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Regulation der *FBP1* Expression und Änderungen im Glukosestoffwechsel sind entscheidend für eine angepasste Antwort auf DNA-Schädigung und Altern in der Hefe *S.cerevisiae*

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Title:

Regulation of *FBP1* expression and changes in glucose metabolism are crucial for an appropriate response to DNA damage and aging in *S.cerevisiae*

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Dedicated to my family

Contents

Summary

Response to DNA damage, lack of nutrients and other stress conditions are the essential properties of all living systems. The coordinated response includes DNA damage repair, DNA damage checkpoints, transcriptional alterations and the activation of alternate biochemical pathways, as well as drastic measures like cellular suicide which prevents proliferation of severely damaged cells. Transcriptional response of cells exposed to DNA damaging agents is a coordinated process which induces transcription of all necessary proteins which are not only involved in the repair process, but also in the general stress response and maintenance of internal homeostasis. The most important aspects of transcriptional modifications upon DNA damage are induction of the environmental stress response program (ESR), repression of genes involved in cell cycle progression and modulation of major energy metabolism pathways.

Exposure of living organisms to smaller amounts of toxic agents and other adverse effects may be more common in natural environments than direct impact of highly cytotoxic doses of the same agents. Occurring over various time spans or as the consequence of repeated exposures, the accumulation of mutations may be as critical for the organism as the immediate cytotoxic effect. Therefore, cellular response to the treatment with DNA-damaging substances at low concentrations which are genotoxic but do not have a strong cytotoxic effect are of special interest. In addition, environmental variations that influence growth conditions, e.g. different media, and individual fitness, e.g. different strains, are likely to influence and modulate the adverse effects of individual DNA-damaging substances.

Investigating the transcriptional response of *S.cereveisiae* to low doses of the alkylating agent methylmethane sulfonate (MMS) we observed that cellular sensitivity to MMS directly depends on their ability to immediately induce the basic, stereotypical stress response program called ESR. Transcriptional response of cells cultivated in nutrient rich

medium significantly differed from those cultivated in minimal medium. In the full medium MMS treatment triggered induction of the ESR programme in a dose and time dependent manner. In contrast, expression of many genes involved in ESR was already elevated when cells are cultivated in minimal, nutrient deprived medium. Additional treatment of those cells with MMS led to the transcriptional regulation of genes more specific and necessary for DNA damage repair, cell cycle arrest or detoxification.

Comparing the response to low level treatment with MMS of two different yeast strains, FF18984 and BY4742, we found that BY4742 is more sensitive to MMS than FF18984. While FF18984 showed an immediate strong adaptation in transcription including a higher and stronger activation of ESR, the response of the BY4742 strain did not result in the transcriptional regulation of many genes. In particularly genes involved in protein biosynthesis, mRNA processing and transcriptional regulation were not affected in the same manner as observed in the FF18984 strain. Among the genes induced in BY4742 not more than three belonged to one functional group. These results led us to the conclusion that in the BY4742 strain more time may be required to response to MMS. Persistent proliferation of this strain during the first hours of MMS treatment most probably leads to a higher accumulation of mutations, conversion of primary lesions to double strand breaks (DSB) and in consequence to a higher sensitivity to the toxic agent.

Modulation of basic metabolic pathways and induction of diauxic shift are other factors that directly contributed to the increased resistance of the FF18984 strain to MMS. Metabolic adaptation and pre-induction of ESR resulting from nutrient deprivation helped this strain to cope better with the toxic effect of genotoxic agents applied later such as MMS. Our results showed that the major stimulus that triggers the adaptive response and the induction of ESR genes upon MMS treatment is an alteration in glucose utilization. These results point to an important correlation between metabolic pathways and the ability of living organisms to

cope with adverse environmental conditions. Moreover, the induction of ESR seemed to be the most important prerequisite for a proper and fast cellular response to DNA damage.

Expression of the key enzyme of gluconeogenesis fructose-1,6-bisphosphatase (*FBP1)* was clearly up-regulated by MMS in glucose-rich medium. Interestingly, deletion of *FBP1* led to reduced sensitivity to MMS, but not to other DNA damaging agents such as 4-nitroquinoline (4-NQO) or phleomycin. The reduced sensitivity of the *Δfbp1* mutant was the result of better recovery of this mutant after a long-term treatment with MMS. Reintroduction of *FBP1* in the knockout strain restored the wild-type phenotype while overexpression increased MMS-sensitivity of wild-type. The fact that terminally GFP-tagged Fbp1p restored the lack of Fbp1p on non-fermentable carbon source, but not wild-type MMS-sensitivity in *Δfbp1* cells, implicated that the function of FBPase in cellular growth on media with alternative carbon sources could be independent, at least in part, from its role in response to MMS treatment.

The connection between Fbp1p and one of the most important DNA damage signalling cascade that starts with the Mec1/Tel1 damage sensors was investigated with the RNR2-GFP reporter assay. These experiments revealed that the deletion of *FBP1* had no effect on induction through the *RNR2* promoter while overexpression of *FBP1* significantly increased the activity of the *RNR2* promoter. These results indicated that the increased intracellular level of Fbp1p after DNA damage caused by MMS probably acts as a signal that mediates cellular response to this toxic agent.

Deletion of *FBP1* reduced the production of reactive oxygen species (ROS) in response to MMS and in untreated aged cells. The mutant cells showed delayed production of ROS in the first fifteen days in aging culture what resulted in better viability in full medium. In minimal medium the lack of Fbp1p was no advantage for cellular survival. In these conditions aged *Δfbp1* mutants survived even less and accumulated similar levels of ROS. Elevated amounts of Fbp1p shortened life-span, but did not have any influence on ROS

accumulation. These results showed that Fbp1p is an important factor that modulates ROS production in response to MMS treatment and aging. However, in media with limited nutrients Fbp1p is a critical factor for cellular survival and its lack is rather a disadvantage.

Based on the above observations, we concluded that *FBP1* influences the connection between DNA damage, aging and oxidative stress either through direct signalling or an intricate adaptation in energy metabolism. In consequence, the tight regulation of *FBP1* expression and age-associated changes in glucose metabolism are not only crucial for the control of gluconeogenesis but also for an appropriate response to aging and DNA damage.

Zusammenfassung

Die Fähigkeit auf DNA Schädigungen, das Fehlen von Nährstoffen oder andere Stressfaktoren zu reagieren, ist eine wichtige Eigenschaft aller lebenden Systeme. Dies beinhaltet sowohl die Reparatur von DNA Schäden, DNA-Schädigung abhängige Kontrolle des Zellzyklus, die Änderung der Transkription und die Aktivierung alternativer biochemische Prozesse, als auch drastischere Maßnahmen, wie zum Beispiel zellulären Selbstmord, der eine Proliferation schwer beschädigter Zellen verhindert. Zellen, die DNA schädigenden Substanzen ausgesetzt werden, reagieren mit einem aufeinander abgestimmten Prozess, der auch die Regulation der Transkription aller benötigter Proteine beinhaltet. Diese Proteine spielen nicht nur bei der Reparatur der DNA, sondern auch bei der allgemeinen Antwort der Zelle auf Stress und der Aufrechterhaltung des inneren Gleichgewichts eine entscheidende Rolle. Die wichtigsten Schritte bei der Anpassung der Transkription nach einer DNA Schädigung sind die Induktion der allgemeine Stress-Antwort (ERS), die verminderte Expression von Genen, die den Zellzyklus steuern, und eine Modulation wichtiger Gene des Energiemetabolismus.

In einer natürlichen Umgebung ist zu vermuten, dass lebende Organismen kleineren Mengen toxischer Agenzien ausgesetzt sind, die nicht direkt zum Zelltod führen. Die Akkumulation von Mutationen, die durch die Einwirkung des toxischen Agens auf die Zellen über längere Zeitspannen oder durch eine wiederholte Exposition zustande kommen, kann für den Organismus genauso kritisch sein, als ein sofortiger zytotoxischer Effekt. Deshalb ist die zelluläre Antwort auf die Behandlung mit DNA schädigenden Substanzen in niedrigen Konzentrationen, in denen sie einen genotoxischen, jedoch keinen stark zytoxischen Effekt besitzen, von besonderem Interesse. Zusätzlich könnten Veränderungen der Umwelt, die einen Einfluss auf Wachstumsbedingungen haben, wie z.B. unterschiedliche Medien und die

individuelle Fitness z.B. verschiedener Stämme, die nachteiligen Effekte einzelner DNA schädigender Substanzen beeinflussen und regulieren.

Bei der Untersuchung der transkriptionalen Antwort von *S. cereveisiae* auf kleine Dosen des alkylierenden Agens Methylmethansulfonat (MMS) haben wir beobachtet, dass die Empfindlichkeit der Zellen gegenüber MMS direkt von ihrer Fähigkeit abhängt, unmittelbar die grundlegende allgemeine Stressantwort (ESR) einzuleiten. Die transkriptionale Antwort von Zellen, die in nährstoffreichem Medium kultiviert werden, unterscheidet sich signifikant von denen, die in Minimalmedium kultiviert werden. In Vollmedium leitet eine MMS Behandlung die Induktion der allgemeinen Stressantwort ESR in einer dosis- und zeitabhängigen Weise ein. Im Gegensatz dazu wurde die Expression vieler Gene, die Teil der ESR sind, erhöht, wenn die Zellen in Medium, dem Nährstoffe entzogen wurden, kultiviert wurden. Eine weitere Behandlung dieser Zellen mit MMS führt zu einer transkriptionalen Regulation von spezifischeren Genen, die für die Reparatur von DNA-Schäden, die Zellzykluskontrolle und die Entgiftung nötig sind.

Beim Vergleich der Antwort zweier unterschiedlicher Stämme auf eine Behandlung mit MMS in niedrigen Konzentrationen zeigte der Stamm BY4742 eine höhere Empfindlichkeit auf MMS als der Stamm FF18984. Während FF18984 eine sofortige starke Anpassung der Transkription, einschließlich einer stärkeren Aktivierung der ESR, zeigte, war im Stamm BY4742 keine transkriptionale Regulation vieler Gene zu beobachten. Besonders Gene, die an der Proteinbiosynthese, der mRNA Prozessierung und der Transkriptionsregulation beteiligt sind, wurden nicht auf die gleiche Weise, wie im Stamm FF18984 beeinflusst. Unter den Genen, die in BY4742 induziert wurden, gehören weniger als drei zu einer funktionellen Gruppe. Diese Ergebnisse führen uns zu der Schlussfolgerung, dass BY4742 für eine ähnliche Antwort mehr Zeit benötigt als FF18984. Die andauernde Proliferation dieses Stammes während der ersten Stunden der MMS Behandlung führt wahrscheinlich zu einer größeren Ansammlung von Mutationen, zur Umwandlung primärer

Veränderungen zu Doppelstrangbrüchen (DSB) und auf diese Weise zu einer höheren Empfindlichkeit gegenüber toxischen Agenzien.

Die Anpassung der grundlegenden Metabolismuswege und die Induktion des „Diauxic Shifts“ sind weitere Faktoren, die direkt an der gesteigerten Widerstandskraft des Stammes FF18984 gegenüber MMS beteiligt sind. Die durch eine Entziehung von Nährstoffen vermittelte Induktion der ESR und die damit verbundene metabolische Anpassung ermöglicht es den Zellen mit toxischen Effekten genotoxischer Agenzien, die wie MMS später zugefügt werden, zurechtzukommen. Unsere Ergebnisse zeigen, dass der wichtigste Stimulus für eine angepasste Antwort und die Induktion der ESR Gene auf eine MMS Behandlung, eine Änderung in der Glukosenutzung ist. Diese Ergebnisse führen zu einer wichtigen Beziehung zwischen den metabolischen Signalwegen und der Fähigkeit lebender Organismen mit ungünstigen Umweltbedingungen umzugehen. Des Weiteren scheint die Induktion der ESR die wichtigste Bedingung für eine korrekte und schnelle zelluläre Antwort auf DNA Schäden zu sein.

Die Expression des Schlüsselenzyms der Glukoneogenese Fructose-1,6-bisphosphatase (*FBP1*) wird durch eine Behandlung mit MMS in glukosereichem Medium hochreguliert. Interessanter Weise führt das Fehlen von *FBP1* zu einer Verringerung der Sensitivität gegenüber MMS, nicht aber gegenüber anderen DNA schädigenden Substanzen, wie 4-Nitroquinolin (4-NQO) oder Phleomycin. Die verringerte Sensitivität des *Δfbp1* Mutanten ist das Ergebnis einer besseren Erholung dieses Mutanten nach einer Langzeitbehandlung mit MMS. Das Wiedereinführen von *FBP1* in einen Knockoutstamm stellt den Phenotyp des Wildtyps wieder her, wohingegen die Überexpression von *FBP1* zu einer gesteigerten MMS Sensitivität des Wildtyps führt. Die Expression eines Fbp1-Fusionsproteins mit terminalem GFP reicht aus, um im Knockoutstamm das Wachstum auf nicht fermentierbaren Kohlenstoffquellen zu ermöglichen; die Empfindlichkeit gegen MMS in *Δfbp1* Zellen kann aber nicht wiederhergestellt werden. Dies impliziert, dass die Funktion

der FBPase für das Zellwachstum auf alternativen Kohlenstoffquellen, unabhängig sein könnte von seiner Funktion bei der Antwort auf eine MMS Behandlung.

Die Verbindung zwischen Fbp1p und einer der wichtigsten Signalkaskaden bei DNA Schäden, die mit den Mec1/Tel1 Sensoren beginnt, wurde mit Hilfe des RNR2-GFP Reporterassays untersucht. Diese Experimente offenbaren, dass die Deletion von *FBP1* keinen Einfluss auf die Induktion des *RNR2* Promotor hat, während eine Überexpression von *FBP1* zu einem signifikanten Anstieg der Aktivität des *RNR2* Promotors führt. Dieses Ergebnis deutet auf die Schlussfolgerung hin, dass der ansteigende zelluläre Fbp1p Spiegel nach einer DNA- Schädigung, durch MMS möglicher Weise als ein Signal fungiert, das die Antwort der Zelle auf dieses toxische Agens vermittelt.

Die Deletion von *FBP1* vermindert die Bildung von Reaktiven Sauerstoff Spezies (ROS) als Antwort auf MMS oder in gealterten unbehandelten Zellen. In Vollmedium zeigten Mutanten eine verzögerte ROS Produktion in den ersten fünfzehn Tagen einer alternden Kultur, was zu einer besseren Lebensfähigkeit führte. In Minimalmedium ist das Fehlen vom Fbp1p kein Vorteil für das Überleben der Zellen. Unter diesen Bedingungen überlebten sogar weniger *Δfbp1* Mutanten und sie akkumulierten ähnliche ROS Spiegel. Erhöhte Mengen Fbp1p verkürzten die Lebenszeit, hatten jedoch keinen Einfluss auf die Ansammlung von ROS. Diese Ergebnisse zeigen, dass Fbp1p ein bedeutender Faktor ist, der die Produktion von ROS als Antwort auf eine MMS Behandlung und das Altern moduliert. In Medien mit eingeschränktem Nährstoffangebot ist Fbp1p jedoch ein kritischer Faktor für das Überleben der Zellen und sein Fehlen ist eher ein Nachteil.

Auf Grundlage dieser Beobachtungen kommen wir zu dem Schluss, dass *FBP1* die Verbindung zwischen der Schädigung der DNA, dem Altern und oxidativem Stress, entweder über einen direkten Signalweg oder durch eine komplexe Anpassung im Energiemetabolismus, beeinflusst. Als Folge sind die enge Regulation der *FBP1* Expression und die mit dem Alter verknüpften Veränderungen im Glukosestoffwechsel nicht nur für die

Kontrolle der Gukoneogenese, sondern auch für eine passende Reaktion auf das Altern und die Schädigung von DNA entscheidend.

1. Introduction

All eukaryotic cells are constantly exposed to exogenous or endogenous agents that damage DNA. DNA is highly reactive and easily altered either by normal cell processes or by exogenous factors. One estimate is that a mammalian genome undergoes about 100,000 modifications per day, each bearing a finite probability of residual damage (Friedberg *et al.,* 1995). Whether DNA damage is only a simple base change or more complex like deletions, fusions, translocations, or aneuploidy, accumulation of unrepaired DNA damage increases genetic instability that can lead, among other things, to elevation of intracellular levels of reactive oxygen species (ROS) or facilitate tumor promotion and progression. Mutational changes of proto-oncogenes which are involved in normal cellular functions can generate an oncogene. The examples are oncogenes derived from the *c-ras* family. Each of the *c-ras* proto-oncogenes can give rise to a transforming oncogene by a single base mutation. Moreover, almost any mutation at either position 12 or 61 can convert a *c-ras* proto-oncogene into an active oncogene (see Lewin, 2000). Increased genetic instability can also lead to activation of proto-oncogenes by elevated insertion, translocation or amplification events in the cell. One such example is the *bcr-abl* fusion protein derived from reciprocal translocation of the 5000 kb region from the end of chromosome 9, carrying *c-abl*, to the *bcr* gene of chromosome 22. This fusion protein appears to activate the Ras pathway for transformation. Depending on the breakpoint in the *bcr* gene the consequence of this translocation is developing of either chronic myelogenous leukemia (CML) or acute lymphoblastic leukaemia (ALL). The translocation can be detected by the presence of the *Philadelphia (PH^1)* chromosome in patients with CML or ALL (see Lewin, 2000).

To ensure that the potentially irreparable damage will not give rise to viable mutants with an instable genome, both prokaryotic and eukaryotic cells developed a complex network to detect and eliminate such changes, known as the DNA damage response. The fact that just a

single double strand DNA brake (DSB) can facilitate cell cycle arrest and subsequently trigger apoptosis (Rich *et al.*, 2000) can illustrate the importance of genome stability maintenance and the need to develop an elaborate mechanism to monitor and keep this stability. Therefore, it is not surprising that these mechanisms and their components have been conserved throughout the evolution from unicellular to mammalian organisms. Thus, budding yeast, *Saccharomyces cerevisiae*, as a model organism provides an exciting possibility of discovering genes that play important role in the DNA damage response.

DNA damage response is a hierarchical process which includes subsequent activation of signalling pathways that culminate in activation of four response pathways: DNA repair, DNA damage checkpoints, transcriptional response, and apoptosis. These pathways may function independently, but frequently a protein primarily involved in one response participates in other responses. Defects in any of these pathways may cause genomic instability (Sancar *et al,* 2004). The magnitudes of these responses and, especially, cell fate choice are proportional to the dose, time and type of damaging agent applied. All of these four processes orchestrate together. DNA damage checkpoints ensure cell cycle arrest giving the repair mechanisms enough time to fix the damage. The transcriptional response induces transcription of proteins involved in the repair process, but also of the general stress response. Moreover, it is also required to maintain the internal homeostasis of cells. Eventually, if the damage cannot be repaired apoptosis is induced to remove the seriously damaged cells.

1.1. DNA lesions and structures that elicit DNA response reactions and DNA damage recognition

DNA molecules, like all other biomolecules, can be damaged in numerous ways. Spontaneous damage due to replication errors, deamination, depurination and oxidation is compounded in the real world by the additional effects of radiation and environmental

chemicals. DNA lesions and structural alterations that induce DNA damage response and binding of recognition factors include (Fig.1):

- Replication, recombination, and repair intermediates that include fork structures, bubbles, Holliday structures, and other nonduplex DNA forms (Cox *et al.*, 2000).
- DNA base damages produced by different agents include chemical modifications and photodamage. Chemical modifications imply deamination, reduction, oxidation, or bases fragmentation. Deamination includes conversion of primary amino groups to keto groups, adenine to hypoxanthine, guanine to xanthine, and 5-methyl cytosine to thymine. The agents that lead to chemical modifications of DNA are, for instance, reactive oxygen species (ROS), alkylating agents like methyl methanesulfonate (MMS), *N*-methyl-*N´*-nitro-*N*-nitrosoguanidine (MNNG) and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), or 4-nitroquinoline 1-oxide (4-NQO) that produce quinoline-purine monoadducts, the so called “bulky adducts”. Photodamage could be induced by ultraviolet radiation (UV) that leads to formation of pyrimidine dimers (most frequent are cyclobutane pyrimidine dimers) and pyrimidine (6-4) pyrimidone photoproducts (6-4PPs) causing extensive distortion of the normal DNA structure (Sancar *et al.*, 2004). Nearly half of chemotherapeutic drugs, including cisplatin, mitomycin C, psoralen, nitrogen mustard, and adriamycin, make different base adducts. In addition to the impact of exogenous chemicals, normal metabolism frequently leads to alkylation. It has been shown that S-adenosylmethionine, the normal biological methyl group donor, reacts accidentally with DNA to produce alkylated bases like 3-methyladenine at a rate of several hundred per day per mammalian haploid genome (Rydberg and Lindahl, 1982).
- DNA backbone damages include abasic sites and single- and double-strand DNA breaks. Abasic sites (apurinic/apyrimidinic (AP) sites) are generated spontaneously, by the formation of unstable base adducts or by base excision repair. Within a typical

mammalian cell, several thousand purines and several hundred pyrimidines are spontaneously lost per haploid genome per day (Smith, 1992). Single-strand breaks are produced directly by damaging agents or as intermediates of base and nucleotide excision repair. Double-strand breaks are formed by ionizing radiation (IR) and other DNA-damaging agents, but they are also essential intermediates in recombination.

- Cross-links that can be produced by bifunctional agents such as cisplatin, nitrogen mustard, mitomycin D or psoralen, but could also be an effect of UV and IR. Bifunctional alkylating agents and radiation can also create crosslinks between DNA and protein molecules.

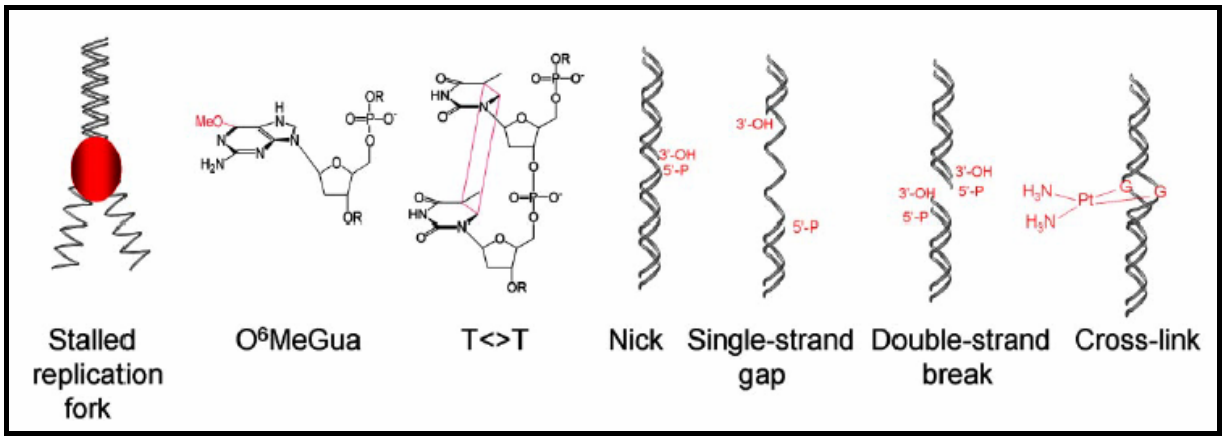

Figure 1. DNA lesions and structures that elicit DNA response reactions. Some of the base backbone lesions and noncanonical DNA structures that elicit DNA response reactions are shown. O6 MeGua indicates O6-methyldeoxyguanosine, T__T indicates a cyclobutane thymine dimer, and the cross-link shown is a cisplatin G-G interstrand cross-link (from Sancar *et al*., 2004).

Based on the type of DNA lesion and of proteins that recognise corresponding structural alterations, DNA damage sensors can utilize several strategies to recognise damage and initiate DNA damage response. The simplest way is a direct recognition based on complementarity of a particular DNA damage and a cognate protein, usually an enzyme. Photolyase and DNA glycosylase illustrate the enzymes that can directly recognise DNA damage. Photlyase is a monomeric protein of 55–65 kDa with two chromophore cofactors, a pterin in the form of methenyltetrapydrofolate and a flavin in the form of $FADH^-$. This

enzyme is able to directly repair UV-induced cyclobutane pyrimidine dimers and photoproducts using blue-light photons as an energy source, but it is absent in many species, including humans (Sancar, 2003).

The second very important way of DNA damage recognition is a multistep recognition which includes molecular matchmakers and combinatorial recognition. A molecular matchmaker is a protein that by itself is not directly involved in the repair process, but rather promotes association of other repair proteins into a complex bound to the damage site and than dissociates from the complex. Those proteins usually utilise energy from ATP hydrolysis. An example for a molecular matchmaker is the eukaryotic replication factor C (RFC) that loads PCNA (Proliferating Cell Nuclear Antigen) onto DNA and then dissociates, allowing PCNA to act as a DNA polymerase clamp and confer high processivity upon the polymerase (Sancar *et al.*, 2004).

Combinatorial recognition implies a synergistic action of two or more proteins for promoting DNA repair (Naar *et al.*, 2001). The example are three human damage recognition proteins, RPA, XPA, and XPC that cooperatively act in nucleotide excision repair in order to achieve a high-specificity in recognition. Each of these is a DNA-binding protein with some preference for damaged DNA. RPA is the most abundant factor, while XPC has the highest specific and non-specific constant. The moderate specificity of cooperative binding of these factors is amplified by the kinetic proofreading function of the transcription/repair factor TFIIH with 3’ to 5’ and 5’ to 3’ helicase activities. TFIIH is recruited by three damage recognition factors. Together they form a preincision complex 1 (PIC1) and the DNA is unwound by about 20 bp at the assembly site. If they assemble at a non-damaged site, ATP hydrolysis by TFIIH leads to the disassembly of the complex (kinetic proofreading). PIC1 formed at a damage site is more stable, and the unwound DNA constitutes a high-affinity binding site for XPG which is followed by disassociation of XPC from the complex and formation of PIC2. Finally, association of XPF•ERCC1 with the complex form PIC3, that

result in irreversible dual incisions and release of the excised oligomer (reviewed in Sancar *et al.*, 2004).

