic processes of DNA repair are highly conserved among both prokaryotes and eukaryotes. Organisms are provided with multiple DNA repair patways, that have contrasting roles in the course of evolution, maintenance of the genome integrity and allowing for a certain level of mutations. The ability of a large number of protein structural motifs to catalyze relevant chemical reactions has moreover played a significant role in the elaboration of repair mechanisms during evolution. The base excision repair pathway (BER) has evolved to protect cells from the deleterious effects resulting from oxidation, alkylation, and deamination of DNA bases. Oxidative damage in DNA, and specifically the 7,8-dihydro-8-oxoguanine (8-oxodG) lesion is removed or prevented by the 8-oxodG-specific base excision repair (BER) enzymes MutY, MutM, and MutT [43]. The MutY and MutM glycosylases are both members of the helix-hairpin-helix (HhH) superfamily. This gene family is the most diverse of the DNA glycosylases, with divergent substrate specificities [81]. The MutT homolog belongs to the group of nudix hydrolases and is not classified as a DNA glycosylase, although a component of the 8-oxodG repair system [112].

Limited phylogenetic data is accessible about the highly diverse and adaptable HhH gene family of repair enzymes among eukaryotes. The availability of complete genome sequences from multiple eukaryotic organisms therefore made it possible to perform a phylogenetic distribution analysis among eukaryotes of the 8-oxodG repair genes, along with a sequence analysis of identified repair genes.

The phylogenetic distribution and sequence analysis of the 8-oxoG repair components included a large number of species from the kingdoms of fungi, animals, and plants. In general, all three homologs of MutY, MutM, and MutT are found in most surveyed subgroups of animals and plants with a few exceptions. Land plants appear to lack a MutT homolog, but do possess MutY and MutM homologs. The MutY repair homolog is not found among insects and annelids. Interestingly, the nematode *C. elegans* harbours none of the three 8-oxodG repair genes, while they are all found in the nematode *T. spiralis*. This indicates a somewhat patchy distribution pattern within certain animal subgroups. Also the molluscs display a scattered distribution of these repair enzymes, with no single species harbouring all three of the repair homologs. However, incomplete genome sequencing cannot be ruled out as a

source of error, and additional 8-oxodG repair genes are likely to be found in some animal subgroups.

In fungi, the distribution looks more phylogenetically diverse. Overall, all three repair homologs are found among basidiomycetes. All ascomycetous fungi were found to harbour the MutM homolog, with the exception of "Schizosaccharomyces", although the single species *S. japonicus* seems to have the MutM protein as well. In the Saccharomycotina group, the MutT homolog seems to be lost among "Saccharomyces" species. The "Candida" subgroup of Saccharomycotina does harbour the MutT homolog, but lacks the MutY repair protein. The MutY homolog appears not to be present at all in the Saccharomycotina group. Interestingly, the MutY component is found in the Sordariomycetes, and in Dothideomycetes of Pezizomycotina, as well as in "Schizosaccharomyces". The Pezizomycotina subgroup Eurotiomycetes harbours the MutM homolog but seems to have lost the MutY protein. Overall, the MutM homolog apparently is the most prevalent repair 8-oxodG component among the eukaryotic domain of life.

Alignments of the MutY, MutM, and the MutT protein sequences show a strong conservation of defined protein domains throughout the majority of eukaryotic organisms in this study. Identified residues, critical for DNA binding and substrate interaction [113-115], are extremely well conserved through all species examined. Interestingly though, again the group of fungi is somewhat different. The "Saccharomyces" subgroup of Saccharomycotina is found to harbour a MutM insert, close to and downstream the HhH-PVD structural domain. Furthermore, the Eurotiomycetes subgroup of Pezizomycotina harbours another MutM insert, located immediately upstream of the HhH-PVD domain. Interestingly, fungi with any of the two identified MutM sequence insertions do not possess either the MutY or the MutT repair homolog, with the exception of the Pezizomycotina group Dothideomycetes, that harbours both the MutM N-terminal insert and a MutY homolog. Conversely, the Saccharomycotina group "Candida" and the Pezizomycotina group Sordariomycetes, that do not harbour any of the two MutM insertions, instead are found to have a small MutT sequence insertion and a longer MutY insertion, respectively. The MutY insertion interestingly is located in the beginning of the specific "adenine recognition site", the substrate binding domain of the MutY protein. While the MutY insertion is located in the important substrate recognition site, the MutT insertion is located outside the highly conserved structural "nudix motif" in the catalytic site of the protein. All identified sequence insertions are notably only found among fungal species, and seem to be highly phylogenetically connected.