In some cases, proteins that are not part of DNA repair machinery provide so called proxy mechanism of recognition. One of them is RNA polymerase that simply arrests at the damage site and thus helps recruit the repair proteins (Friedberg, 1996). Moreover, DNA repair intermediates generated by one repair pathway could initiate another repair mechanism. A gap created by nucleotide excision repair, for instance, could be recognised and further repaired by homologous recombination (Cox, 2001).

1.2. DNA repair pathways

To handle genotoxic stress, cells have evolved a number of mechanisms to either repair or tolerate DNA damage. These pathways include direct repair (DR), base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MR), homologous recombination (HR), nonhomologous end-joining (NHEJ) mechanisms and translation bypass synthesis (TBS) (Fig.2).

1.2.1. Direct repair

Although it might seem that direct reversal of damage would be the simplest way to correct the damage, in most cases the reverse reaction is not possible for thermodynamic or kinetic reasons (Kao *et al.*, 2005; Zang *et al.*, 2005). Beside the aforementioned DNA photolyase which repairs UV-induced cyclobutane pyrimidine dimers, the other enzyme involved in direct repair methylguanine, DNA methyltransferase, has nearly universal distribution in nature. This enzyme recognizes damage by three-dimensional diffusion and forms a low-stability complex with the DNA. O6-methyldeoxyguanosine (O6MeGua) is than flipped-out into the active site cavity, wherein the methyl group is transferred to an active site cysteine. This C-S bond of methylcysteine is stable, and therefore, after one catalytic event the enzyme becomes inactivated (Sancar *et al.*, 2004). To accomplish this, in *E.coli* and

perhaps in other organisms, the alkylated version of the protein induces increased transcription of the gene encoding the protein (Teo *et al.*, 1984). Another example in humans is oxidative methyl transferase (hABH1–3), an alpha-keto-glutarate-dependent and iron-dependent oxygenases (aKG-Fe(II)-oxygenases), which use iron-oxo intermediates to oxidize chemically inert compounds. hABH1–3 is capable of repairing 1-methyladenine and 3-methylcytosine (Sancar *et al.*, 2004).

1.2.2. Base excision repair (BER)

All three excision mechanisms: base excision repair, nucleotide excision repair and mismatch repair cut out and replace damages present in one strand by using the complementary strand as template. The first, base excision repair pathway, for instance, removes incorrect bases present in one strand by employing three common steps. In the first step DNA N-glycosylase removes the damaged base and creates an AP site. This step is followed by cleavage at the abasic site by an apurinic/apyrimidinic (AP) endonuclease and subsequently, extension of the 3'-OH terminus by a DNA polymerase, accompanied by excision of the AP site. In the end, human DNA ligase III and XRCC1 protein ligate the gap (Rich *et al.*, 2000).

A large number of DNA N-glycosylases have been identified that specifically recognise different types of incorrect bases: uracil (uracil-DNA glycosylase), alkylated purines (methyl- purine glycosylase), oxidized/reduced pyrimidines (homologues of *E. coli* endonuclease III), or oxidized purines (homologs of *E. coli* Fapy glycosylase or 8-oxoguanine glycosylase) (Sancar *et al.,* 2004). The general principle of damage recognition by DNA glycosilase is pinching the DNA while scanning it. The result is that the DNA kinks at positions of instability caused by mismatching and binds to the enzyme (Mol *et al.*, 1999). Some DNA glycosylases in addition to glycosilase activity cleave off the base by a lyase mechanism and catalyze a subsequent AP lyase reaction. The major polymerase used for base excision repair in mammalian cells is polymerase beta, which has two distinct enzymatic

activities: polymerase activity and deoxyribose phosphatase activity used to excise the deoxyribose phosphate moiety in a case where glycosylase lack lyase activity (Prasad *et al.*, 1998).

E. coli	Mammals	Yeast	Substrates	Other functions
XTH	APE1 (HAP, APE, hAPE, ref-1)	Absent	AP sites, 3´phosphate, 3´phosphoglycolate	Redox activation of transcription factors and bioreductive drugs, activation of p53, 3´ diesterase activity ~100-fold less than endonuclease activity, 3´mismatch exonuclease
no homologue to date	APE2	APN2	AP sites, 3´phosphate, 3´phosphoglycolate	
NFO	no homologue to date	APN1	AP sites, 3´phosphate, 3´ phosphoglycolate	3´diesterase activity = endonuclease activity
NTH1	NTH1	no homologue to date	Thymine glycol, 5-hydroxy- and 6-hydroxy-dihydrothymine (DHT), uracil glycol, 5-hydroxycytosine, 5-hydroxyuracil, β-ureidoisobutyric acid, urea	
no homologue to date	no homologue to date	NTG1 and NTG2 (endo III homologues)	Thymine glycol, 5-hydroxy-6-hydrothymine, 5-hydroxy-6-hydrouracil, 5-hydroxy-5-methylhydantoin, 5-hydroxyuracil, 5,6-dihydrouracil, 5-hydroxycytosine, DHT, urea, uracil glycol, Formamdiopyrimidine	
NEI	NEIL1	Absent	G (FapyG), Fapy A, 8- oxoG:G Same as NTH1. In addition, endo VIII also recognize 8-oxoG, in particular when 8-oxoG is paired with A or G.	
UNG	UNG	UNG	Uracil in both single and double stranded DNA	
MUG	DUG	Absent	Uracil and thymine (in T/G mismatch) in double stranded DNA. Ethenocytosine	
FPG	OGG1	OGG1	8-oxoG:C, 8-oxoG:G, 8-oxoG:T, FapyG, FapyA	
Mut Y	MYH	Absent	8-oxoG:A	
Mut T	MTH	Absent	8-oxo-dGTPase	

Table 1. Comparison of BER enzymes in *E. coli*, yeast, and mammals

The 1-nucleotide replacement pathway is called a short-patch base excision repair, the alternative mechanism being a long-patch base excision repair. In general, base excision repair initiated by glycosylases is a short patch initiated by AP sites resulting from "spontaneous hydrolysis", while oxidative base loss is a long patch (Sancar *et al.*, 2004). Another difference is that human long patch pathway, for instance, employs enzymes of DNA

replication, DNA polymerase delta and epsilon in combination with PCNA, FEN1 and DNA ligase 1. This mechanism involves synthesis of a new stretch, usually 2-10 nucleotides long, which results in displacement of the strand terminated by deoxyribosephosphate group made by APE1 (Sancar *et al.*, 2004). Although BER pathway in *S.cerevisiae* shares some homologues with the mammalian one, their main players are remarkably divergent (Table 1).

1.2.3. Nucleotide excision repair (NER)

NER is the major repair system for removing bulky DNA lesions formed by exposure to radiation or chemicals, or by protein addition to DNA. It recognizes damaged regions based on their abnormal structure as well as on their abnormal chemistry. The basic steps of nucleotide excision repair are (a) damage recognition, (b) dual incisions of the damaged strand to form a 12–13-nt oligomer in prokaryotes or a 24–32-nt oligomer in eukaryotes, (c) release of the excised oligomer, (d) repair synthesis to fill in the resulting gap, and (e) ligation (Sancar *et al.*, 2004).

In both prokaryotes and eukaryotes, the excision follows a similar path: an ATP-independent, low-specificity recognition complex (XPC/HR23B in humans) recognise the damage, which is followed by an ATP-dependent DNA unwinding by two subunits of transcription factor TFIIH (XPB and XPD in humans) and formation of a long-lived DNA-protein complex (stabilised by three additional proteins XPA, RPA and XPG in humans), and, finally, dual incisions by two nucleases (XPG and ERCC1/XPF in humans) (Petit and Sancar, 1999). The damage-containing oligonucleotide is displaced concomitant with the binding of replicative gap-repair proteins (humans RFC, PCNA, DNA polymerase delta or epsilon) and the final nick is sealed by DNA ligase I. Mutation in any of NER genes give rise to human DNA repair diseases, like Xeroderma pigmentosum (XP), Cockayne's syndrome or trichothiodystrophy. XP is characterized by a very high incidence of light-induced skin cancer, Cockayne's syndrome by growth retardation, photosensitivity, premature aging and

early death, while hallmarks of trichothiodystrophy are hair dysplasia and numerous symptoms affecting mainly organs derived from the neuroectoderm (Cleaver, 2005).

Tanscription-coupled NER and BER are special types of excision repair. They utilise the stalling of RNA polymerase at damaged site of transcribed strain for damage recognition (Mellon, 2005). Generally, removal of certain types of DNA damage is more rapid and more efficient from the transcribed strands of expressed genes in comparison to the non-transcribed strands.

1.2.4. Double-strand break repair

Double-strand DNA breaks are repaired either by homologous recombination (HR) or nonhomologous end-joining (NHEJ) mechanisms. Homologous recombination obtains instructions from the sister chromatide or homologous chromosome for proper repair of breaks consisting of three major steps: strand invasion, branch migration, and Holliday junction formation (Wyman *et al.,* 2004; Krogh and Symington, 2004). Strand invasion and branch migration are initiated by Rad51 in eukaryotes or RecA in prokaryotes. In eukaryotes, Rad52, Rad54, Rad55, Rad57, BRCA1, and BRCA2 are also involved in homologous recombination, but the precise roles of these proteins are unclear. The Mre11/Rad50/NBS1 (M/R/N) complex (Mre11/Rad50/Xrs2 complex in yeast) performs the nucleolytic processing of DSBs before initiation of strand invasion by Rad51. The MUS81-MMS4 heterodimer resolves the Holliday junctions or the topologically equivalent four-strand intermediates arising from replication fork regression.

A transitional pathway between HR and NHEJ is the so-called single-strand annealing (SSA) repair mechanism. In this case, the ends of the duplex are digested by an exonuclease, possibly the M/R/N complex (Paull and Gellert, 1998), until some of homology regions (usually short repeat sequences are abundant in mammals) on the two sides of the break are exposed and paired. Considering that nonhomologous tails are cut away, loss of information is inevitable.

NHEJ permits joining of ends even if there is no sequence similarity between them. In NHEJ the Ku heterodimer (encoded by *HDF1* and *HDF2* in yeast) binds to the two ends of a double-strand break and recruits DNA-PKcs (not present in yeast) and the ligase4-XRCC4 heterodimer (Lig4/Lif1 complex in yeast), which then ligates broken dsDNA molecules after their ends have been properly processed. (Hefferin and Tomkinson, 2005). DNA-PK phosphorylate a special protein Artemis (absent in yeast) which by its endonuclease activity creates blunt double-stranded structures that are good ligase substrates. The M/R/N complex may also participate in NHEJ, particularly when this pathway is utilized for V(D)J recombination, a site-specific DNA rearrangement process which assembles the variable regions of immunoglobulin and T-cell receptor genes from multiple germline V, (D) and J gene segments (Roth, 2000).

There are indications that HR is important for the recovery of collapsed replication forks, while, NHEJ is essential for V(D)J recombination and is thought to be the major pathway for repair of double-strand breaks induced by ionizing radiation and radiomimetic agents (Sancar *et al.*, 2004). Despite its inaccuracy, mammals seem to favour NHEJ as their repeat-ridden genomes make sequence alignment tricky, while HR is dominant double strand break repair pathway in yeast (Rich *et al.*, 2000).

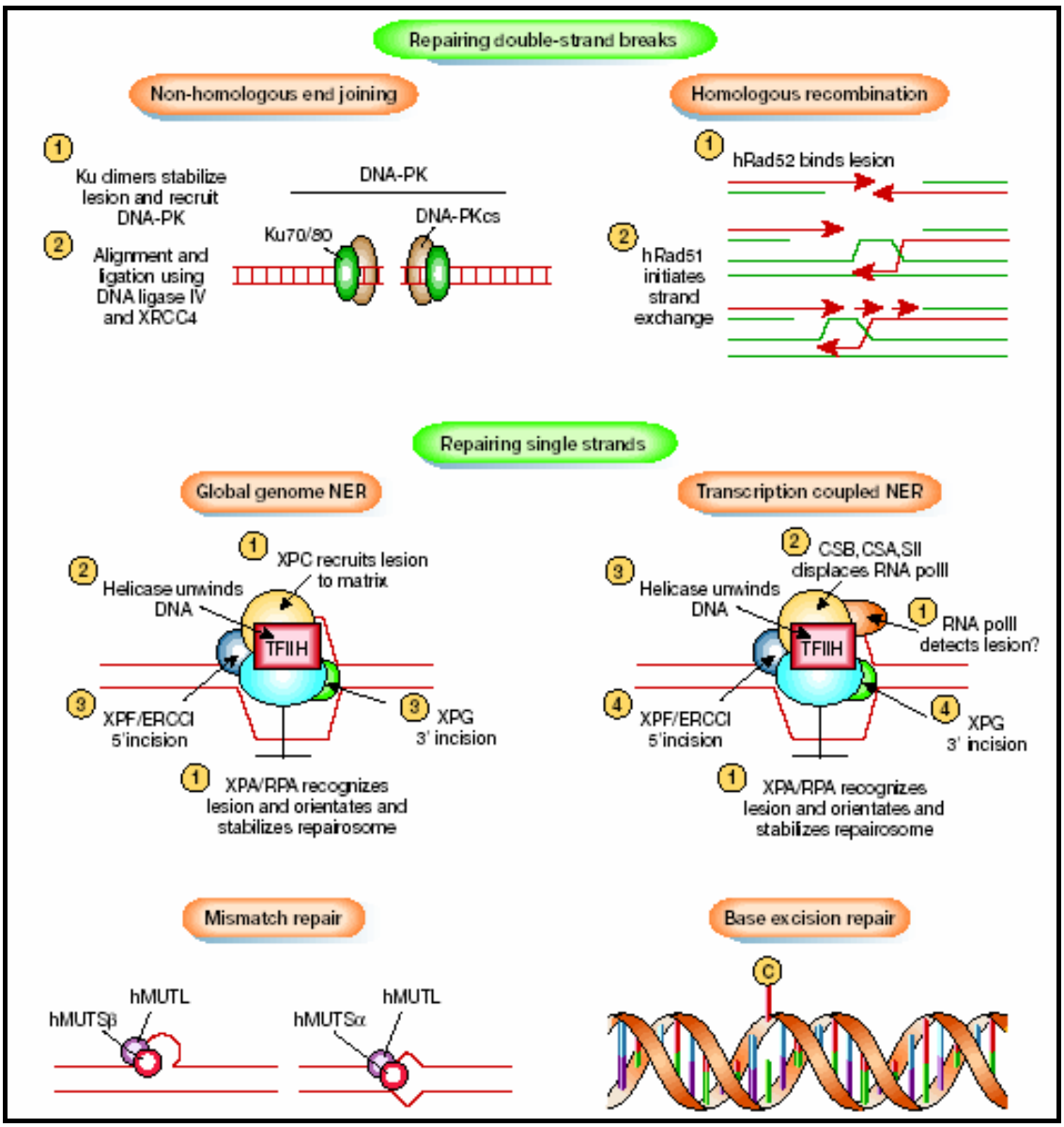

Figure 2. DNA repair mechanisms (from Rich *et al.*, 2000; see text for the detailed explanation).

1.2.5. Mismatch repair

Mismatch repair pathway corrects mismatched nucleotides and small loops. Most of the mismatches occur due to replication errors that result in double helix distortion. Because neither nucleotide is damaged or modified, it is not obvious which strand carries the correct genetic information and which carries the error; thus, the mismatch repair cannot be accomplished by a mechanism such as BER or NER, which simply excise the damaged base, or a short DNA fragment containing the damage, respectively (Stojic *et al.*, 2004). In the initial step of mismatch repair in *E.coli* MutS recognizes mismatches in the DNA and binds to

them. This is followed by binding of second protein MutL that stabilizes the complex (Schofield and Hsieh, 2003). Distinction between old and newly synthesized (presumably incorrect) strand is made based on methylation at GATC sequences of the old strand in *E.coli*, and single-strand nicks in eukaryotes (the presence of gaps between Okazaki fragments on the lagging strand or the free 3´-terminus on the leading strand; Stojic *et al.*, 2004). The MutS-MutL complex activates further MutH which in cooperation with UvrD nicks the newly synthesized strand. Missing nucleotides are resynthesized by polymerase II and ligated at the end of the repair process.

Although lacking the homologous of MutH and UvrD, eukaryotic organisms possess numerous homologous of MutS and MutL, like MSH2-6 (homologous of MutS), PMS1-2, MLH1-3 (both homologous of MutL). Interestingly, the eukaryotic homologous all function as heterodimers. In eukaryots several other proteins are needed for this repair: PCNA, RPA, replication factor C and DNA polymerase delta in humans (Stojic *et al.*, 2004).

1.2.6. DNA damage bypass

In some cases, during the replication damage encountered in template, the strand may not be reparable. In these situations damage could become simply bypassed. In *S.cerevisiae RAD6* epistasis group is responsible for this process (Broomfield *et al.*, 2001). The major alternative bypass pathway, translesion synthesis, can be either non-mutagenic or mutagenic, depending on the type of damage and the repertoire of translesion polymerases available to the cell. In yeast polymerases zeta and eta are involved in this process.

Various repair pathways may share certain enzymes and reaction intermediates. Conversely, particular lesions might be repaired by more than one pathway, in which case, they might compete for the same substrate, interfering with one another's function, or cooperating in removing the lesion. Likewise, it is unclear whether a particular damage-specific binding protein can act as a nucleation site for more than one repair pathway.

1.3. DNA Damage Cell Cycle Checkpoints

Progression through the cell cycle is tightly controlled by cell cycle checkpoint mechanisms. During the transition from one cell cycle phase to another, checkpoint proteins control the integrity of macromolecules, like DNA and proteins, as well as the successful completion of cellular processes prior the initiation of the next cell cycle phase. If the macromolecular damage, especially DNA damage is sensed, checkpoint response mediates cell cycle arrest, DNA damage repair and transcriptional induction of certain genes involved in the general stress response and DNA damage repair. Growth arrest represents an adaptive and integrated part of the cellular stress response. It ensures preservation of energy and reducing equivalents necessary for macromolecular stabilization and repair, and, what is also very important, gives enough time for the repair process. Proliferating cells that actively undergo DNA replication and mitosis are more prone to suffer stress-induced damage to macromolecules than the cells in a resting state (Kültz, 2005). Therefore, the activation of cell cycle checkpoints is the key mechanism in prevention of further cellular damage.

In yeast, there are four checkpoints in the cell cycle in which cellular division could be ceased (Fig.3): at the G1/S transition (the G1 checkpoint), during the S phase to prevent DNA replication (the S-phase progression checkpoint) and mitosis (the S/M checkpoint), and at the G2/M boundary (the G2/M checkpoint) (reviewed in Elledge, 1996; Longhese *et al*., 1998; Weinert, 1998). Which checkpoint will be activated depends on the type of DNA lesion, as well as on the consequences of the damage. For example, ionizing radiation triggers G2/M arrest, preventing loss of DNA fragments during division (Weinert and Hartwell, 1989), whereas base modifications that inhibit DNA replication activate the S-phase progression checkpoint (Paulovich and Hartwell, 1995).

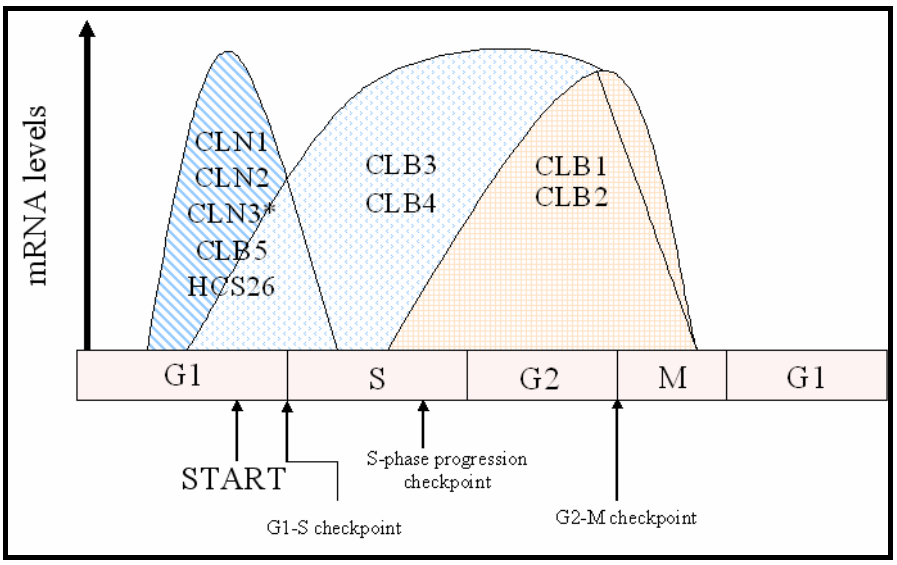

Figure 3. The cell cycle phases in *Saccharomyces cerevisiae*, periodicity of cyclins and DNA damage cell cycle checkpoints. Cyclins are grouped according to suggested times of action during the cell cycle and mRNA periodicity. As indicated by *, CLN3 is the exception to the rule in that its mRNA levels remain constant throughout the cell cycle.

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The DNA damage checkpoint conceptually has three components (Fig.4; Table 2): sensors, signal transducers, and effectors. However, various components of the checkpoint could serve at the same time in several steps. For example, the damage sensor, ATM, also functions as a signal transducer. Moreover, the fourth class of checkpoint mediator proteins, placed between sensors and signal transducers, has been identified. In humans this class includes BRCA1, Claspin, 53BP1, and MDC1. These mediator proteins also appear to participate in more than one step of the checkpoint response (Sancar *et al.*, 2004).

Checkpoint-specific damage sensors can be classified into two groups: phosphoinositide 3-kinase-like kinase (PIKK) family members, presented with ATM (for ataxia telangiectasia mutated) and ATR (ataxia telangiectasia Rad3 related), and the RFC/PCNA (clamp loader/polymerase clamp)-related Rad17- RFC/9-1-1 complex (Melo and Toczyski, 2002). ATM is a sensor and transducer responding to double-strand breaks and ATR serves an analogous role for base damages, at least from UV irradiation. In both *S. cerevisiae* and *S. pombe*, the ATR homologous (scMec1 and spRad3, respectively) were

shown to be in a complex with scDdc2 and spRad26 (Edwards *et al.*, 1999, Paciotti *et al.*, 2000) (low homology protein ATRIP was identified in mammalian cells; Zou and Elledge, 2003). The Rad17-RFC complex is a checkpoint specific structural homolog of the replication factor, RFC. The 9-1-1 (Rad9-Rad1-Hus1) complex is the checkpoint counterpart of PCNA, a homotrimer with a ring-like structure (Lindsey-Boltz *et al.*, 2001). In vivo biochemical experiments show that in budding yeast the 9-1-1 complex equivalent (scDdc1- scRad17-scMec3) is recruited to double-strand breaks introduced by HO endonuclease independently of recruitment of scMec1 (Kondo *et al.*, 2001).

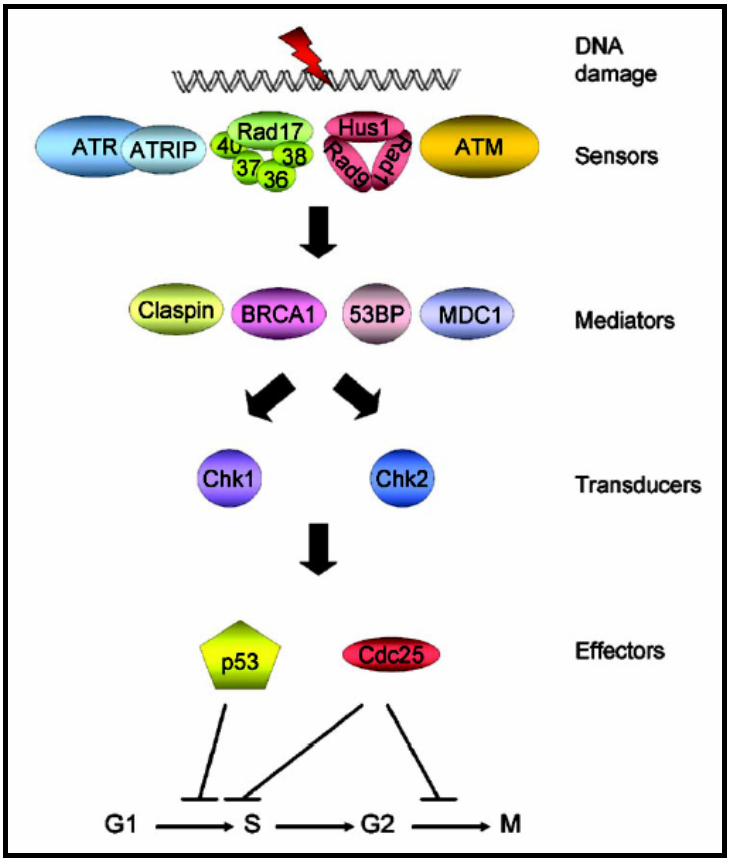

Figure 4. Components of the DNA damage checkpoints in human cells. The damage is detected by sensors that, with the aid of mediators, transduce the signal to transducers. The transducers, in turn, activate or inactivate other proteins (effectors) that directly participate in inhibiting the G1/S transition, S-phase progression, or the G2/M transition (from Sancar *et al.*, 2004; see text for the detailed explanation).

Mutations in any of damage sensors cause chromosome instability, increase risk for tumor development and lead to many immune deficiency diseases. For example, mutations in ATM damage sensor give rise to Ataxia–telangiectasia (A–T), a human autosomal recessive

disorder characterized by progressive neurodegeneration, immunodeficiency and cancer predisposition (Sedgwick and Boder, 1991). The A–T cellular phenotype includes chromosomal instability, radiosensitivity and failure to adequately activate cell cycle checkpoints (Lavin and Shiloh, 1997).

Protein function	Mammals	*S.pombe*	*S.cerevisiae*
Sensors			
RFC-like	Rad17	Rad17	Rad24
PCNA-like	Rad9	Rad9	Ddc1
	Rad1	Rad1	Rad17
	Hus1	Hus1	Mec3
PI3-Kinases (PIKK)	ATM	Tel1	Tel1
	ATR	Rad3	Mec1
PIKK binding partner	ATRIP	Rad26	Ddc2/Lcd1/Pie1
Mediators			
	MDC1		
	53BP1		
	TopBP1	Cut5	Dpb11
	Claspin	Mrc1	Mrc1
	BRCA1	Crb2/Rph9	Rad9
Transducers			
Kinase	Chk1	Chk1	Chk1
	Chk2	Cds1	Rad53

Table 2. DNA damage checkpoint proteins in mammals, *S.pombe* and *S.cerevisiae*.

Checkpoint mediators simultaneously associate with damage sensors and signal transducers at certain phases of the cell cycle and as a consequence help to provide signal transduction specificity. In *S.cerevisiae* the Rad9 protein functions along the signal transduction pathway from scMec1 (ATR) to scRad53 (Chk2) (Vialard *et al.*, 1998). Another mediator, Mrc1 (mediator of replication checkpoint), found in both *S.cerevisiae* and *S.pombe* (Alcasabas *et al.*, 2001; Tanaka and Russell, 2001), is expressed only during the S phase and is essential for S-phase checkpoint signalling from scMec1/spRad3 to scRad53/spCds1. There are three checkpoint mediators identified in humans: the p53 binding protein, 53BP1 (Wang *et al.*, 2002); the topoisomerase binding protein, TopBP1 (Yamane *et al.*, 2002); and the mediator of DNA damage checkpoint 1, MDC1 (Stewart *et al.*, 2003). In addition, other proteins such as H2AX, BRCA1, the M/R/N complex, and SMC1 (structural maintenance of chromatin 1), play essential roles in the activation of checkpoint kinases (Sancar *et al.*, 2004).

Signal transducers are two S/T kinases, Chk1 and Chk2 (yeast Rad53), that transduce the double-strand break signal sensed by ATR and UV-damage signal sensed by ATM, respectively (Sancar *et al.,* 2004).

In humans, three phosphotyrosine phosphatases, Cdc25A, -B, and -C serve as effector proteins downstream from the signal transducers. They dephosphorylate the cyclin-dependent kinases that act on proteins directly involved in cell-cycle transitions. Phosphorylation of these Cdc25 proteins by the checkpoint kinases creates binding sites for the 14-3-3 adaptor proteins, which inactivates the Cdc25 proteins by excluding them from the nucleus, or causing proteolytic degradation. Because active Cdc25 proteins promote the G1/S transition by dephosphorylating Cdk2 or G2/M transition by dephosphorylating Cdc2 phosphotyrosine, its inactivation directly arrests the cell cycle in G1 or G2 phase, respectively (Bartek and Lukas, 2001). In *S.cerevisiae* there are few effectors downstream from Rad53 and Chk1 signal transducers (Wahl and Carr, 2001). Rad53p mediates phosphorylation of Swi6, a part of the SCB (Swi4/6 cell-cycle box) binding factor (SBF) and MCB (MluI cell-cycle box) binding factor (MBF). MBF is required to transcribe a range of genes required for S phase entry and DNA replication. Rad53 also phosphorylates Cdc5 (a polo-like kinase that regulates the anaphase-promoting complex), whereas Chk1 phosphorylates Pds1, an inhibitor of sister chromosome separation and anaphase.

1.4. Transcriptional response to DNA-damaging agents in yeast

An important aspect of each cellular response to DNA damage is the reorganization of gene expression. The first works that monitor transcriptional response of yeast cells to the DNA damage induced by MMS, revealed that ~30% of mRNA species could be regulated by MMS treatment in dose and time dependent manner (Jelinsky and Samson, 1999; Jelinsky *et al.,* 2000). Transcriptional alteration after DNA damage is a coordinate process initiated by damage sensors, like ATR or ATM proteins. Gasch *et al.* (2001) showed that ATM homolog

Mec1 regulates transcription of the whole set of genes in response to DNA damage (Fig.5). Mec1 signalling pathway activates two checkpoint kinases, Rad53 and Chk1. Signal transduced through Chk1 leads to the regulation of cell cycle specific genes and cell cycle arrest. Kinase cascade Mec1-Rad53-Dun1 induce transcription of transcriptional factors, like Msn2 and Msn4, involved in expression of genes part of environmental stress response (ESR). This cluster involves more than 900 genes whose expression is stereotypically altered in response to different stress conditions (Gasch *et al.*, 2000). The group of genes whose transcription is induced in the ESR includes those that encode proteins involved in carbohydrate metabolism, protein folding and degradation, oxidative stress defence, autophagy, cytoskeletal reorganisation, DNA-damage repair. Genes that are repressed in the ESR are mostly those required for ribosome synthesis and processing, RNA polymerase I- and III-dependent transcription and protein translation. The proposed role of transcriptional regulation triggered by DNA damage is energy conservation and maintenance of internal osmolarity, oxidation-reduction potential and integrity of cellular structures. The global and coordinated activation of yeast stress response genes is enabled by bimodal transcriptional regulation of yeast genome. Namely, Huisinga and Pugh (2004) showed that genome of *S.cerevisiae* is divided into genes preferentially targeted by SAGA (Spt-Ada-GCN5-acetyltransferase) transcriptional complex (~10% of the genome) and genes preferentially targeted by the TFIID transcriptional complex (~90% of the genome). Many SAGA-regulated genes are stress inducible, whereas most TFIID regulated genes have housekeeping functions.

Undoubtedly, DNA-damaging agents damage not only DNA, but also other cellular macromolecules, and organelles as well. Those agents that affect protein structure and result in protein unfolding or misfolding induce transcription of genes encoding protein chaperones and proteasome subunits (Fig.5). Moreover, many of DNA-damaging agents alter the cellular redox potential and create high oxidative stress through formation of free radicals. Therefore, these agents often induce transcription of cellular redox sensors, like AP-1 (yeast Yap1) and

its targets, e.g. genes encoding: proteins involved in glutathione synthesis and conjugation, putative transporters required for resistance to various drugs, and proteins involved in thiol oxidation and reduction (Kültz, 2005).

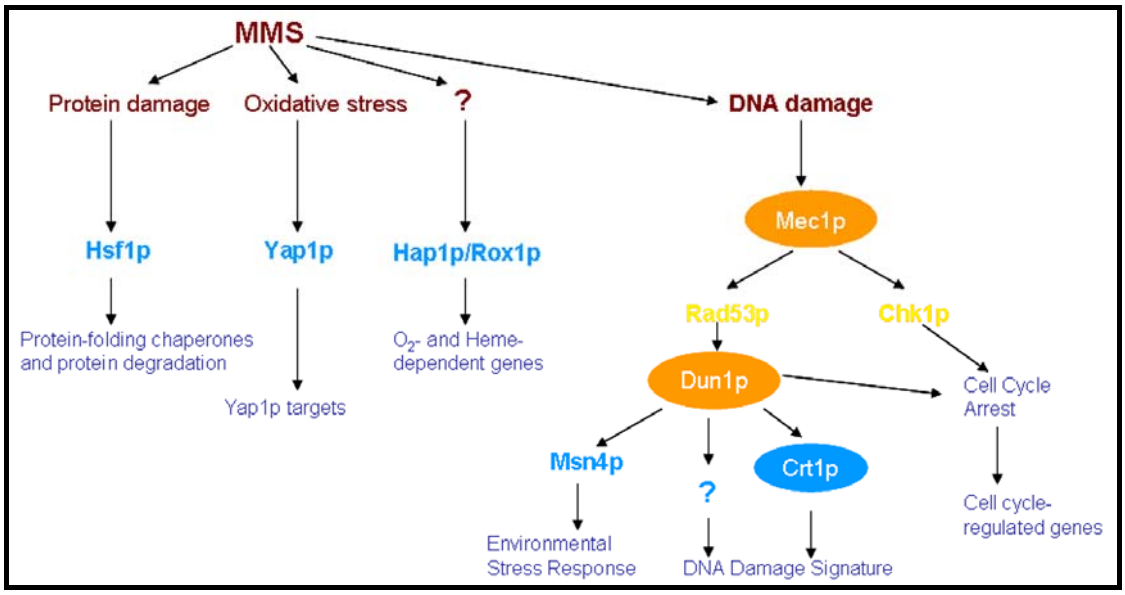

Figure 5. Summary of genomic responses to MMS and ionizing radiation. This diagram summarizes the functional features of the genomic expression responses: observed in the study from Gasch et al. (2001) (purple), transcription factors (blue) and protein kinases (yellow) that have been implicated in those genomic responses, and the hypothetical cellular signals that trigger the responses (orange) (from Gasch *et al*., 2001).

In addition, reactive oxygen and nitrogen species (ROS, RNS) are employed as second messengers that carry signals about alterations of cellular redox potential or DNA damage (Mikkelsen and Wardman, 2003). Salmon *et al.* (2004) showed that in yeast, just like in mammalian cells, ROS are important second messengers in response to DNA damage. Elevated amount of ROS in the cell, referred to as oxidative burst, triggers induction of many genes involved in oxidative stress defence and cell cycle arrest, but could also lead to activation of apoptotic program or senescence. Thus, increased concentrations of free radicals are rather beneficial for cellular stress sensing and signalling, because they enable proper cellular stress response and suicide of seriously damaged cells.

Very important aspect of the DNA damage response is the modulation of major pathways of energy metabolism, which may be closely linked to the oxidative burst in cells

exposed to stress. Induction of many key enzymes involved in glycolysis, pentose phosphate pathway, or the Krebs (citrate) cycle may be necessary for generating reducing equivalents (NADH, NADPH) that are needed for cellular antioxidant systems (Kültz, 2005). The elevated transcription of glycerol-3-phosphate dehydrogenase (G3PDH), 6 phosphogluconate dehydrogenase (6PGDH), enolase, citrate synthase, and isocitrate dehydrogenase (IDH) were observed in response to many stress conditions or DNA damage (Jelinsky and Samson, 1999; Jelinsky *et al.*, 2000; Gasch *et al.*, 2001). Moreover, growth arrest results in redirection of NADPH/NADH and ATP utilization from proliferative processes to macromolecular stabilization and repair (Kültz, 2005). Therefore, another potential reason for inducing these metabolic pathways lies in the energetic requirements of protein degradation, protein chaperoning, and DNA repair.

It is very important to underline that few, if any genes encoding proteins involved in repairing the various potentially lethal DNA lesions, are induced in response to exposure to the agents that produce these lesions (Birrell *et al*., 2002). Only a small cluster of 9 genes, among many considered as specific signature of DNA damage, was identified in the work of Gasch *et al*. (2001) and Jelinsky *et al*. (2000). These include two genes involved in homologous recombination (*RAD51* and *RAD54*) as well as the ribonucleotide reductase subunit genes *RNR2* and *RNR4*. This implicate that endogenous levels of various proteins involved in protecting against DNA damage are at sufficient levels to provide full, immediate and fast response to the lesions produced by the agents used.

Cellular sensitivity to different DNA damaging agents varies widely, depending on species, cell type and differentiation state. It is very well known that cells that were exposed to low doses of certain agent become more resistant to higher doses of the same agent applied later. Further, cells treated with one agent show increased tolerance to another one. These two phenomena, the so called stress-hardening and cross-tolerance, are common and significant (Gasch and Werner-Washburne, 2002; Kültz, 2005). The activation and induction of a

common set of stress proteins is the molecular basis of both phenomena. Depending on species, cell type, history of prior stress exposure, gene-environment interactions during development, and stress severity, proteins activated by one stress remain active/elevated for a certain period, conferring resistance to many different types of stress. Those proteins are usually involved in general aspects of cellular protection like protein stabilization, DNA repair, and free radical scavenging.

1.5. The role of apoptosis in the DNA damage response

Apoptosis is a critical tumour suppressive mechanism. It serves to remove from the cellular population the cells in which damage of macromolecules or organelles is not likely to be efficiently and accurately repaired. The induction of apoptosis is the base of the therapeutic effect of many antitumor drugs, including those that damage DNA or inhibit DNA replication (reviewed in Ding and Fisher, 2002). Therefore, defect in apoptosis can lead to drug resistance (reviewed in Johnstone *et al.,* 2002).

Apoptotic cell death induced by DNA damage is a mitochondrial mediated process that results in realising of cytochrome *c* (Green and Evan, 2002; Wolf and Green, 2002). The release of cytochrome *c* and other apoptosis inducing factors from mytochondria is the initial step in activation of apoptosome. The final outcome of this process is a programmed cell death (Fig.6). The transmision of the damage signal to the apoptosome is under control of checkpoint sensors, ATM, ATR and DNA-PK. These sensors have a critical role in the DNA damage response system as they provide an opportunity to monitor the appropriateness of suicide over repair (Rich *et al.,* 2000). They catalyse phosphorylation cascade which transmits damage signal to repair proteins and checkpoints, but also activates tumor suppressors.

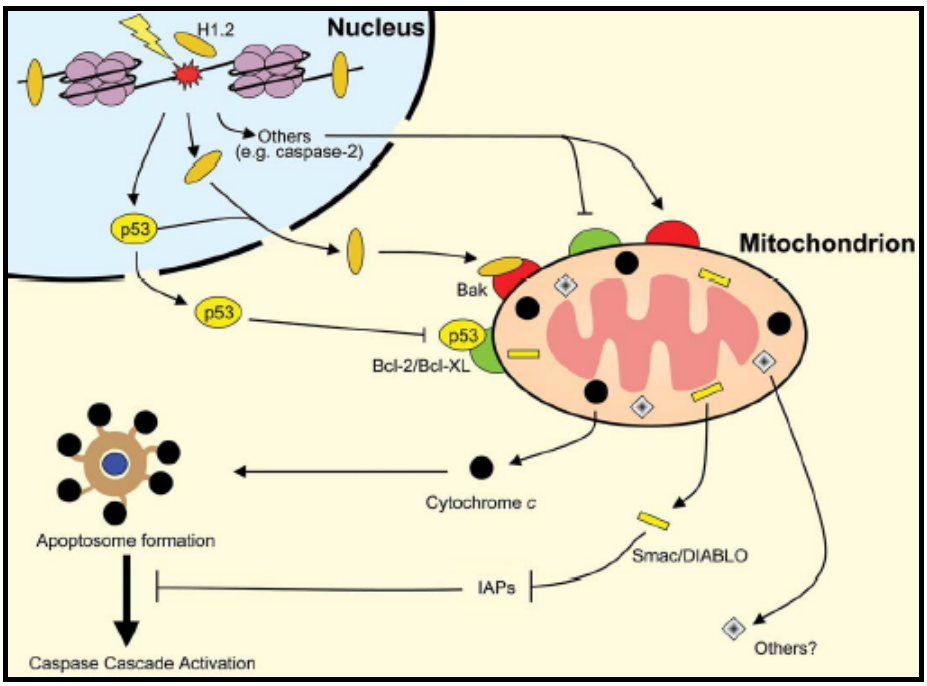

Figure 6. The role of the mitochondria in DSB-specific apoptosis. Some of the principal activities required for regulating DSB-specific apoptosis are shown. Multi-domain Bcl-2 family members (red indicates pro-apoptotic and green pro-survival) are shown at the mitochondrial surface. DSB triggered apoptosis ultimately results in activation of pro-apoptotic or inhibition of pro-survival Bcl-2 family members, as indicated. The resulting release of mitochondrial factors such as cytochrome *c* and Smac/DIABLO (an inhibitor of IAPs, inhibitors of apoptosis proteins) effectively amplifies the apoptotic signal by driving apoptosome formation and activation of the caspase cascade (from Bree et al., 2004).

How the decision between apoptosis and other fates can be made is well illustrated by human p53 tumor suppressor. This very important transcriptional factor in the DNA damage-induced apoptosis is mutated in 50% of all human tumors (Halazonetis, 2004). After DNA damage is sensed, p53 becomes phosphorylated by ATM kinase and regulate further transcription of pro-survival genes involved in the cell cycle arrest and DNA damage repair, as well as pro-apoptotic genes (Bcl-2 family members) (Bree *et al.*, 2004). Which cellular fate will be chosen depends on the levels of p53 expression. Low levels of this protein are known to have anti-apoptotic activity while high levels promote apoptosis (Chen *et al.*, 1996). In addition, a portion of activated p53 can translocate to the mitochondria and by forming a complex with Bcl-XL and Bcl-2 proteins induces permeabilisation of mitochondrial outer

membrane (Mihara *et al.,* 2003). p53 also promote release of histone H1.2 from damaged hromatin and its translocation into the cytosol, where it further induces release of cytochrome *c* (Konishi *et al.,* 2003). The two other human proteins, involved in regulation of cell cycle checkpoint and apoptosis induction following DNA damage, are E2F-1 and c-Abl. These proteins are also phosphorylated by ATM and DNA-PK, and they function independently from p53 (Rich *et al.*, 2000).

The first correlation between DNA damage and apoptosis in yeast was shown by Blanchard and colleges (2002), where exposure of *S.cerevisiae* to lethal levels of DNA-damaging antitumor agent caused the proteasome-dependent destruction of the DNA replication initiation protein Cdc6. Also other DNA damage response genes, like *MEC1* or *RAD9,* have been implicated in programmed cell death in yeast (reviewed in Burhans *et al.,* 2003), linking yeast apoptosis to two major signalling molecules involved in DNA-damage repair. Yeasts were also shown to induce an apoptotic phenotype by UV irradiation (Del Carratore *et al.,* 2002) or by inactivation of the yeast telomere binding protein Cdc13p (Qi *et al.*, 2003). The latter results in abnormal telomeres and in the activation of the DNA damage checkpoint. Budding yeast cells harbouring the *orc2-1*, the mutation in the origin recognition complex required for initiation of DNA replication, also show typical apoptotic features (Watanabe *et al.,* 2002). Salmon and colleagues (2004) presented that DNA damage can trigger an increase in ROS production suggesting that ROS may function as a signal mediating cellular response to unprepared DNA damage. Therefore, despite the lack of many pro-apoptotic proteins, like caspases and Bcl-2 family, yeast cells might contain an intrinsic cell death pathway. Indeed, yeast cells do undergo an apoptotic program in response to many external stimuli other than DNA damage: treatment with acetic acid (Ludovico *et al.,* 2002), hydrogen peroxide (Madeo *et al.,* 1999) or high levels of mating pheromone (Severin and Hyman, 2002).

The important role of ROS in the regulation of yeast apoptosis indicates the origin and primary purpose of the suicide process in unicellular organisms. Cells that continue proliferation even with the seriously damaged DNA endanger genetic stability of the population. In a case of unicellular organisms cells are mostly clonal relatives. Therefore, committing suicide in this kind of populations would save resources for neighbouring cells and enable the healthiest cells to survive (Herker *et al.,* 2004). Via the p53 system higher eukaryotes evaluate cell damage to decide whether suicide is advisable. In a case of unicellular organisms, which miss such a complex system and lack p53 tumor supressor, chemical reactivity of ROS themselves may have been used to trigger cellular suicide. With this simple signalling system that triggers apoptosis, yeast offers the opportunity to easily screen for the substances that are directly involved in committing the programmed cell death without being diverted by a complex upstream network.

1.6. Objectives

Considering that DNA damage is not only associated with immediate cytotoxicity, the response to substances that cause DNA damage is of particular interest. At sub-cytotoxic levels, DNA damaging substances play an important role in the accumulation of genomic mutations. In longer living organisms, like humans and other mammals, exposure to DNA damaging substances over extended period of time is a critical factor that contributes to the development of various diseases and in particular of tumors (Vogelstein and Kinzler, 1993). In previous studies analyzing gene expression profiles of yeast cells in response to DNA damaging agents, including methyl methane sulfonate (MMS), strong cytotoxic concentrations in short-term treatment (up to 2h) were used (Jelinsky and Samson, 1999; Jelinsky *et al.,* 2000; Gasch *et al.,* 2001). Also, in all these studies influence of nutrient availability, aging and strain background on cellular sensitivity and transcriptional response to damaging agent were not taken in consideration. However, exposure of living organisms to

smaller amounts of toxic agents and other adverse effects may be more common in natural environments. Occurring over various time spans or as the consequence of repeated exposures the accumulation of mutations may be as critical for the organism as the immediate cytotoxic effect. Therefore, cellular response to treatment with DNA-damaging substances at low concentrations which are genotoxic but do not have a strong cytotoxic effect are of special interest. In addition, environmental variations that influence growth conditions, e.g. different media, and individual fitness, e.g. different strains, are likely to influence and modulate the adverse effects of individual DNA damaging substances.

Methyl methanesulfonate (MMS) is a methylating agent that methylates DNA at 7-deoxyguanine and 3-deoxyadenine. The resulting 3-methyladenine (3MeA) and 7-methylguanine cause base mispairing and replication blocks which activate DNA damage repair pathways and cell cycle arrest (Evensen and Seeberg, 1982). The major repair pathways involved in the repair of DNA alkylation damage are predominantly base excision repair (BER) and repair by DNA alkyltransferases (Lindahl and Wood, 1999), but all three radiation repair pathways are involved in this process as well (Friedberg, 1988). In yeast Mag1p (3MeA DNA glycosylase) removes the damaged base, than Apn1p (apurinic/apyrimidinic endonuclease) cleaves the DNA strand at the abasic site for subsequent repair (Friedberg *et al.,* 1995). Overexpression of *MAG1* causes a mutator phenotype (Frosina, 2000) as does mutation of *APN1* (Ramotar *et al.,* 1991), suggesting that this AP site produced by Apn1p is the first product of lesion processing that can be converted to a potentially lethal double strand brakes (DSB) and check-point activation (Frosina, 2000). In addition, MMS was shown to cause oxidative cell injury that follows the depletion of intracellular glutathione (GSH) (Mizumoto *et al.*, 1993). A decrease in the intracellular pool of reduced GSH results in an increase of ROS levels. The latter of course depends on the balance of ROS production versus ROS scavenging. However, MMS is not expected to directly cause intracellular ROS formation (Salmon *et al.,* 2004). More likely, ROS may function as a signal which mediates

cellular response to unprepared DNA damage. Therefore, cellular response to MMS treatment includes a complex network of proteins involved in DNA repair, cell cycle control, oxidative stress response and apoptosis. The magnitude of response will, of course, depend on the dose and time of treatment.

The aim of our work was to study how strain background and growth conditions influence respond to DNA damage caused by low doses of MMS and which part of these changes is responsible for their sensitivity to toxic conditions. We analyzed sensitivity of two yeast strains FF18984 and BY4742 to MMS in media with limited and full nutrient availability, as well as in a respiratory induced medium. We also tried to find out if the sensitivity to MMS is influenced by aging and what are the differences in MMS sensitivity in yeast cells that were aged in different media. Furthermore, we analyzed and compared transcriptional response of two yeast strains to short-term treatment with low doses of MMS, as well as of yeast strains growth in full and minimal media. The MMS concentrations used were selected based on their relative toxic effect: either for a selective genotoxic effect (0.00125% MMS); or with a slightly cytotoxic effect (0.0125% MMS) at which nevertheless more than 50% of the cells are viable and continue proliferation. Finally, we investigated the role of glucose metabolism, particularly the key enzyme in gluconeogenesis fructose -1,6-bisphosphatase (Fbp1p) in cellular response to DNA damage and aging.