Evolutionary loss of individual 8-oxoG repair proteins, from early to higher developed eukaryotes, is the most likely event behind the observed distribution pattern of the MutM, MutY, and MutT proteins. The existence of highly conserved protein sequences throughout the lineage of evolution argues for specific gene losses rather than independent origins of repair genes among diverse phylogenetic subgroups of eukaryotic species. In fungi, the loss of specific repair genes in phylogenetic branches is very distinct.

The widespread distribution of the MutM homolog in eukaryotic genomes, and the lack of either the MytY or the MutT homolog, or both, probably indicate 8-oxodG in non-replicated DNA as the most abundant and important oxidative DNA damage to correct. The post-replicative adenine DNA glycosylase MutY mainly serves to excise misincorporated adenines opposite 8-oxodG, in cooperation with MMR oxidative repair [52]. This likely provides redundancy in post-replicative mismatch repair by separate pathways. All three 8-oxodG repair components, however, are highly specific for their substrates [76], and possibly may have evolved from more "promiscuous" BER repair enzymes with catalytic activity toward alternative substrates. The situation of combining "promiscuous" broad substrate enzymes with highly specific ones may provide an advantage in terms of specificity and redundancy within and between separate DNA repair pathways, and from an evolutionary perspective [76]. The organism thereby holds the capacity to deal with all different kinds of DNA damage in a new complex chemical environment.

The identified sequence insertions in MutY, MutM, and MutT, respectively, among subgroups of fungi, do not make up any obvious clues to the phylogenetically correlated distribution of the individual 8-oxodG repair homologs. It is also not possible to predict the importance of these sequence insertions for specific enzymatic activities. Possible disparities in catalytic mechanisms and in DNA repair pathways, by which an organism is processing DNA damage, probably is part of the explanation [81]. The HhH glycosylase diversity is still a puzzle that needs more experimental data in substrate specificity of individual repair enzymes, and in DNA repair pathway redundancy.

A peroxisomal glutathione transferase of *Saccharomyces cerevisiae* is functionally related to sulphur amino acid metabolism (Paper IV)

Glutathione-S-transferases (GSTs) are a family of Phase II detoxification enzymes that catalyse the conjugation of glutathione (GSH) to a wide variety of endogenous and exogenous electrophilic compounds. Members of the GST superfamily are important in cellular defence mechanisms against oxidative stress, anticancer drugs and environmental xenobiotics, in regulation of gene expression and signal transduction [116-118]. Glutathione-S-transferases have been classified into cytosolic, mitochondrial and membrane-associated forms. Most GST classes share a number of substrates, among which CDNB (1-chloro-2,4-dinitro-benzene) is commonly used in standard GST activity determinations. However, omega class GSTs display low or zero activity with standard GST substrates, while they have a significant thiol transferase (glutaredoxin) activity [95,119]. In most GST classes, an N-terminal tyrosine or serine residue is essential for the nucleophilic attack on substrates. In contrast, omega class GSTs form a mixed disulphide involving GSH and an N-terminal domain cysteine residue from the GST molecule [120,121]. These GSTs lack the active site tyrosine/serine residue characteristic of the other GST classes and are active as redox regulators of thiol groups, with GSH as a reductant.