2. Materials and methods

2.1. Materials

2.1.1. Bacterial and yeast strains

<u>*E.coli* strain</u>

DH5α chemically competent cells (Invitrogen)

<u>*Saccharomyces cerevisiae* strains</u>

Strains	***Plasmid***	**Genotype**	**Source**
FF 18984 (WT)		*MATa leu2-3,112 ura3-52, lys2-1, his7-1*	Provided by Prof.Dr. R.Walmsley, Manchester, UK
Δfbp1		*MATa leu2-3,112 ura3-52, lys2-1, his7-1;fbp1::KanMX4*	This study
Δhap4		*MATa leu2-3,112 ura3-52, lys2-1, his7-1;hap4::KanMX4*	— ’’ —
Δmig1		*MATa leu2-3,112 ura3-52, lys2-1, his7-1;mig1::KanMX4*	— ’’ —
Δrad9		*MATa leu2-3,112 ura3-52, lys2-1, his7-1;rad9::KanMX4*	Provided by Prof.Dr. R.Walmsley, Manchester, UK
BY4742		*MATα ; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0*	EUROSCARF yeast strain collection
Transformed strains			
FF 18984 (WT)	pRS426	as FF18984 and *URA3*	This study
FF 18984 (WT)	pGen *ACT*	as FF18984 and *URA3*	— ’’ —
FF 18984 (WT)	pRS426 *FBP1*	as FF18984 and *FBP1, URA3*	— ’’ —
FF 18984 (WT)	pUMGP5	as FF18984 and *GFP* controled by *RNR2* promoter, *URA3*	— ’’ —
FF 18984 (WT)	pRS425 + pUMGP5	as FF18984 and *LEU2, URA3,* and *GFP* controlled by *RNR2* promoter,	— ’’ —
FF 18984 (WT)	pRS425 *FBP1* + pUMGP5	as FF18984 and *LEU2, URA3, FBP1* and *GFP* controlled by *RNR2* promoter,	— ’’ —
Δfbp1	pRS426	as *Δfbp1* and *URA3*	— ’’ —
Δfbp1	pGen *ACT*	as *Δfbp1* and *URA3*	— ’’ —
Δfbp1	pRS426 + *FBP1*	as *Δfbp1* and *FBP1, URA3*	— ’’ —
Δfbp1	pGen *ACT* + *FBP1-GFP*	as *Δfbp1* and *FBP1-GFP, URA3*	— ’’ —
Δfbp1	pUMGP5	as *Δfbp1* and *GFP* controled by *RNR2* promoter, *URA3*	— ’’ —
Δfbp1	pRS425 + pUMGP5	as *Δfbp1*and *LEU2, URA3,* and *GFP* controlled by *RNR2* promoter,	— ’’ —
Δfbp1	pRS425 *FBP1* + pUMGP5	as *Δfbp1*and *LEU2, URA3, FBP1* and *GFP* controlled by *RNR2* promoter,	— ’’ —
Δrad9	pUMGP5	as *Δrad9* and *GFP* controlled by *RNR2* promoter, *URA3*	— ’’ —

2.1.2. Plasmids used in this study

Plasmid	Selection marker in yeast	promoter	Cloned DNA	Source
pRS425 GPD	*LEU2*	GPD	-	Provided by Dr. T.Munder, Jena, Germany
pRS426 GPD	*URA3*	GPD	-	Provided by Dr. T.Munder, Jena, Germany
pGen ACT	*URA3*	ACT	*GFP*	Provided by Prof.Dr. R.Walmsley, Manchester, UK
pRS425 GPD+ *FBP1*	*LEU2*	GPD	*FBP1*	This study
pRS426 GPD+ *FBP1*	*URA3*	GPD	*FBP1*	This study
pGen ACT + *FBP-GFP*	*URA3*	ACT	*FBP1-GFP*	This study
pUMGP5	*URA3*	RNR2	*GFP*	Provided by Prof.Dr. R.Walmsley, Manchester, UK
pUG6	-	-	Kan MX	Template for PCR, EUROSCARF

2.1.3. Primers used in this study

Gene	Forward primer	Reverse primer	Fragment
RT_PCR primers			
ACT	cat cta tcg tcg gta gac	ttc tac cgg aag agt aca	348 bp
FBP1	tcg agc acc aga agc aat	agt ctc gtt cca gta gag	614 bp
YAP1	agg ata gcg agc aac cga	tat cag tgc tac cag tgc	1068 bp
YCF1	ctg agg ata gag cat tga	gtg tca gct ctc gca taa	658 bp
Plasmid construction primers			
***FBP1* overexpression**	tgc aga cca cta gta tgc caa ctc tag taa atg g	tgc aga ccc tcg agc tac tgt gac ttg cca ata tgg	-
***FBP1-GFP* fusion**	tgc aga cct taa tta agc caa ctc tag taa atg gac c	tgc aga cct taa tta a ct gtg act tgc caa tat ggt	-
Gene disruption primers			
***FBP1* disruption**	cta aca aat gta cgt ata tat atg gag caa caa gta gtg cca gct gaa gct tcg tac gc	cgc gat cat tga act act gtg act tgc caa tat ggt cta agc ata ggc cac tag tgg atc tg	-
***MIG1* disruption**	tgt aac tac acg aga gtt gag tat agt gga gac gac ata cca gct gaa gct tcg tac gc	tga ttt atc tgc acc gcc aaa aac ttg tca gcg tat cag tgc ata ggc cac tag tgg atc tg	-
***HAP4* disruption**	ggt ctc cta gta cat caa aga gca ttt taa tgg gtt gct gca gct gaa gct tcg tac gc	aag gaa aag gac gcc taa gca ggc gaa gaa tac tat cat tgc ata ggc cac tag tgg atc tg	-

All primers were purchased from MWG-Biotech AG (Germany).

2.1.4. Media

F1 Medium

Salts:

$(NH_4)_2SO_4$	3.13 g/l
KH_2PO_4	2.00 g/l
$MgSO_4$ * 7 H_2O	0.55 g/l
$CaCl_2$ * $2H_2O$	0.09 g/l
NaCl	0.10 g/l

Trace elements:

$ZnSO_4$ * 7 H_2O	0.07 mg/l
$FeCl_3$ * $6H_2O$	0.05 mg/l
$CuSO_4$ * 5 H_2O	0.01 mg/l
H_3BO_3	0.01 mg/l
KI	0.01 mg/l

Vitamins:

Inositol	31 mg/l
Thiamine-HCl	14 mg/l
Pyridoxine	4 mg/l
Ca-Pantothenat	4 mg/l
Biotin	0.3 mg/l

Phosphate buffer:

Na_2HPO_4	13.8 g/l
KH_2PO_4	9.08 g/l

Amino acids:

L-Lysine HCl	100 mg/l
L-Leucine	100 mg/l
L-Histidine	100 mg/l

Other components:

Glucose	20 g/l
Uracil	20 mg/l

YPD Medium (Ready-made "YEPD Broth" – Invitrogen)

gives medium with:

Yeast extracts	10 g/l
Peptone	20 g/l
Glucose	20 g/l

for plates: 2% agar

selection: 300 µg/ml G418

SD Medium

Yeast nitrogen base	6.7 g/l
Glucose	20 g/l
L-Lysine HCl	100 mg/l
L-Histidine	100 mg/l
L-Leucine	100 mg/l
Uracil	20 mg/l

For plates: 2% agar

YPKG Medium

Yeast extracts	10 g/l
Peptone	20 g/l
Potassium acetate	10 g/l
Glucose	5 g/l

SDEG Medium

Yeast nitrogen base	6.7 g/l
Glycerol	2%
Ethanol	2%
L-Lysine HCl	100 mg/l
L-Histidine	100 mg/l
L-Leucine	100 mg/l
Uracil	20 mg/l

For plates: 2% agar

LY medium for growth of *E.coli*

Bacto-Trypton	10 g/l
Yeast extracts	5 g/l
NaCl	5 g/l

For plates: 2% agar

For selection: ampicillin 100 µg/ml of medium

2.1.5. Buffers

TES solution

Tris-HCl	10 mM
EDTA	10 mM
SDS	0.5%

pH 7.5

Denaturing solution

NaOH	1 M
EDTA	10 mM

Hybridisation buffer

SDS	7%
EDTA	1 mM
Sodium phosphate	0.5 M
BSA	1%
Herring Sperm DNA	500 µg
Yeast t-RNA	250 µg
pH 7.2	

Wash buffer I

SDS	1%
EDTA	1 mM
Sodium phosphate	40 mM
pH 7.2	

Wash buffer II

Sodium phosphate	100 mM
pH 7.2	

Stripping buffer

Sodium phosphate	5 mM
SDS	0.1%
pH 7.2	

Phosphate-buffered saline (PBS)

$Na_2HPO_4 \cdot 7\ H_2O$	4.3 mM
KH_2PO_4	1.4 mM
NaCl	137 mM
KCl	2.7 mM
pH 7.4	

2.1.6. Other reagents and chemicals

Tag Polymerase (Qiagen)

Herring Sperm DNA (Sigma)

QIAquickGel Extraction Kit (Qiagen)

Plasmid Mini Kit (Qiagen)

Methyl methanesulfonate (MMS; Sigma-Aldrich)

[α-^{33}P]dATP (2500 Ci/mmol; Amersham Pharmacia)

$(dT)_{18}$ primer (MWG-Biotech, Germany)

$p(dN_6)$ random primer (Roche)

Super Script™II (Invitrogen)

NucleoSpin Extraction Columns (Clontech Laboratories, Inc)

Imaging Plates (FUJIFILM Medical Systems USA, Inc)

AMV Reverse Transcriptase (Promega)

G-50 Sephadex columns (Amersham Pharmacia)

Fluorescein diacetate (FDA; ICN Biomedicals, Inc.)

Propidium iodide (PI; Sigma-Aldrich)

Restriction enzymes (New England Biolabs)

FM 4-64 (Molecular Probes)

Dihydroethidium (DHE; Molecular Probes)

DNase I, RNase free (Roche)

3,3,5,5,-tetramethyl-pyrroline *N*-oxide (TMPO; Sigma-Aldrich)

Yeast t-RNA (Invitrogen)

G418 disulfate (Sigma-Aldrich)

2.2 Methods

2.2.1. Gene disruption

Gene disruption was performed as described by Güldener *et al.* (1996) and Lorenz *et al.* (1995) using the KanMX4 marker from the plasmid pUG6. Briefly, KanMX4 cassette was amplified by PCR using the primers with 40 bp homologue sequences up- and down-stream from the disrupted gene. PCR reaction mix contained polymerase buffer (Qiagen) at 1x concentration, 3 mM magnesium chloride, 0.2 mM deoxyribonucleotides, 100 pmol PCR primers and 2.5 units Tag polymerase in total volume of 50 µl. The PCR cycling was performed using an Eppendorf Mastercycler. The PCR reaction was denatured at 95°C for 2 min, followed by 40 cycles of 30 s denaturation at 95°C, 30 s annealing at 54°C, and 90 s extension at 72°C. PCR products were separated by electrophoresis in 1% agarose gels containing ethidium bromide (EtBr; 0.5 mg/ml) and visualised by UV transillumination. Disruption cassettes were extracted from the gel by using the Gel Extraction Kit (Qiagen). For the gene disruption protocol wild-type cells were grown until mid-log phase (OD_{600} 0.6-0.8), cells were harvested, washed once with sterile water and resuspended in 100 mM lithium acetate. Cells were incubated for 20 min at 30°C with shaking. 100 µl of cells were mixed with 0.5-1 µg of disruption cassette and 5 µg of herring sperm DNA (denatured for 10 min at 98°C). After 20 min incubation at 30°C 600 µl of 100 mM lithium acetate/40%PEG 3350 solution was added and tubes were incubated at 30°C for additional 20 min. 71 µl of 100% DMSO was added and heat shock was performed at 42° for 15 min. Cells were harvested and resuspended in YPD medium and grown for 3h at 30°C. At the end cells were harvested and plated on selective medium, YPD with 300 µg/ml of G418. To prove correct gene replacement colony PCR was performed with flanking region and internal primers.

2.2.2. Low-level treatment with MMS and RNA isolation

YPD and F1 media were inoculated with overnight pre-cultures and grown at 30°C to mid log-phase (OD_{600} 0.6 to 0.8). Cultures were split into three parts: the first aliquot was mock-treated and used as control; the second and third aliquots were treated with low concentration of MMS (0.00125% and 0.0125%). All cultures were incubated at 30° C. Samples were collected after 30 min and 1h incubation. Cells were pelleted, frozen in liquid nitrogen and stored at -80°C until RNA preparation.

Total RNA was isolated with the hot-phenol method described by Schmitt *et al.* (1990). Cell pellets were resuspended in 400 µl TES solution and 400 µl acidic phenol. Samples were incubated at 65°C for 1h. The upper phase was extracted twice with one volume of chloroform and subsequently ethanol precipitated. Samples were digested with DNAse and ethanol precipitated again. RNA pellet was dissolved in RNAse free water and stored at –20°C.

2.2.3. Hybridisation probe synthesis

Probes were generated by a first-strand cDNA synthesis. 4 µg of total RNA was mixed with 100 pmol $(dT)_{18}$ and $p(dN_6)$ random primer and heated at 70°C for 5 min. Reverse transcription was performed in a total volume of 20 µl using Super Script^{TM}II (Invitrogen) and 25 µCi of [α-^{33}P]dATP (2500 Ci/mmol; Amersham, UK) and incubated at 42° for 1.5 h. Subsequently, probes were denatured with 1/10 volume denaturing solution at 68°C for 20 min and afterwards neutralized with 1M NaH_2PO_4, pH 7.0 at 68°C for 10 min The unincorporated nucleotides were removed using Atlas NucleoSpin Extraction Columns (Clontech).

2.2.4. Hybridisation and image analysis

All hybridisations were performed using complementary DNA arrays produced with PCR fragments of 6116 open reading frames (ORFs) of *Saccharomyces cerevisiae* spotted onto nylon membranes (Hauser *et al.*, 1998). Arrays were pre-hybridised for 2h at 68° in hybridisation buffer. Probe hybridisation was done in the same buffer at 68° for 16-20 h in 5 ml. Subsequently, filters were washed 5 times at 68° with Wash buffer I and 3 times at room temperature with Wash buffer II. Filters were stripped washing twice with boiled stripping buffer (5mM sodium phosphate, pH 7.2, 0.1% SDS), rinsing membranes in 200 mM KOH for 1h and repeated washing for three times with boiled stripping buffer. Samples from the same medium and the same time point, but different treatments (mock-treated; 0.00125%; 0.0125% MMS) were hybridized on the same membrane (membranes were stripped in between two hybridizations).

Signals were detected by exposition to Imaging Plates (Fuji) for 16h and scanning on Storm 860 Phosphor Imager (Molecular Dynamics, USA). All images were analyzed with the AIDA Image Analyser Software version 3.22 (Raytest, Germany). Data analysis was performed for each set of results from one filter separately. Datasets from one experiment were ranked and compared in a rank intensity plot (Kroll and Wölfl, 2002). The curves varied as expected not only in scaling but as well in an additional offset. The real offset cannot be determined, so the average intensity of each rank was calculated. To this curve all pooled experiments were mapped rank wise (Kroll and Wölfl, 2002; Bolstad *et al.*, 2003). After normalization, spots with hybridization signals at least two times higher than the maximal background level were selected for further analysis. Changes in expression of particular gene after MMS treatment, greater than 3-fold were considered significant. Using these stringent criteria the changes in gene expression subscribed solely to MMS-induction were determined. Genes with significantly changed expression were grouped according to their function and pathway they belong to (according to Saccharomyces Genome Database – SGD).

2.2.5. Semi-quantitative RT-PCR

Reverse transcription was performed using AMV Reverse Transcriptase (Promega). 10 µg of total RNA was mixed with 100 pmol $(dT)_{18}$ and 100 pmol random hexamer primer and heated at 70°C for 5 min. Master mix, containing 5 µl AMV RT 5x buffer, 2.5 µl 10 mM dNTP's and 2.5 µl AMV Reverse Transcriptase (300 u/µl), was added, volume was adjusted with RNAsee free water to 25 µl and probes were incubated at 42° for 1.5 h. cDNA was purified with G-50 Sephadex columns (Amersham Pharmacia), measured on spectrophotometer and concentrations were equalised. Semi-quantitative RT-PCR protocol was performed as described by Halford *et al*. (1999) and Spadoni *et al*. (2003). Briefly, 5-fold or 10-fold serial dilutions of cDNA, prepared from total RNA, were used as RT-PCR templates. One twentieth volume of the resulting dilutions was subjected to PCR amplification using polymerase buffer (Qiagen) at 1x concentration containing 1.5 mM magnesium chloride, supplemented with 0.2 mM dNTP's, 50 pmol PCR of each primer and 2.5 units Tag polymerase (Qiagen) in total volume of 50 µl. The PCR cycling was performed using an Eppendorf Mastercycler. The cDNA mixture was denatured at 95°C for 2 min, followed by 25 or 30 cycles (as described in the text) of 30 s denaturation at 95°C, 30 s annealing at the temperature specific for each primer set, and 45 s extension at 72°C. PCR products were separated by electrophoresis in 1% agarose gels containing EtBr (0.5 mg/ml) and visualised by UV transillumination. As an internal control of cDNA, PCR was performed on the same cDNA using primers for *ACT1*. For every RNA sample three separate cDNA preparations were performed and analysed by PCR amplifications with each set of primers.

2.2.6. Drug sensitivity assay

Strains were grown in YPD, SD, SD-URA or YPKG medium (as indicated in the text) until mid log phase (OD_{600} = 0.6-0.8) or stationary phase (one or six days old). OD_{600} was

adjusted to 0.5 and five additional 5-fold serial dilutions were made. Four microliters of each serial dilution were spotted onto the indicated media and incubated at 30°C for 3 days.

2.2.7. Survive test and viability staining

Cells cultures were started at time point zero with equal number of cells (OD_{600} 0.2), grown until mid log phase at 30°C, split and treated with MMS (concentrations are indicated in the text). Samples were taken after 2h, 4h, 24h and 48 h. OD was adjusted to 0.5 and 0.1 ml from 10-fold serial dilutions (10^{-4} dilutions for control samples or 10^{-2} dilutions for MMS treated samples) were plated on YPD or SD-URA in triplicate. After 2 days numbers of colonies were scored and calculated according to the dilution factor. For viability test aliquots of the same samples were stained with fluorescein diacetate (FDA) in concentration of 0.04 µg per 100 µl of cell culture and propidium iodide (PI) in concentration of 25 µg per 100 µl of cell culture according to the protocol described in Nikolova *et al.* (2000-2002). Cells were incubated for 15 min at room temperature in the dark, washed and resuspended in PBS. Fluorescence was monitored by fluorescence microscope (AxioCam HR/Axioplan 2, Carl Zeiss, Germany) or stained cells were counted using a FACS® Calibur (Becton Dickinson) and CellQuest Pro analysis software. Excitation and emission settings were 488 nm and 525–550 nm (FL1 filter) for FDA and 488 nm and 564–606 nm (FL2 filter) for PI staining, respectively.

2.2.8. Plasmid construction and overexpression assay

Full-length *FBP1* (1070-bp) was amplified by PCR using the sense primer containing the *Spe*I site upstream the start codon and the antisense primer with *Xho*I site downstream the stop codon. The PCR product was digested with *Spe*I/*Xho*I and the resulting product was ligated into the *Spe*I/*Xho*I sites of the pRS426 or pRS425 plasmids with GPD promoter (Mumberg *et al.*, 1995; kindly provided by Thomas Munder). Competent *E.coli* DH5α cells were used for cloning. Plasmids were isolated from bacteria cells grown in selective LB

medium using Qiagen plasmid purification kit. Sequencing of obtained plasmids was done by JenaGen (Jena, Germany). Resulting plasmids, encoding *FBP1* under the control of the GPD promoter as well as the "empty" pRS426 and pRS425 plasmids were transformed into wild-type and *Δfbp1* mutant strains using the lithium acetate protocol and *URA3* or *LEU2* as selection markers, respectively.

2.2.9. RNR2 reporter plasmid assay

Wild-type, *Δfbp1* and *Δrad9* mutants were transformed with only pUMGP5 RNR2 reporter plasmid (Walmsley *et al.*, 1983) or with both pUMGP5 RNR2 reporter plasmid and either pRS425 empty plasmid (Mumberg *et al.*, 1995) or pRS425 containing the *FBP1* cassette under the control of the GPD promoter (this work). Cells were grown until mid log phase in selective F1 (F1-URA for transformants with only reporter plasmid or F1-URA-LEU for double transformants). F1 is a minimal medium optimised for fluorescence measurements (Afanassiev *et al.*, 2000). Afterwards, cells were resuspended in F1 medium containing increasing MMS concentrations (0%-0.045%) to final OD_{600} of 0.1 and grown at 30°C for 16h. Every sample was run in triplicate. For all experiments we used black 96-well microtiter plates with transparent bottoms (Greiner). Fluorescence intensity was measured at 0h and 16h of treatment using the Tecan Ultra plate reader (Tecan, Germany) with excitation and emission at 485 nm and 535 nm, respectively. Absorbance values were measured using the same plate reader through a 620 nm filter. For every MMS-treated sample each fluorescence value was normalised by the absorption value and the normalised fluorescence of the non-treated sample.

2.2.10. Expression of GFP fused proteins

Full-length (excluding start and stop codon) *FBP1* (1070-bp) was amplified by PCR using primers with *Pac*I sites on both ends. The PCR products were digested with *Pac*I and

the resulting products were ligated after start codon into *Pac*I site of pGen ACT plasmid with actin promoter (2μ plasmid with upstream non-coding DNA sequence of the *S.cerevisiae ACT1* promoter, Walmsley *et al*., 1997, kindly provided by Richard Walmsley). Competent *E.coli* DH5α cells were used for cloning. Plasmids were isolated from bacteria cells grown in selective LB medium using Qiagen plasmid-purification kit. Sequencing of obtained plasmids was done by JenaGen (Jena, Germany). The constructs were used to transform wild-type and *Δfbp1* strains using the lithium acetate protocol. The integration resulted in the expression of an Fbp1-GFP fusion protein.

2.2.11. Fluorescence microscopy and vacuolar staining

Intracellular localisation of fused proteins was monitored by fluorescence microscopy (AxioCam HR/Axioplan 2, Carl Zeiss, Germany) in cells grown until mid-log phase in selective SD-URA medium before and after 1h treatment with 0.03% MMS. For vacuolar staining aliquots from the same cultures were incubated with 30 μM FM 4-64 (Molecular Probes) for 30 min at 4°C, washed with PBS and further incubated at room temperature for 1h.

2.2.12. Detection of ROS production

Cells were grown in YPD or SD-URA medium until mid log phase and treated with 0.03% MMS for 1h. Samples collected before and after MMS treatment were incubated for 10 min with dihydroethidium added to the medium (DHE; Molecular Probes) at final concentration of 5 μg/ml. Cells were washed and resuspended in PBS. ROS production was quantified with a laser-scanning cytometer (LSC; Olympus) and visualised by fluorescence microscopy (AxioCam HR/Axioplan 2, Carl Zeiss, Germany).

2.2.13. Chronological aging experiment

Cells from overnight culture were diluted to the same density in full (YPD) or selective minimal (SD-URA) medium and incubated at 30°C. Every day (SD-URA) or every third day (YPD) aliquots of the cultures were adjusted to OD_{600} 0.5 and 0.1 ml of 10^{-4} and 10^{-3} dilutions were plated on YPD or SD-URA plates in triplicate. After 2 days numbers of colonies were scored and calculated according to the dilution factor. The number of colonies on day 0 (SD-URA) or day 3 (YPD) is considered to denote 100% survival. The assay was performed for 12 (SD-URA) or 21 (YPD) days (described in Bitterman *et al.*, 2003). Aliquots of cells were taken and incubated with dihydroethidium for 10 min as described above. ROS accumulation was analyzed with flow cytometry by using a FACS® Calibur (Becton Dickinson). Excitation and emission settings were 488 nm and 564–606 nm (FL2 filter), respectively.

3. Results

3.1. Cytotoxicity of low doses of MMS

Sensitivity of yeast cells to the metylating agent MMS was tested by spotting serial dilution of cells onto YPD medium containing different concentrations of MMS. The concentrations used were chosen based on those that were used in the work of Gasch *et al.* (2001), where 0.02% of MMS was producing significant transcriptional changes of >750 genes after 15 min of treatment. Therefore, we tested the cellular sensitivity to concentrations between 0.02 and 0.03%. All experiments were performed in parallel with cells of two *Saccharomyces cerevisiae* strains, FF18984 and BY4742 grown to mid-log phase. The results showed a strong cytotoxic effect of MMS concentrations starting from 0.025% in the FF18984 strain and from 0.02% in the BY4742 strain (Fig.7). Also, strong differences in sensitivity to MMS between the two strains were observed. The BY4742 strain was much more sensitive to MMS than the FF18984 strain showing reduced biomass yield already on plates with 0.02% of MMS. The sensitivity test is performed with constant exposure of cells to toxic agent and thus can indicate the percentage of cells that can continue proliferation and form colonies in the constant presence of strong cytotoxic agents. In the natural environment a more important issue could be: how many cells survive short or repeated treatment with toxic agents and are they able to recover and continue proliferation.

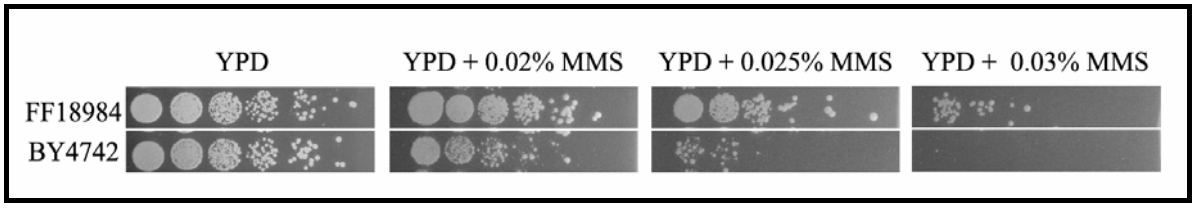

Figure 7. Sensitivity of FF18984 and BY4742 strains to MMS. Cultures were grown in YPD overnight at 30°C. Serial 5-fold dilutions were spotted onto YPD or YPD + MMS and incubated for 48h at 30°C. Reduced growth at higher dilutions reflects higher sensitivity of BY4742 to MMS.

To address this question we measured the survival rate of cells that were treated with MMS and afterwards released from treatment and plated on full medium without toxic agent. The time-course MMS treatment was done in liquid medium in which the access of toxic agents to the cells is much easier than on plates. Therefore, we used lower MMS concentrations than used for the sensitivity assay on plates, the higher 0.0125% and the second ten times lower 0.00125%. Cells from mid-log phase were treated for 72h and samples were taken at 0h, 2h, 24h and 48h. We also measured the growth rate of these cultures during the 72h time-course. In addition to the two strains used before, FF18984 and BY4742, we also tested the survival of the *Δrad9* mutant (FF18984 *Δrad9).* The *Δrad9* mutant is impaired for checkpoint-induced cell cycle arrest but able to process primary lesions (Toh and Lowndes, 2003). Thus, DNA damage induced by a genotoxic substance will persist through the cell cycle and accumulate, and in turn will lead to an enhanced cytotoxicity in the *Δrad9* mutant. Both doses of MMS reduced biomass yield in all strains measured as cell density (Fig. 8A). With the lower MMS concentration (0.00125%) both wild-type strains showed reduced proliferation but reached 80-100% of the cell density of the mock-treated culture after 48h. At the higher MMS concentration (0.0125%) the block of proliferation was more efficient and cultures reached only 50-60% density of the mock-treated reference. Proliferation of the *Δrad9* mutant was impaired even in medium without MMS. This strain stopped proliferation after approximately 24h in all conditions. Treatment of the *Δrad9* mutant with the higher MMS concentration also significantly reduced the cell density, while treatment with the lower concentration did not considerably influence growth.

Colony forming capacity was assessed after plating aliquots of cells on MMS free YPD plates and calculated as percentage of colonies from the untreated culture at time point zero (0 h). Amounts of viable cells were lower in all strains treated with the higher concentration of MMS (Fig.8B). The number of viable cells in the FF18984 strain strongly decreased already after 2h of treatment, while in the BY4742 strain a clear decrease occurred

only after 24h. Only 10% of the *Δrad9* mutant cells formed colonies after 2h of treatment, while there were no viable cells after 24h of treatment. With the lower MMS concentration the number of viable cells was significantly reduced in both wild-type strains. Interestingly, after 2h only FF18984 showed a sharp drop in viability. After 24h viability was comparably reduced in both strains, while after 48h survival was significantly reduced in all untreated cells showing no additional effect of the lower MMS concentration. Apparently, at this time point nutrient deprivation and relatively high culture density severely reduced the number of viable cells even in untreated cultures.

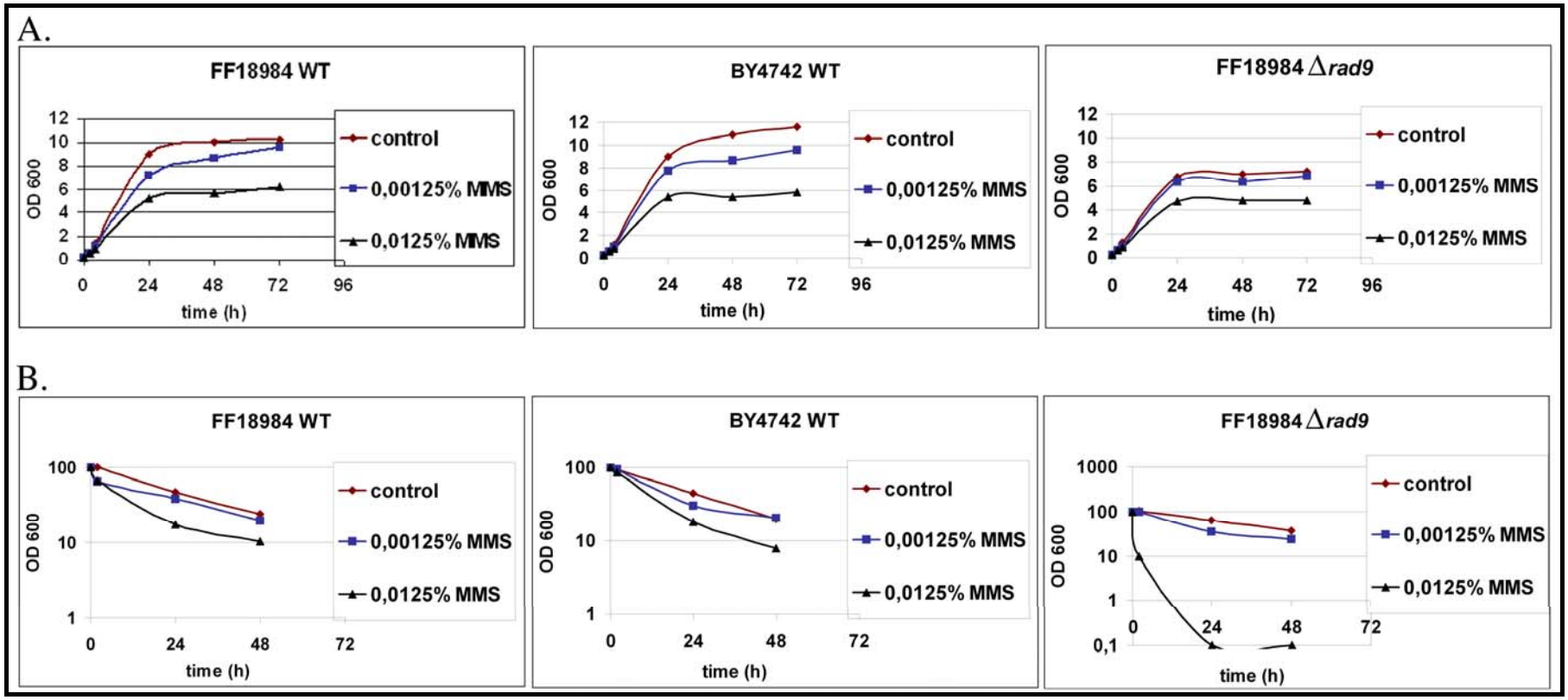

Figure 8. Cytotoxic effect of 0.00125% and 0.0125% of MMS. FF18984, BY4742 and FF18984 *rad9* mutant were treated with 0.00125% or 0.0125% MMS in YPD medium during a 72h time course (control indicates mock-treated sample). (A) Optical densities of the cultures (OD_{600}) measured after 2h, 24h, 48h or 72h. (B) Survival of MMS treated cells. Sample aliquots were taken at 0h, 2h, 24h and 48h, ODs were adjusted to 0.5 and 0.1 ml of 10^{-4} dilutions was plated on YPD in triplicate. After 2 days the numbers of colonies were scored. Relative survival was estimated as the percentage of cells capable of forming colonies in reference to the time point zero from control sample. The results are presented as logarithmic mean values. Standard deviations are indicated. Experiment was repeated three times with similar results at every repetition.

3.2. Transcriptional response after treatment with low doses of MMS

In the survival experiment both concentrations of MMS (0.0125% and 0.00125%) had a clear genotoxic effect and showed a high potential to inhibit cellular proliferation. The sensitivity assay showed that higher doses are needed for a clear cytotoxic effect. Therefore, treatment with these doses can be considered as a low level treatment and used to investigate sensing and first line response to non-cytotoxic concentrations of the DNA damaging agent MMS. To identify genes that are immediately regulated in response to MMS treatment, we analyzed gene expression profile of the FF18984 strain after 30 min and 1 h treatment with 0.00125% and 0.0125% MMS. Experiments were done in full (YPD) and minimal medium (F1) in order to include the effect of growth conditions on the response. In the following analysis only genes with hybridization signals at least two times higher than maximum background and a greater than 3-fold change in one comparison were considered significant. Classification of these ORFs into functional groups is based on their molecular function and biological process in which they are involved according to the Saccharomyces Genome Database (SGD). Significantly induced/down-regulated ORFs with unknown function and without any homology to known eukaryotic genes are not presented in this work.

The lower 0.00125% of MMS induced significant changes in transcription only in YPD medium after 1h. At this time point we observed significant up-regulation of genes involved in carbohydrate metabolism and fermentation, which implicates that even such a small dose of MMS provokes metabolic adaptation in the cell (Table 3). However, there was no up-regulation of genes from this functional group in F1 medium. The next large up-regulated gene groups include those that encode proteins involved in: protein biosynthesis (with some translation elongation factors, *EFT1*, *EFT2* and *TEF1* and ribosomal proteins), as well as protein folding and degradation (like heat shock proteins *SSA1* and *SSA2* and proteasome genes *DOA1* and *SHP1*). The induction of protein chaperons and proteasome subunits is a result of MMS induced protein damage and was already observed in the work of

Jelinsky *et al.* (2000) and Gasch *et al.* (2001). However, in their work the higher doses of MMS that were used rather down-regulated the transcription of protein biosynthesis genes. Connected with these functional groups is also the up-regulation of some genes involved in regulation of transcription and RNA processing as well as induction of some amino acid synthesis genes. With this MMS concentration we also identified induction of genes responsible for cell wall biosynthesis and organization. In between are some mannoproteins, glycoproteins and ergosterol biosynthesis genes, as well as induction of some membrane transporters, like regulators of cytoplasmic pH and multidrug resistance genes. These groups of genes may prevent the alkylating agent from entering the cells and reacting with target molecules. With exception of the *LRP1* gene, there were no other genes directly involved in DNA repair which were induced with this MMS concentration. Basically, most of the functional groups whose genes are induced and down-regulated in this experiment belong to the big superfamily of genes stereotypically regulated by different stress conditions called environmental stress response (ESR; Gasch *et al.,* 2000). In the group of down-regulated genes with exception of a protein biosynthesis group with 5 genes, other groups did not include more than two genes the transcription of which was reduced by 0.00125% of MMS and therefore, we did not consider those as significantly down-regulated processes in the cell (Table 4). However, the important down-regulation was detected for two cell cycle genes, *CDC53* and *KEL2*, and two protein sorting genes, *YIP4* and *WSC4*. It seems that this modest down-regulation of gene expression serves to slow-down cellular division until alkylation exposure is diminished.

The higher MMS concentration, 0.0125%, caused a much stronger response in both induced and down-regulated groups of genes, as well as in both media (Table 5, Table 6). Certain important facts regarding this treatment should be underlined. First of all, cells grown in F1 medium elicited faster and stronger response which was observed already after 30 min of treatment. In YPD medium 30 min treatment was not sufficient to produce significant

transcriptional response of the cells. Second, in F1 medium MMS partially promoted response of different functional groups or, if the same groups were involved in response as in YPD medium, in many cases different genes represented those groups. Third, what could be at least observed in F1 medium, the global changes in transcript abundance were largely transient. Most of the genes regulated after 30 min of treatment were not regulated any longer after 1h. An explanation could be that as soon as the protein level of the important components of cellular defence machinery is adjusted to a new steady-state level, the transcription of these genes is again reduced to basal one. In the group of up-regulated genes in YPD medium we could detect the same functional groups as in the treatment with 0.00125% of MMS: genes involved in carbohydrate metabolism and fermentation, amino acid metabolism, protein biosynthesis regulation of transcription and RNA processing, cell wall biosynthesis and organisation, protein folding and degradation, membrane transporters. This time more genes part of these functional groups were involved in cellular response. An important fact is also that from the 46 genes up-regulated in YPD medium after 30 min of treatment with 0.00125% MMS 33 of them showed similar regulation with 0.0125% of MMS. Interestingly, it was only in F1 medium that the higher MMS concentration after 30 min of treatment induced transcription of many genes involved in DNA synthesis/repair (the exceptions are *RNR2* and *MAG2* up-regulated also in YPD medium and *RNR4* up-regulated only in YPD), stress response/detoxification, cell cycle control (with the exception of *CDC39* up-regulated also in YPD after 30 min of treatment) as well as in the mitochondrial biogenesis and aerobic respiration. Additionally, some important stress induced transcription factors, like *POL2*, *YAP1* and *YRR1*, were only up-regulated in F1 medium. In contrast, in cells cultivated in F1 medium we again could not detect induction of genes that are part of carbohydrate metabolism and fermentation group. As already mentioned, some of the functional groups were presented with different genes in F1 and YPD media. This especially stands for the

signalling kinases/phosphatases, membrane transporters, and genes involved in protein sorting, folding and degradation.

With 0.0125% MMS treatment in F1 we found only 24 down-regulated genes after either 30 min or 1h of treatment, while in YPD medium this number reached 60 genes (Table 6). The functional groups that were included in this response belong to those involved in protein biosynthesis, regulation of transcription and RNA processing, cell wall biogenesis and organisation, protein folding and degradation, protein sorting, chromosomes maintenance, mitochondrial biogenesis and aerobic respiration. Out of the 25 down-regulated genes with 0.00125% of MMS in YPD medium after 30 min of treatment, 13 of them were regulated in a similar way with the 10-fold higher MMS concentration.

Table 3. ORFs whose transcripts are induced in FF18984 strain by 3-fold by 0.00125% MMS in F1 and/or YPD media (n=52)

Up-regulated ORFs	Gene short name	F1 0.00125% 30 min	YPD 0.00125% 30 min	F1 0.00125% 1h	YPD 0.00125% 1h	Molecular function	Biological process
Carbohydrate metabolism and fermentation, pentose phosphate shunt (9 ORFs)							
YOL086C	**ADH1**	n.d.	n.d.	n.d.	4.53	Alcohol dehydrogenase	Fermentation
YMR083W	**ADH3**	n.d.	n.d.	n.d.	5.06	Alcohol dehydrogenase isoenzyme III	Fermentation
YGR254W	**ENO1**	2.05	n.d.	n.d.	6.13	Enolase I	Glycolysis
YKL060C	**FBA1**	n.d.	n.d.	n.d.	3.98	Aldolase	Glycolysis
YLR134W	**PDC5**	n.d.	n.d.	2.36	6.18	Pyruvate decarboxylase	Ethanol fermentation
YBR196C	**PGI1**	n.d.	n.d.	n.d.	3.68	Phosphoglucoisomerase	Glycolysis
YHR163W	**SOL3**	3.43	n.d.	n.d.	n.d.	Weak multicopy suppressor of los1 1	Pentose phosphate shunt, oxidative branch
YJL052W	**TDH1**	n.d.	n.d.	n.d.	3.53	Glyceraldehyde 3 phosphate dehydrogenase 1	Glycolysis
YGR192C	**TDH3**	n.d.	n.d.	n.d.	4.87	Glyceraldehyde 3 phosphate dehydrogenase 3	Glycolysis
DNA synthesis/repair (1 ORF)							
YHR081W	**LRP1**	2.91	n.d.	n.d.	3.45	Substrate-specific nuclear cofactor for exosome activity in the processing of stable RNAs	Double-strand break DNA repair
Signaling/kinases/phosphatases (1 ORF)							
YHR005C	**GPA1**	n.d.	n.d.	n.d.	5.94	Involved in the mating pheromone signal transduction pathway	Signal transduction of mating signal (sensu Saccharomyces)
Amino acid metabolism (3 ORFs)							
YLR089C	**ALT1**	4.49	n.d.	n.d.	n.d.	Putative alanine transaminase	Biological process unknown
YJR016C	**ILV3**	n.d.	n.d.	2.23	3.04	Dihydroxy-acid dehydratase activity	Branched chain family amino acid biosynthesis
YBR263W	**SHM1**	n.d.	n.d.	n.d.	6.97	Serine hydroxymethyltransferase, mitochondrial	Serine and glycine biosynthesis
Protein biosynthesis (8 ORFs)							
YOR133W	**EFT1**	n.d.	n.d.	n.d.	5.19	Translation elongation factor 2 (EF 2)	Protein synthesis elongation
YDR385W	**EFT2**	n.d.	n.d.	n.d.	6.47	Translation elongation factor 2 (EF 2)	Protein synthesis elongation
YNL069C	**RP23**	n.d.	n.d.	n.d.	3.28	Homology to rat ribosomal protein L13a	Protein biosynthesis
YLR344W	**RPL33A**	3.56	n.d.	n.d.	n.d.	Homology to rat L26	Protein biosynthesis
YIL018W	**RPL5A**	n.d.	n.d.	n.d.	3.61	Homology to rat L8 and E. coli L2	Protein biosynthesis
YPL081W	**RPS13B**	n.d.	n.d.	n.d.	3.05	Homology to rat S9 and E.coli S4	Protein biosynthesis
YNL209W	**SSB2**	n.d.	n.d.	n.d.	3.09	Stress seventy subfamily B	Protein biosynthesis
YPR080W	**TEF1**	n.d.	n.d.	n.d.	3.91	Translational elongation factor EF 1 alpha	Protein synthesis elongation
Regulation of transcription, RNA metabolism and processing (8 ORFs)							
YKR036C	**CAF4**	n.d.	2.01	n.d.	3.12	CCR4 associated factor	Regulation of transcription
YHR187W	**IKI1**	n.d.	n.d.	n.d.	5.00	RNA polymerase II Elongator associated protein	Regulation of transcription from RNA polymerase II promoter

Up-regulated ORFs	Gene Short name	F1 0.00125% 30 min	YPD 0.00125% 30 min	F1 0.00125% 1h	YPD 0.00125% 1h	Molecular function	Biological process
YBR167C	**POP7**	n.d.	n.d.	n.d.	12.98	Processing of Precursors	rRNA processing
YLR039C	**RIC1**	n.d.	n.d.	n.d.	3.41	Involved in transcription of ribosomal protein genes and ribosomal RNA	Regulation of transcription from Pol II promoter
YJL148W	**RPA34**	3.96	n.d.	n.d.	n.d.	RNA polymerase I subunit, not shared (A34.5)	Transcription from Pol I promoter
YGR013W	**SNU71**	2.04	n.d.	n.d.	5.31	Associated with U1 snRNP	mRNA splicing
YKL058W	**TOA2**	n.d.	n.d.	2.18	3.66	Transcription factor IIA, small chain	Transcription initiation from Pol II promoter
YHR196W	**UTP9**	9.29	n.d.	n.d.	n.d.	Part of small (ribosomal) subunit (SSU) processosome (contains U3 snoRNA)	Processing of 20S pre rRNA
Cell wall biosynthesis and organisation (8 ORFs)							
YLR110C	**CCW12**	n.d.	n.d.	n.d.	4.87	Cell wall mannoprotein	Agglutination during conjugation with cellular fusion
YBR078W	**ECM33**	n.d.	n.d.	n.d.	4.43	Extra Cellular Mutant	GPI anchored protein
YGL012W	**ERG4**	n.d.	n.d.	n.d.	3.07	Sterol C 24 reductase	Ergosterol biosynthesis
YJL159W	**HSP150**	n.d.	n.d.	n.d.	5.89	Heat shock protein, secretory glycoprotein	Cell wall organization and biogenesis
YJL062W	**LAS21**	3.27	n.d.	n.d.	n.d.	Involved in the attachment of glycosylphosphatidylinositol (GPI) anchors to proteins.	Integral plasma membrane protein
YOR298W	**MUM3**	n.d.	n.d.	n.d.	3.12	Acyltransferase activity	Phospholipid biosynthesis
YDR077W	**SED1**	n.d.	n.d.	2.01	4.36	Putative cell surface glycoprotein	Cell wall organization and biogenesis
YOR247W	**SRL1**	n.d.	n.d.	n.d.	3.19	Suppressor of Rad53 null Lethality	Mannoprotein that exhibits a tight association with the cell wall
Protein folding, degradation and translocation (6 ORFs)							
YKL213C	**DOA1**	n.d.	n.d.	n.d.	3.06	Required for normal rates of proteolysis of ubiquitin dependent proteolytic substrates in vivo	Ubiquitin dependent protein degradation
YPL240C	**HSP82**	n.d.	n.d.	n.d.	3.91	82 kDa heat shock protein; homolog of mammalian Hsp90	Stress response
YKL201C	**MNN4**	n.d.	n.d.	n.d.	3.93	Involved in mannose metabolism	Protein amino acid glycosylation
YBL058W	**SHP1**	n.d.	n.d.	n.d.	3.13	Protein phosphatase type 1 regulator activity	Proteasomal ubiquitin dependent protein catabolism
YAL005C	**SSA1**	n.d.	n.d.	n.d.	6.51	Stress seventy subfamily A	Protein folding
YLL024C	**SSA2**	n.d.	n.d.	n.d.	5.64	Member of 70 kDa heat shock protein family	Protein folding
Membrane transport (5 ORFs)							
YGL008C	**PMA1**	n.d.	n.d.	n.d.	8.13	Major regulator of cytoplasmic pH	Regulation of pH
YPL036W	**PMA2**	n.d.	2.05	n.d.	6.72	Plasma membrane H+-ATPase, isoform of Pma1p	Regulation of pH
YEL0171	**PMP2**	n.d.	n.d.	n.d.	8.00	Proteolipid associated with plasma membrane H(+)-ATPase (Pma1p); regulates plasma membrane H(+)-ATPase activity	Cation transport
YLL048C	**YBT1**	n.d.	n.d.	n.d.	3.03	Yeast bile transporter, similar to mammalian bile transporter	Bile acid transport
YDR135C	**YCF1**	n.d.	n.d.	n.d.	3.09	Metal resistance protein with similarity to human cystic fibrosis protein CFTR and multidrug resistance proteins	Bilirubin transport
Protein sorting (1 ORF)							
YCL001W	**RER1**	n.d.	n.d.	n.d.	5.59	Protein involved in retention of membrane proteins in the ER	ER to Golgi transport

Up-regulated ORFs	Gene Short name	F1 0.00125% 30 min	YPD 0.00125% 30 min	F1 0.00125% 1h	YPD 0.00125% 1h	Molecular function	Biological process
Chromatin arrangement, chromosomes maintenance (1 ORF)							
YFR031B	**SMC2**	n.d.	n.d.	n.d.	5.64	Component of the condensin complex	Mitotic chromosome condensation
Others (1 ORF)							
YJL039C	**NUP192**	3.27	n.d.	n.d.	n.d.	Large yeast nucleoporin	Nuclear pore complex subunit

Table 4. ORFs whose transcripts are repressed in FF18984 strain by 3-fold by 0.00125% MMS in F1 and/or YPD media (n=25)

Down-regulated ORFs	Gene short name	F1 0.00125% 30 min	YPD 0.00125% 30 min	F1 0.00125% 1h	YPD 0.00125% 1h	Molecular function	Biological process
Signaling/kinases/phosphatases (1 ORFs)							
YKL048C	**ELM1**	n.d.	n.d.	n.d.	-3.10	Serine/threonine protein kinase that regulates cellular morphogenesis, septin behavior, and cytokinesis	Cell morphology
DNA synthesis/repair (2 ORFs)							
YLR245C	**CDD1**	-2.32	n.d.	-5.42	-4.36	Involved in cytidine and deoxycytidine metabolism	Ribose and deoxyribose phosphate metabolism
YHR164C	**DNA2**	n.d.	n.d.	-3.80	n.d.	DNA replication helicase	DNA repair
Protein biosynthesis (5 ORFs)							
YLR069C	**MEF1**	-2.24	n.d.	-5.42	-2.16	Mitochondrial elongation factor G-like protein	Protein synthesis elongation
YDL184C	**RPL47A**	n.d.	n.d.	n.d.	-3.50	Homology to human L41	Protein biosynthesis
YLR333C	**RPS31B**	n.d.	n.d.	n.d.	-4.01	Homology to rat S25; belongs to the S25E family of ribosomal proteins	Protein biosynthesis
YOR294W	**RRS1**	n.d.	n.d.	n.d.	-4.05	Regulator for ribosome synthesis	Ribosome biogenesis
YDL191W	**SOS1**	-2.55	-3.60	n.d.	n.d.	Homology to rat L35	Protein biosynthesis
Regulation of transcription, RNA metabolism and processing (3 ORFs)							
YLR068W	**FYV7**	n.d.	n.d.	-5.75	n.d.	involved in processing the 35S rRNA primary transcript to generate the 20S and 27SA2 pre-rRNA transcripts	Processing of 20S pre-rRNA
YPL082C	**MOT1**	-3.30	n.d.	n.d.	n.d.	Involved in TBP (TATA-binding protein) regulation	Transcription
YLR335W	**NUP2**	n.d.	n.d.	-4.35	-5.60	Probably functions in transport through nuclear pore	mRNA-nucleus export
Cell wall biosynthesis and organisation (2 ORFs)							
YKL096W	**CWP1**	-3.49	n.d.	n.d.	n.d.	Cell wall protein, involved in O and N glycosylation, acceptor of B1-6 glucan.	Cell wall organization and biogenesis
YKR004C	**ECM9**	n.d.	n.d.	-7.37	n.d.	Extra Cellular Mutant	Not yet annotated
Protein sorting (2 ORFs)							
YGL198W	**YIP4**	n.d.	n.d.	n.d.	-3.67	Protein that interacts with Rab GTPases	Possible role in vesicle-mediated transport
YHL028W	**WSC4**	n.d.	n.d.	n.d.	-7.19	ER membrane protein involved in the translocation of soluble secretory proteins and insertion of membrane proteins into the ER membrane	SRP-dependent cotranslational protein targeting to membrane, translocation
Chromatin arrangement, chromosomes maintenance (1 ORFs)							
YLR357W	**RSC2**	n.d.	n.d.	n.d.	-3.03	RSC2 is a member of RSC complex, which remodels the structure of chromatin	Chromatin modelling
Cell cycle (2 ORFs)							
YDL132W	**CDC53**	n.d.	n.d.	n.d.	-4.27	Acts together with Cdc4p and Cdc34p to control the G1-S phase transition	Ubiquitin-dependent protein degradation
YGR238C	**KEL2**	n.d.	n.d.	n.d.	-4.36	Protein that functions in a complex with Kel1p to negatively regulate mitotic exit	Protein that functions in a complex with Kel1p to negatively regulate mitotic exit
Mitochondrial biogenesis, maintance and aerobic respiration (1 ORF)							
YGR028W	**MSP1**	n.d.	n.d.	n.d.	-4.77	Mitochondrial protein involved in sorting of proteins in the mitochondria; putative membrane-spanning ATPase	Mitochondrial translocation

Down-regulated ORFs	Gene short name	F1 0.00125% 30 min	YPD 0.00125% 30 min	F1 0.00125% 1h	YPD 0.00125% 1h	Molecular function	Biological process
Others (6 ORFs)							
YHR146W	**CRP1**	n.d.	n.d.	n.d.	-4.55	Crucifo-rm DNA Binding Protein 1	Protein that binds to cruciform DNA structures
YHR055C	**CUP1B**	n.d.	n.d.	n.d.	-3.20	Copper-binding metallothionein	Copper sensitivity/resistance
YDL227C	**HO**	n.d.	n.d.	n.d.	-3.73	Homothallic switching	Mating-type switching/recombination
YGL197W	**MDS3**	n.d.	n.d.	n.d.	-3.85	Mck1 Dosage Suppressor 3; negative regulator of early meiotic gene expression	Meiosis
YGL211W	**NCS6**	n.d.	n.d.	n.d.	-3.14	Protein with a role in urmylation and in invasive and pseudohyphal growth	Biological process unknown
YER009W	**NTF2**	n.d.	n.d.	n.d.	-3.13	May coordinate the Ran-dependent (GSP1/GSP2) association and disassociation reactions of nuclear import; human homolog complements yeast mutants	Nuclear envelope protein

Table 5. ORFs whose transcripts are induced in FF18984 strain by 3-fold by 0.0125% MMS in F1 and/or YPD media (n=146)