Compared with GSTs from other organisms, those from fungal species are both more functionally and structurally diverse, and only a very few GST proteins have been studied to some extent. The yeast *Saccharomyces cerevisiae* has two GSTs, Gtt1 and Gtt2, that both act on standard GST substrates [93]. Gtt1 is associated with the endoplasmic reticulum. More recently it has been shown that Gtt1 and Gtt2 provide defence against oxidants and other stresses [122]. Overlapping functions are likely to exist between Gtt1 and Gtt2 and they are suggested to have a role in cadmium stress through the possible formation of a Cd-GSH complex [123]. A role in defence against hydroperoxides has moreover been described for the *Schizosaccharomyces pombe* homologues of Gtt1 and Gtt2 [124]. *Saccharomyces cerevisiae* cells contain three omega class GSTs with glutaredoxin activity, Gto1, Gto2, and Gto3, in addition to glutathione transferases Gtt1 and Gtt2, not classifiable into standard classes. Gto1 is located at the peroxisomes, whereas Gto2 and Gto3 are cytosolic GSTs.

S. cerevisiae GST mutants display a number of phenotypic defects under different stress conditions. By using single and multiple *gto* and *gtt* mutants exposed to oxidants and other toxic agents, a phenotypic analysis indicates the importance of yeast GSTs in protection

against oxidative stress. Lack of a single GTT gene or both together causes hypersensitivity to oxidants such as diethylmaleate, diamide, and *t*-BOOH. The *gtt2* single mutant alone is hypersensitive to 4-nitroquinolone. These observations are consistent with the proposed antioxidant role of Gtt1 and Gtt2. The hypothesis of GTO gene induction in response to oxidants is supported by the sensitivity profile of the *gto1* single mutant. The absence of GTO1 causes significant sensitivity against diamide and a modest sensitivity to diethylmaleate and *t*-BOOH compared to wildtype cells. Moreover, a *gto1 gto2 gto3* triple mutant with a combined lack of the two GTT genes is hypersensitive to cadmium. This indicates a synergistic effect between functions of Gto and Gtt in cadmium detoxification. Surprisingly, this quintuple mutant is hyperresistant to *t*-BOOH compared to any other mutant strain tested. The absence of the three Gto proteins also partially relieved the hypersensitivity of the *gtt2* and *gtt1 gtt2* mutants to 4-nitroquinolone. Further analysis of a *gtt1 gtt2* double mutant background, together with mutations in the different GTO genes, revealed that only the Gto1 protein functions to protect *S. cerevisiae* against Cd toxicity in cooperation with Gtt1/Gtt2. In addition, *S. cerevisiae* cells lacking *GTO1* are defective for growth on oleic acid medium and are affected in the metabolism of sulphur amino acids.

Gto1 most likely has a specific role in *S. cerevisiae* peroxisomes related to sulphur amino acid metabolism. Peroxisomes are a source of reactive oxygen species with an oxidizing environment inside the organelle. Under theses conditions, essential cysteine residues would become oxidized, leading to transient protein inactivation in the amino acid metabolism pathway. Gto1 acts as a glutaredoxin regulating the redox state of target cysteine residues through its deglutathionylating activity. The absence of Gto1 causes growth defects and depleted levels of intracellular GSH. The hypersensitivity observed of a triple *gtt1 gtt2 gto1* mutant exposed to Cd would result from the combination of reduced GSH levels and the lack of functional Gtt proteins with GSH-conjugating activity. Defects in a peroxisomal GST/glutaredoxin function causes a number of phenotypes, probably as a consequence of the alteration in the sulphur amino acid pathway.

A complex relationship most likely exists between Gto and Gtt proteins, with respect to the decreased sensitivity to 4-nitroquinolone and hydroperoxides, of mutants lacking all Gto and Gtt proteins. The induction of other detoxification pathways may explain these observations. Alternatively, Gto proteins could possibly convert the Gtt2 mediated GSH conjugate to a more toxic derivative. The complex relationships between GSTs in general protection against oxidative stress and in specific protein redox regulation need to be further elucidated.

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