Up-regulated ORFs	Gene short name	F1 0.0125% 30 min	YPD 0.0125% 30 min	F1 0.0125% 1h	YPD 0.0125% 1h	Molecular function	Biological process
Carbohydrate metabolism and fermentation, pentose phosphate shunt (12 ORFs)							
YOL086C	**ADH1**	n.d.	n.d.	2.74	5.64	Alcohol dehydrogenase	Fermentation
YMR303C	**ADH2**	n.d.	n.d.	2.09	17.89	Alcohol dehydrogenase II	Fermentation
YMR083W	**ADH3**	n.d.	n.d.	n.d.	5.03	Alcohol dehydrogenase isoenzyme III	Fermentation
YPL061W	**ALD6**	3.20	n.d.	n.d.	n.d.	Aldehyde dehydrogenase activity	Glucose fermentation
YGR254W	**ENO1**	n.d.	n.d.	n.d.	9.69	Enolase I	Glycolysis
YHR174W	**ENO2**	n.d.	n.d.	n.d.	3.21	Enolase	Glycolysis
YKL060C	**FBA1**	n.d.	n.d.	n.d.	4.35	Aldolase	Glycolysis
YLR134W	**PDC5**	n.d.	n.d.	n.d.	6.86	Pyruvate decarboxylase	Ethanol fermentation
YCR012W	**PGK1**	n.d.	n.d.	n.d.	25.29	3 phosphoglycerate kinase	Glycolysis
YJL052W	**TDH1**	n.d.	n.d.	n.d.	6.37	Glyceraldehyde 3 phosphate dehydrogenase 1	Glycolysis
YGR192C	**TDH3**	n.d.	n.d.	n.d.	4.27	Glyceraldehyde 3 phosphate dehydrogenase 3	Glycolysis
YLR070C	**XYL2**	4.25	n.d.	n.d.	n.d.	Xylitol dehydrogenase	Monosaccharide metabolism
DNA synthesis/repair (11 ORFs)							
YGR061C	**ADE6**	13.52	n.d.	n.d.	n.d.	5' phosphoribosylformyl glycinamidine synthetase	Purine nucleotide biosynthesis
YCL050C	**APA1**	16.16	2.01	n.d.	n.d.	Diadenosine 5',5''' P1,P4 tetraphosphate phosphorylase I	Nucleotide metabolism
YLR245C	**CDD1**	8.49	n.d.	n.d.	n.d.	Involved in cytidine and deoxycytidine metabolism	Cytidine catabolism
YLR233C	**EST1**	4.85	n.d.	n.d.	n.d.	Telomere elongation protein	Telomere maintenance
YLR427W	**MAG2**	6.55	4.02	n.d.	n.d.	DNA 3 methyladenine glycosidase II that catalyzes of the hydrolysis of alkylated DNA	DNA dealkylation
YLR154C	**RNH203**	3.32	n.d.	n.d.	n.d.	Ribonuclease H2 subunit	DNA replication
YJL026W	**RNR2**	2.25	3.98	4.39	8.06	Small subunit of ribonucleotide reductase	DNA replication
YGR180C	**RNR4**	n.d.	n.d.	n.d.	3.57	Ribonucleotide reductase, small subunit (alt)	DNA replication
YLR135W	**SLX4**	4.88	n.d.	n.d.	n.d.	Subunit of a complex, with Slx1p, that hydrolyzes 5' branches from duplex DNA in response to stalled or converging replication forks	DNA replication
YKR031C	**SPO14**	3.50	n.d.	n.d.	n.d.	Phospholipase D activity	Phospholipid metabolism
YKR010C	**TOF2**	4.34	n.d.	n.d.	n.d.	Topoisomerase I interacting factor 2	DNA topological change
Signaling/kinases/phosphatases (4 ORFs)							
YHR005C	**GPA1**	n.d.	n.d.	n.d.	5.50	Involved in the mating pheromone signal transduction pathway	Signal transduction of mating signal
YHR082C	**KSP1**	n.d.	n.d.	n.d.	3.68	Serine/threonine kinase similar to casein kinase II	Protein amino acid phosphorylation
YFL033C	**RIM15**	3.28	n.d.	n.d.	n.d.	Involved in signal transduction during cell proliferation in response to nutrients	Meiosis

Up-regulated ORFs	Gene short name	F1 0.0125% 30 min	YPD 0.0125% 30 min	F1 0.0125% 1h	YPD 0.0125% 1h	Molecular function	Biological process
YJR066W	**TOR1**	9.89	5.90	n.d.	n.d.	Phosphatidylinositol 3-kinase activity	Signal transduction
Amino acid metabolism (13 ORFs)							
YDR127W	**ARO1**	7.23	3.09	n.d.	n.d.	Pentafunctional arom protein, catalyzes steps 2 through 6 in the biosynthesis of chorismate, which is a precursor to aromatic amino acids	Phenylalanine, tyrosine and tryptophan biosynthesis
YPR145W	**ASN1**	n.d.	n.d.	n.d.	4.82	Asparagine synthetase	Asparagine biosynthesis
YAL012W	**CYS3**	n.d.	n.d.	4.73	3.20	Catalyzes one of the two reactions involved in the transsulfuration pathway that yields cysteine from homocysteine	Sulfur amino acid metabolism
YJR139C	**HOM6**	n.d.	n.d.	n.d.	3.15	Catalyzes third step in common pathway for methionine and threonine biosynthesis	Homoserine biosynthesis
YJR016C	**ILV3**	n.d.	n.d.	3.13	3.72	Dihydroxy-acid dehydratase activity	Branched chain family amino acid biosynthesis
YCL009C	**ILV6**	n.d.	n.d.	n.d.	3.35	Acetolactate synthase regulatory subunit	Branched chain family amino acid biosynthesis
YLR451W	**LEU3**	6.01	n.d.	n.d.	n.d.	Regulates genes involved in branched chain amino acid biosynthesis and in ammonia assimilation	Leucine biosynthesis
YBR115C	**LYS2**	13.90	3.99	n.d.	n.d.	Involved in the key step in fungal biosynthesis of lysine	Amino acid biosynthesis
YNL076W	**MKS1**	6.80	n.d.	n.d.	n.d.	Pleiotropic regulatory factor involved in Ras CAMP and lysine biosynthetic pathways and nitrogen regulation	Regulation of nitrogen utilization
YER099C	**PRS2**	13.12	n.d.	n.d.	n.d.	Ribose phosphate pyrophosphokinase 2	Histidine biosynthesis
YBR263W	**SHM1**	n.d.	n.d.	n.d.	6.39	Serine hydroxymethyltransferase	One-carbon compound metabolism
YHR025W	**THR1**	3.51	n.d.	n.d.	n.d.	Homoserine kinase	Threonine and methionine biosynthesis
YBR166C	**TYR1**	n.d.	n.d.	n.d.	4.79	Step of tyrosine biosynthesis pathway	Tyrosine metabolism
Protein biosynthesis (11 ORFs)							
YOR133W	**EFT1**	n.d.	n.d.	n.d.	7.49	Translation elongation factor 2 (EF 2)	Protein synthesis elongation
YDR385W	**EFT2**	n.d.	n.d.	2.23	10.07	Translation elongation factor 2 (EF 2)	Protein synthesis elongation
YLR069C	**MEF1**	10.50	n.d.	n.d.	n.d.	Mitochondrial elongation factor G like protein	Protein synthesis elongation
YGR076C	**MRPL25**	14.49	6.68	n.d.	n.d.	Mitochondrial ribosomal protein MRPL25 (YmL25)	Protein biosynthesis
YLR048W	**NAB1B**	n.d.	n.d.	n.d.	3.49	Required for translation and contributes to the assembly and/or stability of the 40S ribosomal subunit	Protein biosynthesis
YBR079C	**RPG1**	4.72	n.d.	n.d.	n.d.	Sequence similarity with a subunit of the mammalian translation initiation factor 3	Protein synthesis initiation
YIL018W	**RPL5A**	n.d.	n.d.	n.d.	3.96	Homology to rat L8 and E. coli L2	Protein biosynthesis
YDL229W	**SSB1**	17.39	n.d.	n.d.	n.d.	Involved in translation, perhaps by guiding the nascent chain through the ribosome	Protein biosynthesis
YPR080W	**TEF1**	n.d.	n.d.	n.d.	4.05	Translational elongation factor EF 1 alpha	Protein synthesis elongation
YKL081W	**TEF4**	n.d.	n.d.	n.d.	3.13	Translation elongation factor EF 1gamma	Protein synthesis elongation
YKR059W	**TIF1**	n.d.	2.27	n.d.	3.27	Translation initiation factor eIF4A	Protein synthesis initiation
Regulation of transcription, RNA metabolism and processing (20 ORFs)							
YPL217C	**BMS1**	3.13	n.d.	n.d.	n.d.	Essential conserved nucleolar GTP-binding protein required for synthesis of 40S ribosomal subunits and for processing of the 35S pre-rRNA	35S primary transcript processing

Up-regulated ORFs	Gene short name	F1 0.0125% 30 min	YPD 0.0125% 30 min	F1 0.0125% 1h	YPD 0.0125% 1h	Molecular function	Biological process
YKR036C	**CAF4**	n.d.	n.d.	n.d.	3.64	CCR4 associated factor	Regulation of transcription
YKL011C	**CCE1**	n.d.	n.d.	2.69	3.35	Cruciform cutting endonuclease	tRNA processing
YLR323C	**CWC24**	3.12	n.d.	n.d.	n.d.	Complexed with Cef1p; spliceosome complex	Biological_process unknown
YFL031W	**HAC1**	n.d.	n.d.	n.d.	3.31	Transcription factor that is required for the unfolded protein response pathway; binds to CRE motif; homologous to ATF/CREB 1	Regulation of transcription from Pol II promoter
YHR187W	**IKI1**	n.d.	n.d.	n.d.	9.87	RNA polymerase II Elongator associated protein	Regulation of transcription from RNA polymerase II promoter
YJR042W	**NUP85**	n.d.	n.d.	n.d.	3.15	May function in nuclear envelope integrity; may also be involved in tRNA biogenesis	mRNA nucleus export
YML107C	**PML39**	12.99	n.d.	n.d.	n.d.	Protein required for nuclear retention of unspliced pre mRNAs along with Mlp1p and Pml1p	mRNA nucleus export
YNL262W	**POL2**	4.43	n.d.	n.d.	n.d.	DNA polymerase II	Lagging strand elongation
YBR167C	**POP7**	n.d.	n.d.	n.d.	11.31	Processing of Precursors	rRNA processing
YOR207C	**RPC128**	8.45	n.d.	n.d.	n.d.	Second largest subunit of RNA polymerase III	Transcription from Pol III promoter
YLR141W	**RRN5**	3.18	n.d.	n.d.	n.d.	Involved in transcription of rDNA by RNA polymerase I.	Transcription from Pol I promoter
YML049C	**RSE1**	5.31	n.d.	n.d.	n.d.	RNA splicing and ER to Golgi transport	Nuclear mRNA splicing, via spliceosome
YGR013W	**SNU71**	2.10	n.d.	n.d.	5.00	Associated with U1 snRNP	mRNA splicing
YLR316C	**TAD3**	3.21	n.d.	n.d.	n.d.	tRNA specific adenosine 34 deaminase subunit Tad3p	tRNA processing
YAL001C	**TFC3**	4.62	n.d.	n.d.	n.d.	Transcription factor tau (TFIIIC) subunit 138	Transcription initiation from Pol III promoter
YKL058W	**TOA2**	n.d.	n.d.	3.32	4.81	Transcription factor IIA, small chain	Transcription initiation from Pol II promoter
YMR093W	**UTP15**	3.71	n.d.	n.d.	n.d.	Part of small (ribosomal) subunit (SSU) processosome (contains U3 snoRNA)	Processing of 20S pre-rRNA
YML007W	**YAP1**	7.96	6.47	n.d.	n.d.	Jun like transcription factor	Response to oxidative stress
YOR162C	**YRR1**	4.68	n.d.	n.d.	n.d.	Zn2-Cys6 zinc-finger transcription factor that activates genes involved in multidrug resistance	Positive regulation of transcription from RNA polymerase II promoter
Cell wall biosynthesis and organisation (15 ORFs)							
YLR110C	**CCW12**	n.d.	n.d.	n.d.	5.68	Cell wall protein, mutants are defective in mating and agglutination	Agglutination during conjugation with cellular fusion
YLR391W	**CCW14**	n.d.	n.d.	n.d.	3.68	Covalently linked cell wall glycoprotein, present in the inner layer of the cell wall	Cell wall organization and biogenesis
YKL046C	**DCW1**	n.d.	n.d.	2.42	3.09	Putative mannosidase, GPI-anchored membrane protein required for cell wall biosynthesis in bud formation	Cell wall biosynthesis
YBL043W	**ECM13**	7.78	n.d.	n.d.	n.d.	Extra Cellular Mutant	Not yet annotated
YLR436C	**ECM30**	4.49	2.02	n.d.	n.d.	Extra Cellular Mutant	Not yet annotated
YBR078W	**ECM33**	n.d.	n.d.	n.d.	6.55	GPI-anchored protein of unknown function, has a possible role in apical bud growth	Not yet annotated
YBR177C	**EHT1**	5.80	n.d.	n.d.	n.d.	Alcohol acyl transferase	Lipid metabolism
YKL182W	**FAS1**	7.95	2.65	n.d.	n.d.	Pentafunctional enzyme consisting of the following domains : acetyl transferase, enoyl reductase, dehydratase and malonyl/palmityl transferase	Fatty acid biosynthesis
YMR306W	**FKS3**	7.55	n.d.	n.d.	n.d.	Protein of unknown function, has similarity to 1,3-beta-D-glucan synthase catalytic subunits Fks1p and Gsc2p	Not yet annotated

Up-regulated ORFs	Gene short name	F1 0.0125% 30 min	YPD 0.0125% 30 min	F1 0.0125% 1h	YPD 0.0125% 1h	Molecular function	Biological process
YML075C	**HMG1**	5.87	n.d.	n.d.	n.d.	3 hydroxy 3 methylglutaryl coenzyme A (HMG CoA) reductase isozyme	Ergosterol biosynthesis
YJL159W	**HSP150**	n.d.	n.d.	n.d.	3.95	Heat shock protein, secretory glycoprotein	Cell wall organization and biogenesis
YGR166W	**KRE11**	14.06	7.71	n.d.	n.d.	Involved in biosynthetic pathway for cell wall beta glucans	ER to Golgi transport
YGR014W	**MSB2**	14.07	7.08	n.d.	n.d.	Putative integral membrane protein	Establishment of cell polarity (sensu Saccharomyces)
YHR102W	**NRK1**	3.53	n.d.	n.d.	n.d.	Protein kinase of the PAK/Ste20 kinase family, required for cell integrity possibly through regulating 1,6-beta-glucan levels in the wall	Cellular morphogenesis during vegetative growth
YER093C	**TSC11**	13.24	n.d.	n.d.	n.d.	Subunit of TORC2 (Tor2p Lst8p Avo1 Avo2 Tsc11p Bit61p), a membrane associated complex that regulates actin cytoskeletal dynamics during polarized growth and cell wall integrity	Establishment and/or maintenance of actin cytoskeleton polarity
Protein folding, degradation and translocation (10 ORFs)							
YDL141W	**BPL1**	6.09	n.d.	n.d.	n.d.	Biotin:apoprotein ligase	Protein modification
YMR186W	**HSC82**	n.d.	n.d.	n.d.	4.41	Constitutively expressed heat shock protein	Stress response
YDR258C	**HSP78**	4.37	n.d.	n.d.	n.d.	Involved in folding of some mitochondrial proteins	Stress response
YPL240C	**HSP82**	n.d.	n.d.	2.81	6.36	82 kDa heat shock protein; homolog of mammalian Hsp90	Stress response
YKL201C	**MNN4**	n.d.	n.d.	n.d.	4.57	Involved in mannose metabolism	Protein amino acid glycosylation
YGR199W	**PMT6**	9.19	n.d.	n.d.	n.d.	Transfers mannose residues from dolichyl phosphate D mannose to specific serine/threonine residues of proteins in the secretory pathway	O linked glycosylation
YAL005C	**SSA1**	n.d.	n.d.	n.d.	12.78	Stress seventy subfamily A	Protein folding
YLL024C	**SSA2**	n.d.	n.d.	2.13	6.65	Member of 70 kDa heat shock protein family	Protein folding
YJR045C	**SSC1**	n.d.	n.d.	n.d.	3.15	Nuclear encoded mitochondrial protein; acts as a chaperone for protein import across the inner membrane	Protein folding
YLR024C	**UBR2**	3.68	n.d.	n.d.	n.d.	Ubiquitin protein ligase (E3)	Polyubiquitination
Membrane transport (11 ORFs)							
YNL270C	**ALP1**	3.96	n.d.	n.d.	n.d.	Basic amino acid transporter, involved in uptake of cationic amino acids	Basic amino acid transport
YLL052C	**AQY2**	7.19	2.03	n.d.	n.d.	Aquaporin water channel in yeast	Water transport
YOR011W	**AUS1**	5.83	n.d.	n.d.	n.d.	ABC(ATP binding cassette) protein involved in uptake of sterols	Sterol transport
YGR217W	**CCH1**	7.28	n.d.	n.d.	n.d.	Calcium channel	Calcium ion transport
YHR175W	**CTR2**	n.d.	n.d.	n.d.	4.25	Putative low affinity copper transport protein	Copper ion import
YGL008C	**PMA1**	n.d.	n.d.	3.39	12.65	Major regulator of cytoplasmic pH	Regulation of pH
YPL036W	**PMA2**	n.d.	n.d.	2.01	5.45	Plasma membrane H+-ATPase, isoform of Pma1p	Regulation of pH
YEL0171	**PMP2**	n.d.	n.d.	n.d.	6.11	Proteolipid associated with plasma membrane H(+)-ATPase (Pma1p); regulates plasma membrane H(+)-ATPase activity	Cation transport
YKR050W	**TRK2**	7.13	n.d.	n.d.	n.d.	Membrane protein; low affinity potassium transport	Potassium ion homeostasis
YLL048C	**YBT1**	n.d.	n.d.	n.d.	3.28	Yeast bile transporter, similar to mammalian bile transporter	Bile acid transport

Up-regulated ORFs	Gene short name	F1 0.0125% 30 min	YPD 0.0125% 30 min	F1 0.0125% 1h	YPD 0.0125% 1h	Molecular function	Biological process
YDR135C	**YCF1**	n.d.	n.d.	n.d.	4.23	Metal resistance protein with similarity to human cystic fibrosis protein CFTR and multidrug resistance proteins	Bilirubin transport
Protein sorting (6 ORFs)							
YCL001W	**RER1**	n.d.	n.d.	n.d.	10.28	Protein involved in retention of membrane proteins in the ER	ER to Golgi transport
YKL002W	**DID4**	3.05	n.d.	n.d.	n.d.	Required for sorting of integral membrane proteins into lumenal vesicles of multivesicular bodies	Golgi retention
YDL145C	**RET1**	3.46	n.d.	n.d.	n.d.	Alpha subunit of the coatamer complex; gamma alpha COP	Retrograde transport, Golgi to ER
YBR214W	**SDS24**	n.d.	n.d.	n.d.	3.13	May play an indirect role in fluid phase endocytosis	Biological_process unknown
YNR006W	**VPS27**	3.27	n.d.	n.d.	n.d.	Required for recycling Golgi proteins, forming lumenal membranes and sorting ubiquitinated proteins destined for degradation	Golgi retention
YLR181C	**VTA1**	n.d.	n.d.	2.14	3.48	Multivesicular body (MVB) protein involved in endosomal protein sorting	Late endosome to vacuole transport
Stress response/detoxification (5 ORFs)							
YPR128C	**ANT1**	4.95	n.d.	n.d.	n.d.	Adenine nucleotide transporter	Peroxisome organization and biogenesis
YJL101C	**GSH1**	4.42	n.d.	n.d.	n.d.	Gamma glutamylcysteine synthetase, catalyzes the first step in the gamma-glutamyl cycle for glutathione (GSH) biosynthesis	Glutathione biosynthesis
YML014W	**TRM9**	5.95	n.d.	n.d.	n.d.	tRNA methyltransferase, catalyzes the esterification of modified uridine nucleotides in tRNAs	Stress response
YBR216C	**YBP1**	5.37	n.d.	n.d.	n.d.	Protein required for oxidation of specific cysteine residues of the transcription factor Yap1p	Response to oxidative stress
YGR234W	**YHB1**	n.d.	n.d.	n.d.	3.40	Nitric oxide oxidoreductase, flavohemoglobin involved in nitric oxide detoxification	Stress response
Chromatin arrangement, chromosomes maintenance (3 ORFs)							
YOR304W	**ISW2**	4.37	n.d.	n.d.	n.d.	Nitric oxide oxidoreductase, flavohemoglobin involved in nitric oxide detoxification	Chromatin silencing at telomere
YFR031c	**SMC2**	n.d.	n.d.	n.d.	5.13	Component of the condensin complex, essential SMC chromosomal ATPase family member that forms a complex with Smc4p to form the active ATPase	Mitotic chromosome condensation
YGR002C	**SWC4**	6.95	2.26	n.d.	n.d.	Component of the Swr1p complex that incorporates Htz1p into chromatin	Chromatin remodeling
Cell cycle (6 ORFs)							
YDL220C	**CDC13**	8.08	n.d.	n.d.	n.d.	Required for the G2/M transition in mitosis	Telomere capping
YAR019C	**CDC15**	6.35	n.d.	n.d.	n.d.	Required for mitosis and sporulation, cell division cycle blocked at 36 degrees	Cell cycle
YDR168W	**CDC37**	3.24	n.d.	n.d.	n.d.	Cell cycle protein necessary for passage through START	Regulation of cell cycle
YCR093W	**CDC39**	15.97	3.89	n.d.	n.d.	Required for Start B in mitosis and spindle pole body separation at meiosis I	Regulation of cell cycle
YCR094W	**CDC50**	4.90	n.d.	n.d.	n.d.	Cell division cycle mutant, transcription regulator activity	G1 phase of mitotic cell cycle
YGR098C	**ESP1**	4.78	n.d.	n.d.	n.d.	Esp1 promotes sister chromatid separation by mediating dissociation from the chromatin of the cohesin Scc1	Regulation of exit from mitosis

Up-regulated ORFs	Gene short name	F1 0.0125% 30 min	YPD 0.0125% 30 min	F1 0.0125% 1h	YPD 0.0125% 1h	Molecular function	Biological process
Mitochondrial biogenesis, maintance and aerobic respiration (7 ORFs)							
YPL170W	**DAP1**	5.20	n.d.	n.d.	n.d.	Heme binding protein involved in regulation of cytochrome P450 protein Erg11p	Biological_process unknown
YPL040C	**ISM1**	7.09	n.d.	n.d.	n.d.	Nuclear encoded mitochondrial isoleucyl tRNA synthetase	Isoleucyl-tRNA aminoacylation
YLR163C	**MAS1**	5.39	n.d.	n.d.	n.d.	Mitochondrial processing protease subunit	Mitochondrial processing
YMR177W	**MMT1**	4.68	n.d.	n.d.	n.d.	Protein involved in mitochondrial iron accumulation	Iron homeostasis
YPR100W	**MRPL51**	6.65	n.d.	n.d.	n.d.	Mitochondrial ribosomal protein of the large subunit	Aerobic respiration
YNL055C	**POR1**	n.d.	n.d.	n.d.	3.06	Outer mitochondrial membrane porin (voltage dependent anion channel, or VDAC)	Transport
YGR181W	**TIM13**	4.71	2.03	n.d.	n.d.	Translocase of the inner membrane; mitochondrial intermembrane space protein mediating import and insertion of polytopic inner membrane proteins	Protein import into mitochondrial inner membrane
Others (11 ORFs)							
YJL020C	**BBC1**	5.04	n.d.	n.d.	n.d.	Protein possibly involved in assembly of actin patches	Actin cytoskeleton organization and biogenesis
YDL238C	**GUD1**	4.85	6.32	n.d.	n.d.	Guanine deaminase, a catabolic enzyme of the guanine salvage pathway producing xanthine and ammonia from guanine	Hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds, in linear amides
YMR207C	**HFA1**	9.92	n.d.	n.d.	n.d.	Mitochondrial acetyl coenzyme A carboxylase, catalyzes the production of malonyl CoA in mitochondrial fatty acid biosynthesis	Fatty acid biosynthesis
YLR347C	**KAP95**	3.05	n.d.	n.d.	n.d.	Required for the docking of import substrate to the nuclear membrane/pore	Protein import into nucleus
YNR008W	**LRO1**	3.15	n.d.	n.d.	n.d.	Lecithin cholesterol acyl transferase (LCAT) Related Orf	Triacylglycerol biosynthesis
YGL183C	**MND1**	7.41	2.43	n.d.	n.d.	Forms a complex with Hop2p, which is involved in chromosome pairing and repair of meiotic double-strand breaks	Meiotic recombination
YLR315W	**NKP2**	3.87	n.d.	n.d.	n.d.	Non essential Kinetochore Protein	Biological_process unknown
YDR356W	**NUF1**	10.38	2.80	n.d.	n.d.	May be involved in connecting nuclear microtubules to the spindle pole body	Microtubule nucleation
YLR146C	**SPE4**	3.03	n.d.	n.d.	n.d.	Spermine Synthase	Spermine biosynthesis
YLR045C	**STU2**	5.70	n.d.	n.d.	2.09	May play a role in attachment, organization, and/or dynamics of microtubule ends at the spindle pole body	Microtubule nucleation
YPR004C		11.80	3.74	n.d.	n.d.	Electron transfer flavoprotein complex subunit ETF alpha; contains a FAD binding domain	Biological_process unknown

Table 6. ORFs whose transcripts are repressed in FF18984 strain by 3-fold by 0.0125% MMS in F1 and/or YPD media (n=69)

Down-regulated ORFs	Gene short name	F1 0.0125% 30 min	YPD 0.0125% 30 min	F1 0.0125% 1h	YPD 0.0125% 1h	Molecular function	Biological process
DNA synthesis/repair (3 ORFs)							
YJR057W	**CDC8**	n.d.	n.d.	n.d.	-3.13	Thymidylate kinase	DNA repair
YHR164C	**DNA2**	n.d.	n.d.	-3.8	-2.17	DNA replication helicase	DNA repair
YLR265C	**NEJ1**	n.d.	n.d.	n.d.	-3.85	Mating-type regulated component of NHEJ	DNA repair
Amino acid metabolism (2 ORFs)							
YLR158C	**ASP3C**	n.d.	n.d.	n.d.	-3.21	Nitrogen catabolite-regulated cell-wall L-asparaginase II	Asparagine catabolism
YLR160C	**ASP3D**	n.d.	n.d.	-2.12	-4.00	Nitrogen catabolite-regulated cell-wall L-asparaginase II	Asparagine catabolism
Protein biosynthesis (15 ORFs)							
YKL191W	**DPH2**	n.d.	n.d.	-3.45	n.d.	Protein required for synthesis of diphthamide, which is a modified histidine residue of Eft1p or Eft2p	Peptidyl-diphthamide biosynthesis from peptidyl-histidine
YLR172C	**DPH5**	n.d.	n.d.	-2.07	-3.19	Diphthamide biosynthesis	Peptidyl-diphthamide biosynthesis from peptidyl-histidine
YGL195W	**GCN1**	-2.61	n.d.	n.d.	-3.24	Translational activator of GCN4 through activation of GCN2 in response to starvation	Regulation of translational elongation
YEL034W	**HYP2**	n.d.	-3.87	n.d.	n.d.	Translation initiation factor eIF-5A	Protein synthesis initiation
YGL099W	**LSG1**	n.d.	n.d.	n.d.	-4.33	Putative GTPase involved in 60S ribosomal subunit biogenesis	Ribosome export from nucleus
YCR024C	**PMP1**	-3.07	n.d.	n.d.	n.d.	Asparagine-tRNA ligase	Protein biosynthesis
YHR141C	**RPL41B**	n.d.	n.d.	n.d.	-4.22	Ribosomal protein L42B (YL27) (L41B) (YP44)	Protein biosynthesis
YDL184C	**RPL47A**	n.d.	n.d.	n.d.	-5.09	Ribosomal protein L41A (YL41) (L47A)	Protein biosynthesis
YKR057W	**RPS25A**	n.d.	n.d.	n.d.	-3.72	Ribosomal protein S21A (S26A) (YS25)	Protein biosynthesis
YKL156W	**RPS27A**	n.d.	n.d.	n.d.	-3.44	Ribosomal protein S27A (rp61) (YS20)	Protein biosynthesis
YGR027C	**RPS31A**	n.d.	n.d.	n.d.	-3.06	Ribosomal protein S25A (S31A) (rp45) (YS23)	Protein biosynthesis
YLR333C	**RPS31B**	n.d.	n.d.	n.d.	-3.77	Ribosomal protein S25B (S31B) (rp45) (YS23)	Protein biosynthesis
YLR264W	**RPS33B**	n.d.	n.d.	n.d.	-4.56	Ribosomal protein S28B (S33B) (YS27)	Protein biosynthesis
YJR007W	**SUI2**	-2.06	n.d.	n.d.	-3.90	Translation initiation factor eIF-2 alpha subunit	Protein synthesis initiation
YIL052C		n.d.	n.d.	-2.11	-3.18	Ribosomal protein L34B	Protein biosynthesis
Regulation of transcription, RNA metabolism and processing (6 ORFs)							
YER045C	**ACA1**	-2.34	-3.11	-2.66	n.d.	Basic leucine zipper (bZIP) transcription factor of the ATF/CREB family	Transcription initiation from RNA polymerase II promoter
YOR046C	**DBP5**	n.d.	n.d.	n.d.	-3.09	RNA helicase	mRNA-nucleus export
YEL015W	**EDC3**	n.d.	n.d.	-2.20	-3.06	Plays a role in mRNA decapping by specifically affecting the function of Dcp1p	Deadenylylation-independent decapping
YMR129W	**POM152**	n.d.	n.d.	n.d.	-3.34	Membrane glycoprotein, nuclear pore complex subunit	mRNA-nucleus export
YOR294W	**RRS1**	n.d.	n.d.	n.d.	-6.64	Regulator for ribosome synthesis	Ribosome biogenesis

Down-regulated ORFs	Gene short name	F1 0.0125% 30 min	YPD 0.0125% 30 min	F1 0.0125% 1h	YPD 0.0125% 1h	Molecular function	Biological process
YIL143C	**SSL2**	n.d.	n.d.	n.d.	-3.79	DNA helicase, human XPBC, ERCC3 homolog	Transcription initiation from Pol II promoter
Cell wall biosynthesis and organisation (5 ORFs)							
YLR342W	**FKS1**	n.d.	n.d.	n.d.	-3.19	1,3-beta-D-glucan synthase	Cell wall organization and biogenesis
YDL049C	**KNH1**	n.d.	n.d.	n.d.	-3.69	Protein with similarity to Kre9p, which is involved in cell wall beta 1,6-glucan synthesis	Beta-1,6 glucan biosynthesis
YLR332W	**MID2**	n.d.	n.d.	n.d.	-3.33	O-glycosylated plasma membrane protein that acts as a sensor for cell wall integrity signaling and activates the pathway	Cell wall organization and biogenesis
YOR010C	**TIR2**	-3.26	n.d.	n.d.	n.d.	Putative cell wall mannoprotein of the Srp1p/Tip1p family of serine-alanine-rich proteins	Cell wall organization and biogenesis
YHL028W	**WSC4**	n.d.	n.d.	-2.35	-7.59	Integral membrane protein (putative)|similar to SLG1 (WSC1), WSC2 and WSC3	Cell wall organization and biogenesis
Protein folding, degradation and translocation (4 ORFs)							
YDL143W	**CCT4**	n.d.	n.d.	n.d.	-4.00	Cytoplasmic chaperonin subunit	Protein folding
YOR020C	**HSP10**	n.d.	n.d.	n.d.	-3.03	Heat shock protein 10	Protein folding
YJR143C	**PMT4**	-3.05	-2.32	n.d.	n.d.	Dolichyl phosphate-D-mannose:protein O-D-mannosyltransferase	O-linked glycosylation
YLL039C	**UBI4**	n.d.	n.d.	n.d.	-5.99	Ubiquitin, becomes conjugated to proteins	Stress response
Membrane transport (1 ORF)							
YGL255W	**ZRT1**	-3.45	n.d.	n.d.	n.d.	High-affinity zinc transport protein	High-affinity zinc ion transport
Protein sorting (7 ORFs)							
YLR078C	**BOS1**	n.d.	n.d.	n.d.	-3.29	necessary for vesicular transport from the ER to the Golgi complex	ER to Golgi transport
YLR093C	**NYV1**	n.d.	n.d.	n.d.	-3.51	Vacuolar v-SNARE	Non-selective vesicle fusion
YLR026C	**SED5**	n.d.	n.d.	n.d.	-5.17	Sed5p is a t-SNARE (soluble NSF attachment protein receptor) required in ER to Golgi transport.	ER to Golgi transport
YGL104C	**VPS73**	n.d.	n.d.	n.d.	-3.52	Mitochondrial protein of unknown function involved in vacuolar protein sorting	Protein targeting to vacuole
YER072W	**VTC1**	n.d.	n.d.	n.d.	-3.11	Involved in distributing V-ATPase and other membrane proteins	Vacuole fusion (non-autophagic)
YHR161C	**YAP1801**	n.d.	n.d.	n.d.	-3.42	Yeast Assembly Polypeptide, member of AP180 protein family	Endocytosis
YGL198W	**YIP4**	n.d.	n.d.	n.d.	-4.63	Protein that interacts with Rab GTPases; a possible role in vesicle-mediated transport	Vesicle-mediated transport
Stress response/detoxification (1 ORF)							
YDR032C	**PST2**	n.d.	n.d.	n.d.	-3.25	similarity to members of a family of flavodoxin-like proteins; induced by oxidative stress in a Yap1p dependent manner	Biological_process unknown
Chromatin arrangement, chromosomes maintenance (4 ORFs)							
YLR318W	**EST2**	n.d.	n.d.	n.d.	-3.14	Telomerase catalytic subunit	Telomere maintenance
YBR010W	**HHT1**	-3.14	n.d.	n.d.	n.d.	Histone H3 (HHT1 and HHT2 code for identical proteins)	Chromatin assembly/disassembly
YNL031C	**HHT2**	-3.76	n.d.	n.d.	n.d.	Histone H3 (HHT1 and HHT2 code for identical proteins)	Hhromatin assembly/disassembly
YLR357W	**RSC2**	n.d.	n.d.	n.d.	-3.36	RSC complex member	Chromatin modeling

Down-regulated ORFs	Gene short name	F1 0.0125% 30 min	YPD 0.0125% 30 min	F1 0.0125% 1h	YPD 0.0125% 1h	Molecular function	Biological process
Cell cycle (2 ORFs)							
YDL132W	**CDC53**	n.d.	n.d.	n.d.	-4.97	Acts together with Cdc4p and Cdc34p to control the G1-S phase transition, assists in mediating the proteolysis of the Cdk inhibitor Sic1p in late G1	G1/S and G2/M transition of mitotic cell cycle
YGR238C	**KEL2**	n.d.	n.d.	-2.18	-6.42	Negatively regulate mitotic exit, interacts with Tem1p and Lte1p	Negative regulation of exit from mitosis
Mitochondrial biogenesis, maintance and aerobic respiration (4 ORFs)							
YHR051W	**COX6**	n.d.	n.d.	n.d.	-3.48	Cytochrome c oxidase subunit	Mitochondrial electron transport, cytochrome c to oxygen
YJR048W	**CYC1**	n.d.	n.d.	-2.51	-3.87	Iso-1-cytochrome c	Oxidative phosphorylation
YGR028W	**MSP1**	n.d.	n.d.	n.d.	-4.77	40 kDa membrane-spanning ATPase	Mitochondrial translocation
YHR050W	**SMF2**	n.d.	n.d.	n.d.	-3.41	Suppressor of Mitochondria import Function, divalent metal ion transporter involved in manganese homeostasis	Manganese ion transport
Others (15 ORFs)							
YOR198C	**BFR1**	n.d.	n.d.	n.d.	-3.54	Multicopy suppressor of BFA (Brefeldin A)-induced lethality; implicated in secretion and nuclear segregation	Meiosis
YHR055C	**CUP1B**	-2.57	n.d.	n.d.	-3.20	Copper binding metallothionein	Copper sensitivity/resistance
YLR206W	**ENT2**	n.d.	n.d.	-2.63	n.d.	Epsin-like protein required for endocytosis and actin patch assembly and functionally redundant with Ent1p	Actin filament organization
YDR437W	**GPI19**	-2.32	-3.41	n.d.	n.d.	Subunit of GPI-GlcNAc transferase involved in synthesis of N-acetylglucosaminyl phosphatidylinositol (GlcNAc-PI)	GPI anchor biosynthesis
YDL227C	**HO**	n.d.	n.d.	n.d.	-4.00	Homothallic switching endonuclease	Mating-type switching/recombination
YDR017C	**KCS1**	n.d.	n.d.	n.d.	-4.82	Inositol hexaphosphate kinase, phosphorylates inositol hexakisphosphate (InsP6) to diphosphoinositol polyphosphates	Vacuole organization and biogenesis
YGL197W	**MDS3**	n.d.	n.d.	n.d.	-5.30	Mck1 Dosage Suppressor 3; negative regulator of early meiotic gene expression	Meiosis
YGL211W	**NCS6**	n.d.	n.d.	n.d.	-3.14	Protein with a role in urmylation and in invasive and pseudohyphal growth	Biological_process unknown
YER009W	**NTF2**	n.d.	n.d.	n.d.	-3.58	Nuclear transport factor, similar to mammalian cytosolic nuclear import factor NTF2	Protein-nucleus import
YDL090C	**RAM1**	n.d.	n.d.	n.d.	-3.40	Farnesyltransferase beta subunit	Protein amino acid farnesylation
YHL024W	**RIM4**	n.d.	n.d.	n.d.	-3.36	Putative RNA-binding protein required for the expression of early and middle sporulation genes	Meiosis
YPR007C	**SPO69**	n.d.	n.d.	n.d.	-3.05	Meiosis-specific component of sister chromatid cohesion complex	Meiosis
YER046W	**SPO73**	-3.68	n.d.	n.d.	n.d.	Meiosis-specific protein of unknown function, required for spore wall formation during sporulation	Spore wall assembly
YOL154W	**ZPS1**	n.d.	n.d.	-5.05	n.d.	Putative GPI-anchored protein; transcription is induced under low-zinc conditions, as mediated by the Zap1p transcription factor, and at alkaline pH	Biological_process unknown
YGL160W		-3.52	n.d.	n.d.	n.d.	Protein with sequence similarity to iron/copper reductases (FRE1-8), possibly involved in iron homeostasis	Biological_process unknown

The results obtained from transcriptional profiling after treatment with very low doses of MMS revealed a possible important role of metabolic adaptation in response to DNA damage and oxidative stress conditions. Expression of genes encoding enzymes involved in the glycolysis/gluconeogenesis (reactions between ethanol and pyruvate, and between pyruvate and glucose-6-phosphate) was similarly induced in YPD medium after 1h of incubation with both concentrations of MMS (Fig.9). Fold-induction by two MMS concentrations is compared in Table 7.

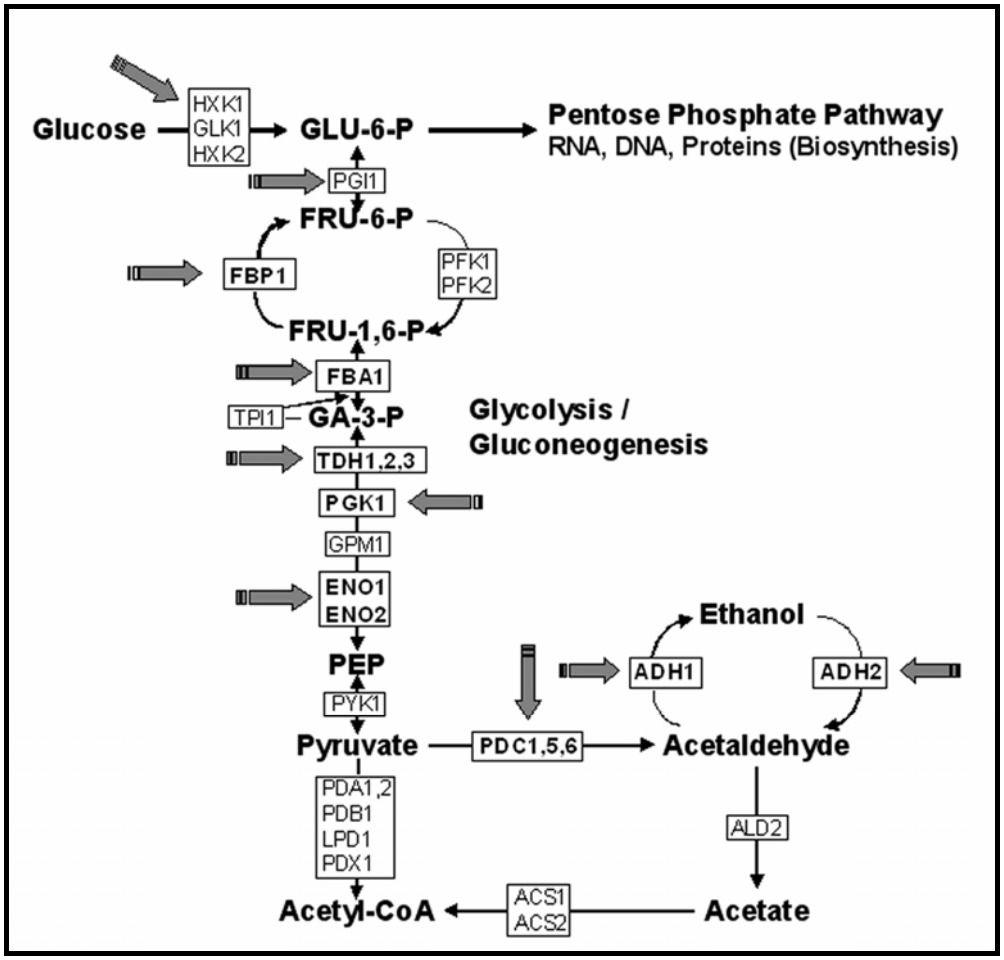

Figure 9. **Genes of the glycolysis/gluconeogenesis pathway induced by MMS.** Outline of the glycolysis/gluconeogenesis pathway. Genes significantly induced after 1 and 4h of MMS treatment are marked with arrows.

Dumond *et al.* (2000) demonstrated a link between oxidative stress and carbohydrate metabolism. They showed that carbohydrate metabolism is up-regulated by Yap1p and

concluded that under oxidative stress preferential utilization of the pentose phosphate pathway leads to a more efficient generation of the redox reaction cofactor NADPH. To check if a shift towards gluconeogenesis occurred after MMS treatment, we analyzed the expression of the key enzyme of gluconeogenesis fructose-1,6-bisphosphatase (*FBP1*), although its expression and regulation was not detectable on the gene expression array. The more sensitive RT-PCR showed a clear induction for *FBP1* after 30 min and 1h in F1 medium and - albeit much weaker - after 1h in YPD with both concentration of MMS (Fig.10). Together with the earlier notion that genes of the carbohydrate metabolism are part of ESR in yeast (Godon *et al.*, 1998; Dumond *et al.*, 2000; Gasch *et al.*, 2000), our result indicates that under oxidative stress and DNA damage caused by MMS, the carbohydrate metabolism is shifted towards an "anti-oxidative-stress" condition, requiring a slight adjustment of balance between glycolisis/gluconeogenesis, energy storage and the pentose phosphate pathway.

Gene/ORF	1h 0.00125%	1h 0.0125%
HXK1	2.3	2.4
PGI1	3.7	4.8
FBA1	4.0	4.3
TDH1	3.5	6.4
TDH2	0.0	2.5
TDH3	4.9	4.3
PGK1	11.4	25.3
ENO1	6.1	9.7
ENO2	2.4	3.2
PDC1	0.0	2.8
PDC5	6.2	6.9
PDC6	3.0	3.0
ADH1	4.5	5.6
ADH2	11.1	17.9

Table 7 Fold induction of genes of the glycolysis/gluconeogenesis pathway induced after 1h treatment with 0.00125% and 0.0125% MMS in YPD medium.

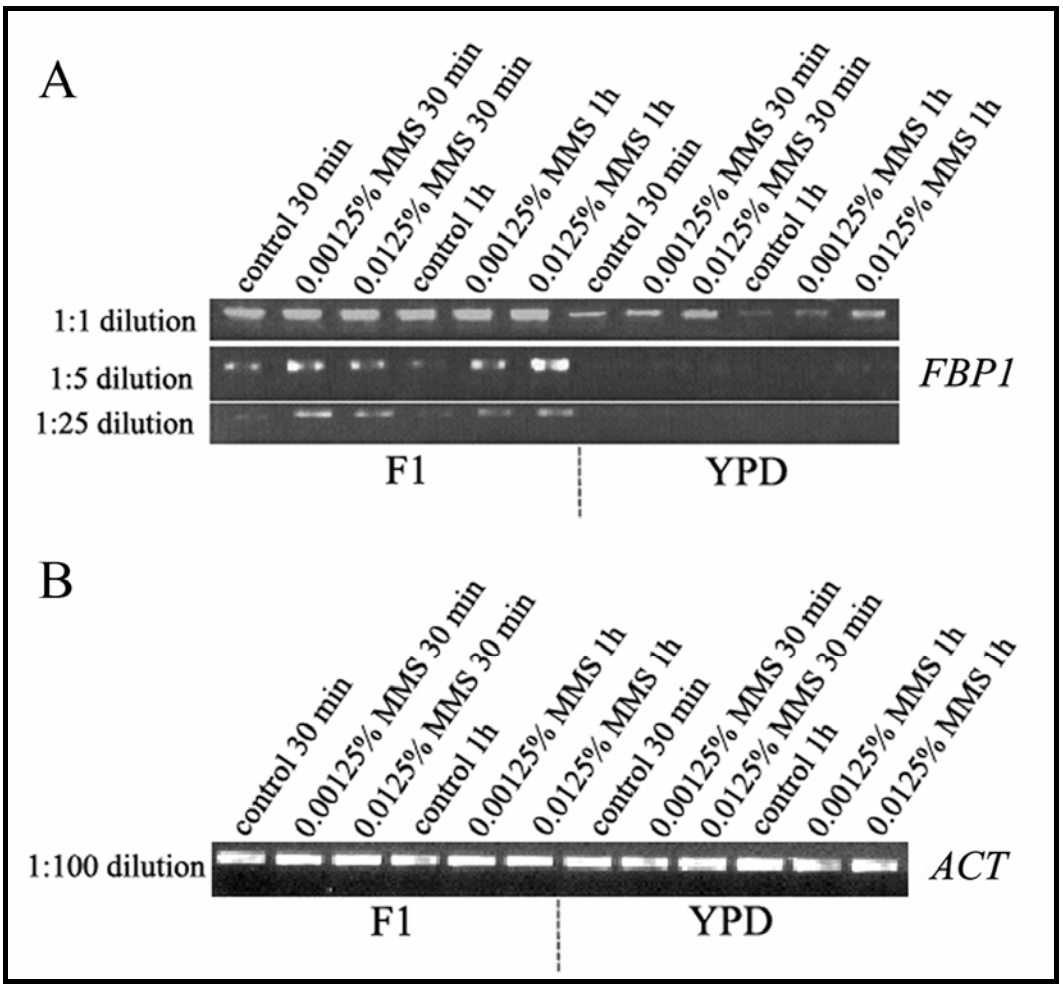

Figure 10. **Induction of *FBP1* expression upon MMS treatment.** (A) Expression of *FBP1* in wild-type after treatment with MMS in minimal (F1) and full medium (YPD). RT-PCR from 5-fold serial dilutions of cDNA preparations was performed and *FBP1* was amplified for 30 cycles. (B) RT-PCR expression analysis of *ACT1* amplified for 25 cycles.

The absence of transcriptional induction of genes involved in carbohydrate metabolism by MMS in cells grown in F1 medium could be a consequence of an already high basal expression of these genes in the cells. The same explanation can stand for the induction of different genes from the same functional group in F1 and YPD media. To confirm these data, we performed RT-PCR analysis of the stress-induced genes *YAP1* and *YCF1*. *YAP1* and *YCF1* are characterized as oxidative stress responsive genes (Gounalaki and Thireos, 1994; Jungwirth *et al.*, 2000). In all cases RT-PCR results showed a significantly higher basal expression in cells grown in F1 medium which was only slightly increased upon induction by

MMS. In YPD these genes were lower expressed and clearly induced already after 30 min by both MMS concentrations (Fig.11). The same effect is visible for *FBP1* discussed above (Fig.10). This observation correlates with hybridization data for other members of the heat shock and stress response gene families, membrane transporters (*HSC*, *HSP*, *SSA* and *SSB;* Fig.12) and genes involved in the carbohydrate metabolism, which also showed elevated basal expression in cells grown in minimal medium.

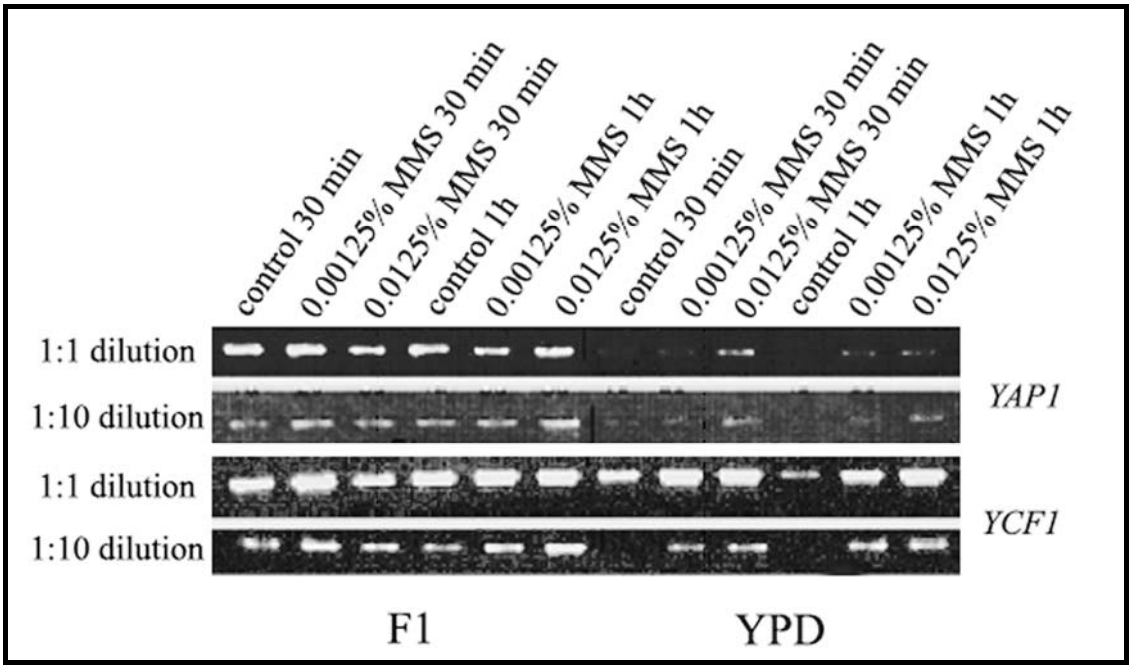

Figure 11. Elevated basal expression of ESR genes in F1 medium. Comparative RT-PCR for *YAP1* and *YCF1*, which is regulated by Yap1p, was performed from 10-fold serial dilutions of cDNA. For both genes a 30-cycle PCR protocol was used. Corresponding control RT-PCR for *ACT1* is presented in Fig. 10B.

Therefore, although the cultivation in F1 did not induce changes in cell morphology and growth rate (data not shown), the limited amount of nutrients produced stress conditions, which are reflected in the expression profiles. In given conditions metabolic reorganization is not necessary, as well as the induction of many genes involved in detoxification and maintenance of cellular homeostasis. In this case cells can further activate more specific and stronger transcriptional response to MMS.

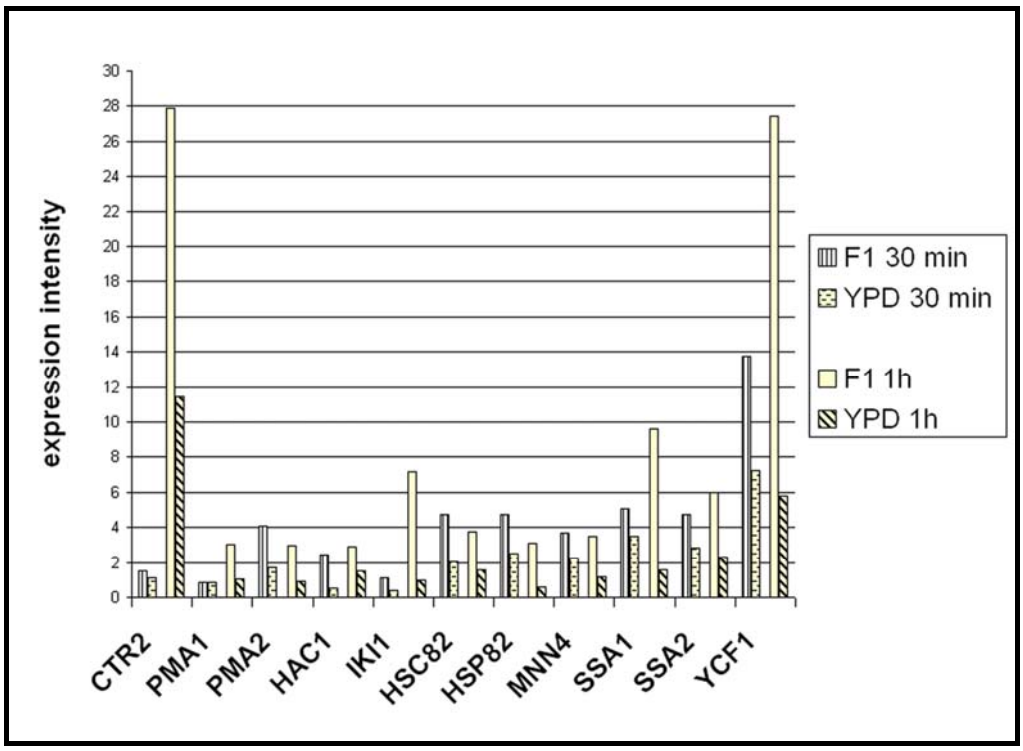

Figure 12. Elevated basal expression of ESR genes in F1 medium. Expression level of some ESR genes in non-treated YPD or F1 cultures. The data represent hybridisation signal intensity obtained from the DNA arrays.

3.3. Differences in transcriptional profiles after low level MMS treatment between strains

The transcription profile of cells grown in media with different nutrient availability showed that in F1 medium MMS triggers the expression of more specific groups involved in defense against this drug. In YPD medium the adjustment in the basic cellular processes like metabolic pathways, proteins biosynthesis or regulation of internal homeostasis is a prerequisite for a further specific response. By analyzing changes in the transcriptional profile of BY4742 in response to a 1h treatment with 0.0125% MMS we wanted to see how strain background influences this basic cellular response.

Selecting for genes regulated at least 3 fold in BY4742 we found 44 up-regulated genes and 11 down-regulated genes (Table 8, Table 9). Only 2 ORF2 were also induced in the FF18984 strain: *RNR2* involved in DNA damage repair and *SHM1* involved in amino acids metabolism. In the group of down-regulated genes there were no similarly regulated genes as

in the FF18984 strain. Although some of the functional groups regulated in the FF18984 strain are also present in the response of the BY4742 strain, many of them are represented with only one to tree genes. This is especially the case for the groups of carbohydrate metabolism and fermentation, amino acid metabolism, protein biosynthesis, regulation of transcription, RNA metabolism and processing, as well as for membrane transporters. All of these groups are strongly induced in the FF18984 strain, in which they are represented with more than tree ORFs. Notably up-regulated in this experiment were genes involved in protein folding, degradation and sorting which implicates high protein damage caused by applied MMS concentrations. The group of genes involved in chromatin rearrangement and chromosomes maintenance was also strongly up-regulated and presented with four ORFs all required for maintaining the chromosome stability. The groups of stress response, cell cycle regulation, mitochondrial biogenesis and aerobic respiration were also not included in the response of the BY4742 strain with significant number of genes. Two genes were highly up-regulated exclusively in this strain, DNA damage-induced *RAD9* required for G2 arrest in mitosis and glutathione reductase *TRR1*. Both of them are hallmarks of DNA damage and oxidative stress response. What could be even more important is that the transcription response of the BY4742 strain did not include significant down-regulation of any specific functional groups. Transcription of only 11 ORFs was down-regulated for more than 3-fold, but no functional group was represented with more than 2 ORFs (Table 9). These results implicate that the higher sensitivity of the BY4742 strain to MMS could be a result of inefficient induction of genes involved in the protection of critical aspects of the internal milieu, like energy conservation, cell wall integrity or export of drugs from the cells.

Table 8. ORFs whose transcripts are induced in BY4742 strain by 3-fold by 0.0125% MMS in F1 and/or YPD media (n=44)

Up-regulated ORFs	Gene short name	YPD 0.0125% 1h	Molecular function	Biological process
Carbohydrate metabolism and fermentation, pentose-phosphate shunt (3 ORFs)				
YBR149W	**ARA1**	3.53	D-arabinose dehydrogenase	Carbohydrate metabolism
YGR256W	**GND2**	3.33	6-phosphogluconate dehydrogenase (decarboxylating), catalyzes an NADPH regenerating reaction in the pentose phosphate pathway	Glucose metabolism
YOL136C	**PFK27**	4.25	6-phosphofructo-2-kinase, has negligible fructose-2,6-bisphosphatase activity	Regulation of glycolysis
DNA synthesis/repair (3 ORFs)				
YDR408C	**ADE8**	6.15	Phosphoribosyl-glycinamide transformylase, catalyzes a step in the 'de novo' purine nucleotide biosynthetic pathway	Purine nucleotide biosynthesis
YDR217C	**RAD9**	3.91	Required for DNA damage-induced G2 arrest in mitosis, required for ionizing radation-induced G1 arrest, and other cdc13-induced G2 arrest in meiosis	DNA repair
YJL026W	**RNR2**	5.53	Small subunit of ribonucleotide reductase	DNA replication
Signaling/kinases/phosphatases (1 ORF)				
YHR030C	**SLT2**	3.24	Serine/threonine MAP kinase involved in regulating the maintenance of cell wall integrity and progression through the cell cycle; regulated by the PKC1-mediated signaling pathway	Signal transduction
Amino acid metabolism (2 ORFs)				
YDR037W	**KRS1**	3.79	Lysyl-tRNA synthetase; also identified as a negative regulator of general control of amino acid biosynthesis	Lysyl-tRNA aminoacylation
YBR263W	**SHM1**	3.76	Serine hydroxymethyltransferase, mitochondrial	One-carbon compound metabolism
Protein biosynthesis (1 ORF)				
YPR163C	**TIF3**	3.15	Translation initiation factor eIF-4B	Protein synthesis initiation
Regulation of transcription, RNA metabolism and processing (3 ORFs)				
YLR117C	**CLF1**	3.03	Essential splicesome assembly factor; contains multiple tetratricopeptide repeat (TPR) protein-binding motifs and interacts specifically with many spliceosome components, may serve as a scaffold during splicesome assembly	Nuclear mRNA splicing, via spliceosome
YLR266C	**PDR8**	3.11	Transcription factor; targets include ATP-binding cassette (ABC) transporters, major facilitator superfamily transporters, and other genes involved in the pleiotropic drug resistance	Positive regulation of transcription from RNA polymerase II promoter
YGR006W	**PRP18**	3.99	RNA splicing factor associated with U5 snRNP	mRNA splicing
Cell wall biosynthesis and organisation (3 ORFs)				
YOR382W	**FIT2**	3.34	Mannoprotein that is incorporated into the cell wall via a glycosylphosphatidylinositol (GPI) anchor, involved in the retention of siderophore-iron in the cell wall	Siderophore transport
YOR383C	**FIT3**	7.87	Mannoprotein that is incorporated into the cell wall via a glycosylphosphatidylinositol (GPI) anchor, involved in the retention of siderophore-iron in the cell wall	Cell wall protein involved in iron transport
YBR205W	**KTR3**	4.25	Putative alpha-1,2-mannosyltransferase	Cell wall organization and biogenesis
Protein folding, degradation and translocation (6 ORFs)				
YGL038C	**OCH1**	3.32	Initiates the polymannose outer chain elongation of N-linked glycans	N-linked glycoprotein maturation
YDR313C	**PIB1**	3.42	RING-type ubiquitin ligase of the endosomal and vacuolar membranes, binds phosphatidylinositol(3)-phosphate; contains a FYVE finger domain	Protein ubiquitination
YMR297W	**PRC1**	3.49	Vacuolar carboxypeptidase Y (proteinase C), involved in protein degradation in the vacuole and required for full protein degradation during sporulation	Vacuolar protein catabolism
YGL048C	**SUG1**	3.90	Member of the 26 S proteasome	Ubiquitin-dependent protein degradation
YEL002c	**WBP1**	3.09	Beta subunit of the oligosaccharyl transferase (OST) glycoprotein complex; required for N-linked glycosylation of proteins in the endoplasmic reticulum	Protein amino acid N-linked glycosylation
YBR075W		4.55	Putative metalloprotease	Proteolysis
Membrane transport (1 ORF)				
YKR050W	**TRK2**	3.11	Membrane protein; low affinity potassium transport	Potassium ion homeostasis
Protein sorting (5 ORFs)				
YGL002W	**ERP6**	3.49	Protein with similarity to Emp24p and Erv25p, member of the p24 family involved in ER to Golgi transport	Secretory pathway
YDL226C	**GCS1**	4.70	Zn-finger-containing protein that functions as ADP-ribosylation factor GTPase-activating protein and is involved in regulating vesicle transport	ER to Golgi transport
YDL100c	**GET3**	3.39	ATPase, subunit of the GET complex; required for the retrieval of HDEL proteins from the Golgi to the ER in an ERD2 dependent fashion	Retrograde transport, Golgi to ER

Up-regulated ORFs	Gene short name	F1 0.0125% 30 min	Molecular function	Biological process
YGL257C	**MNT2**	3.88	MaNnosylTransferase; involved in adding the 4th and 5th mannose residues of O-linked glycans	O-linked glycosylation
YJR033C	**RAV1**	3.22	Subunit of the RAVE complex (Rav1p, Rav2p, Skp1p), which promotes assembly of the V-ATPase holoenzyme	Early endosome to late endosome transport
Stress response/detoxification (1 ORF)				
YDR513W	**TTR1**	3.31	Glutaredoxin (thioltransferase) (glutathione reductase)	Oxidative stress response
Chromatin arrangement, chromosomes maintenance (4 ORFs)				
YDR254W	**CHL4**	3.27	Outer kinetochore protein required for chromosome stability, interacts with kinetochore proteins Ctf19p, Ctf3p, and Iml3p	Chromosome segregation
YBL002W	**HTB2**	5.53	Histone H2B (HTB1 and HTB2 code for nearly identical proteins)	Chromatin assembly/disassembly
YLR033W	**RSC58**	5.50	Remodels the structure of chromatin complex 58KDa subunit	Chromatin remodeling
YDR082w	**STN1**	3.27	Involved in telomere length regulation, function in telomere metabolism during late S phase	Telomere capping
Cell cycle (1 ORF)				
YKL203C	**TOR2**	4.48	Putative protein/phosphatidylinositol kinase involved in signaling activation of translation initiation, distribution of the actin cytoskeleton, and meiosis	Regulation of cell cycle
Mitochondrial biogenesis, maintenance and aerobic respiration (2 ORFs)				
YPR020W	**ATP20**	3.36	Protein associated with mitochondrial ATP Synthase; essential for dimeric state of ATP synthase	ATP synthesis coupled proton transport
YPL078C	**ATP4**	3.52	ATP synthase F0 sector subunit 4; analogous to the bovine b subunit	ATP synthesis coupled proton transport
Others (8 ORFs)				
YER026c	**CHO1**	3.02	Phosphatidylserine synthase	Phosphatidylserine biosynthesis
YJL196C	**ELO1**	3.42	Elongation enzyme 1, required for the elongation of the saturated fatty acid tetradecanoic acid (14:0) to that of hexadecanoic acid (16:0)	Fatty acid metabolism
YDR174W	**HMO1**	7.44	High mobility group (HMG)-like protein	Plasmid maintenance
YDR142C	**PEX7**	3.04	Peroxisomal signal receptor for the N-terminal nonapeptide signal (PTS2) of peroxisomal matrix proteins	Peroxisome organization and biogenesis
YLR084C	**RAX2**	3.25	Involved in the maintenance of bipolar pattern	Maintenance of cell polarity (sensu Saccharomyces)
YIL016W	**SNL1**	3.07	Protein of unknown function proposed to be involved in nuclear pore complex biogenesis and maintenance as well as protein folding	Nuclear pore organization and biogenesis
YER024w	**YAT2**	3.20	The Yat2p protein shows significant homology with the known carnitine acetyltransferase associated with the outer-mitochondrial membrane, Yat1p, and also functions as a carnitine acetyltransferase.	Carnitine metabolism
YDL124w		3.07	NADPH-dependent alpha-keto amide reductase; reduces aromatic alpha-keto amides, aliphatic alpha-keto esters, and aromatic alpha-keto esters	Metabolism

Table 9. ORFs whose transcripts are repressed in BY4742 strain by 3-fold by 0.0125% MMS in F1 and/or YPD media (n=11)

Down-regulated ORFs	Gene short name	YPD 0.0125% 1h	Molecular function	Biological process
Signaling/kinases/phosphatases (1 ORF)				
YNR031C	**SSK2**	-4.89	MAP kinase kinase kinase of the HOG1 mitogen-activated signaling pathway	Protein amino acid phosphorylation
Amino acid metabolism (1 ORF)				
YOR108W	**LEU9**	-3.06	Alpha-isopropylmalate synthase II (2-isopropylmalate synthase), catalyzes the first step in the leucine biosynthesis pathway	Lleucine biosynthesis
Protein biosynthesis (2 ORFs)				
YPL179W	**PPQ1**	-4.75	Putative protein serine/threonine phosphatase; null mutation enhances efficiency of translational suppressors	Regulation of translation
YNL178W	**RPS3**	-4.76	Ribosomal protein S3 (rp13) (YS3)	Protein biosynthesis
Cell wall biosynthesis and organisation (1 ORF)				
YMR307W	**GAS1**	-4.40	Beta-1.3-glucanosyltransferase, required for cell wall assembly	Cell wall organization and biogenesis
Protein folding, degradation and translocation (1 ORF)				
YIL046W	**MET30**	-3.15	F-box protein involved in sulfur metabolism and protein ubiquitination	Ubiquitin-dependent protein degradation
Membrane transport (2 ORFs)				
YHR175W	**CTR2**	-4.72	Putative low-affinity copper transport protein	Transport
YGL008C	**PMA1**	-3.05	Major regulator of cytoplasmic pH	Regulation of pH

Down-regulated ORFs	Gene short name	YPD 0.0125% 1h	Molecular function	Biological process
Chromatin arrangement, chromosomes maintenance (1 ORF)				
YKL049C	**CSE4**	-6.99	Centromere protein that resembles histones, required for proper kinetochore function	Mitotic sister chromatid segregation
Others (2 ORFs)				
YCL050c	**APA1**	-3.10	Diadenosine 5',5'''-P1,P4-tetraphosphate phosphorylase I	Nucleotide metabolism
YOR190W	**SPR1**	-3.72	Glucan 1,3-beta-glucosidase	Sporulation

3.4. Induction of gluconeogenesis and oxidative metabolism has a direct influence on DNA damage response

Taking all aforementioned results into consideration, sensitivity to MMS seems to be dependent on the ability of the cells to induce genes of the ESR and the carbohydrate metabolism. Many of these genes are higher expressed when cells are grown in minimal medium. To check if pre-induction of these genes would decrease sensitivity to MMS we performed survival tests under MMS after pre-incubation in three media: full medium (YPD), synthetic minimal medium (SD), and oxidative metabolism induced medium (YPKG). Both strains, FF18984 and BY4742, were incubated in parallel in these media and aliquots from log phase, early stationary phase (1 day culture) and six days old cultures were spotted in serial dilutions on YPD agar plates with 0.018%-0.0225% MMS. The optical density of all cultures measured after 4h, 1 day and 6 days of cultivation showed no differences between the two strains cultivated in the same medium. The highest optical density was reached in YPD, growth was slower in YPKG and slowest in SD (Fig.13). These results correlate with the availability of nutrients.

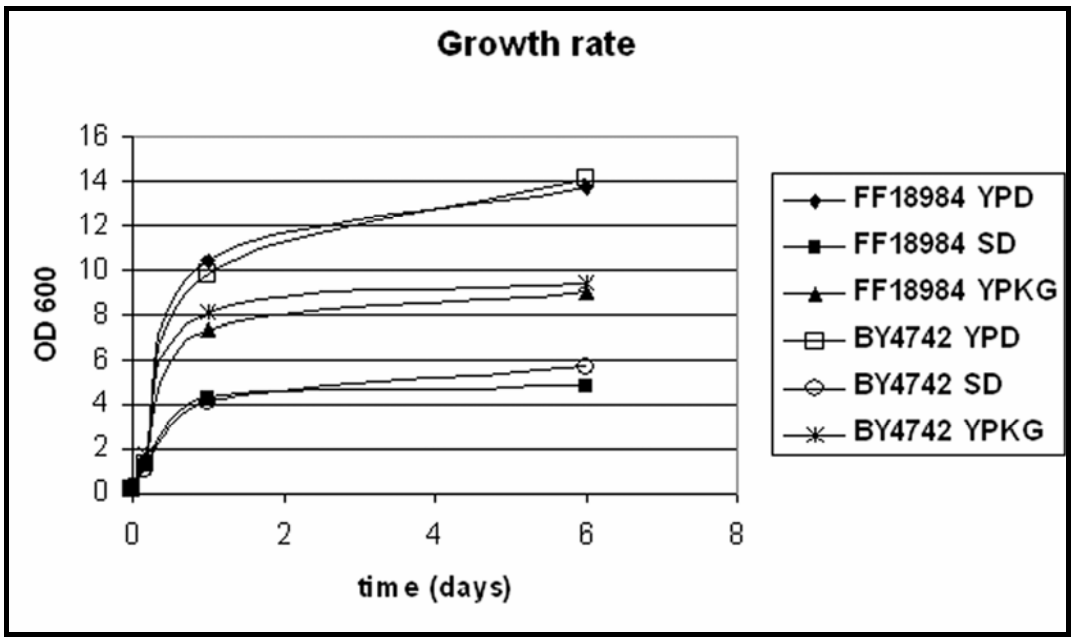

Figure 13. Growth rate of FF18984 and BY4742 strains cultivated in different media. Pre-cultures were grown overnight in full (YPD), minimal (SD) or respiratory induced (YPKG) medium. Cultures were prepared in the same medium by inoculation with pre-cultures, and grown for 6 days. Optical density of the cultures (OD_{600}) was measured after 4h, 24h and 6 days.

After being spotted on MMS plates the BY4742 cells were again more sensitive to MMS than the FF18984 cells. Cells from both strains taken from 1 day or 6 day stationary cultures were less sensitive to MMS than cells taken from the log phase (Fig.14). Although the difference was more striking for BY4742, this increase is also visible for the FF18984 cells. This shows that replicating cells are more sensitive to DNA damage than cells in stationary phase that ceased dividing and already induced ESR. Interestingly, for both strains, cells cultivated in SD or YPKG media were less sensitive to MMS compared with the cells cultivated in YPD. Cells cultivated in YPKG medium, in which genes involved in gluconeogenesis and oxidative metabolism were highly expressed, showed the lowest sensitivity to MMS. This effect is better visible in young cells from mid-log phase and again more striking in BY4742. It also should be noted, that the FF18984 strain showed reduced viability when pre-cultured in SD for six days.

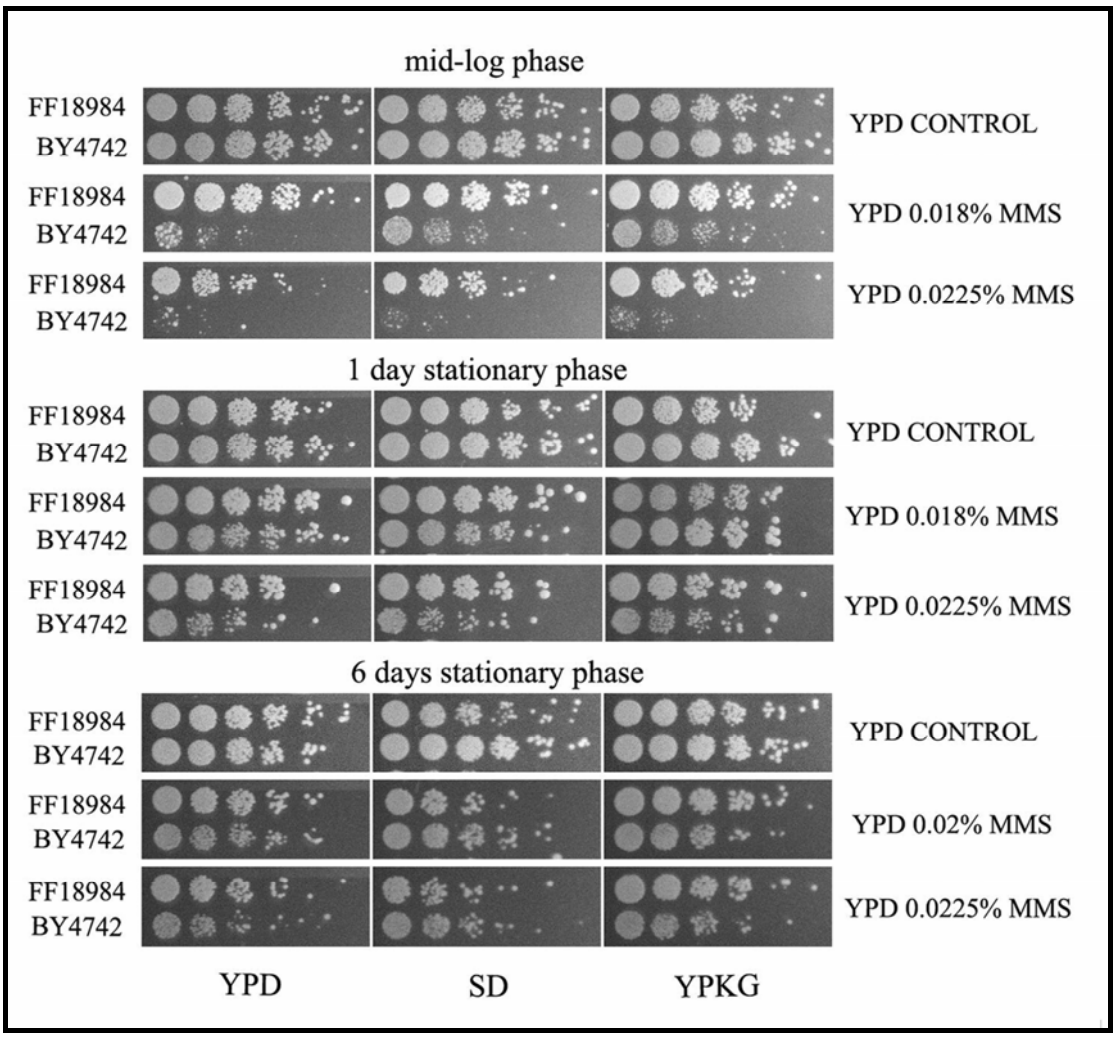

Figure 14. **MMS sensitivity of cells pre-cultivated in different media**. Pre-cultures were grown overnight in full (YPD), minimal (SD) or respiratory induced (YPKG) medium. Cultures were prepared in the same medium by inoculation with pre-cultures, and grown for 6 days. Aliquots of cultures were taken after 4h, 24h and 6 days. Serial 5-fold dilutions were prepared and spotted onto YPD, YPD + 0.018% MMS, YPD + 0.02% MMS or YPD + 0.0225% MMS and incubated for 48h at 30°C. Reduced growth at higher dilutions reflects higher sensitivity to MMS.

These results show that pre-induction of genes involved in ESR, gluconeogenesis and oxidative metabolism by changing the availability of nutrients decreases sensitivity to MMS and allows more cells to survive the treatment with the toxic agent.

3.5. Deletion of **FBP1** *influences sensitivity of* **S.cerevisiae** *to DNA damage caused by MMS*

The results obtained from expression profiling and sensitivity assays with cells grown in different media revealed that metabolic adaptation could play an important role in defence against DNA damage. To test how the deletion of some important regulators of oxidative metabolism and gluconeogenesis influence sensitivity of yeast cells to DNA damage, we constructed the following deletion mutants: *Δfbp1, Δhap4* and *Δmig1*. Fbp1 is the key regulator of gluconeogenesis and its transcription is known to be strongly repressed in the presence of glucose (Polakis and Bartley, 1965; Eraso and Gancedo, 1984). Hap4p enhances transcription of a large set of mitochondrial protein genes during transition from fermentative to non-fermentative metabolism (Lascaris *et al.*, 2002), while Mig1p is a transcription factor that negatively regulates transcription of genes involved in gluconeogenesis and energy generation. Mig1 binds to the promoter of *FBP1* and represses its transcription under high glucose conditions (Zaragoza *et al.*, 2001), although additional Mig1p-independent *FBP1* repression by glucose is described (Balciunas and Ronne, 1995). First we tested sensitivity of these strains to various DNA damaging agents: MMS, 4-nitroquinolin-N-oxide (4-NQO) and phleomycin and their vitality on non-fermentable carbon sources. The test was done by spotting serial 5-fold dilutions of cells from mid-log phase onto YPD plates containing different toxic substances (as indicated in the figures) or SD plates containing ethanol and glycerol (Fig.15). In addition, the *Δrad9* mutant was again used as a positive control to detect a substance specific genotoxic effect.

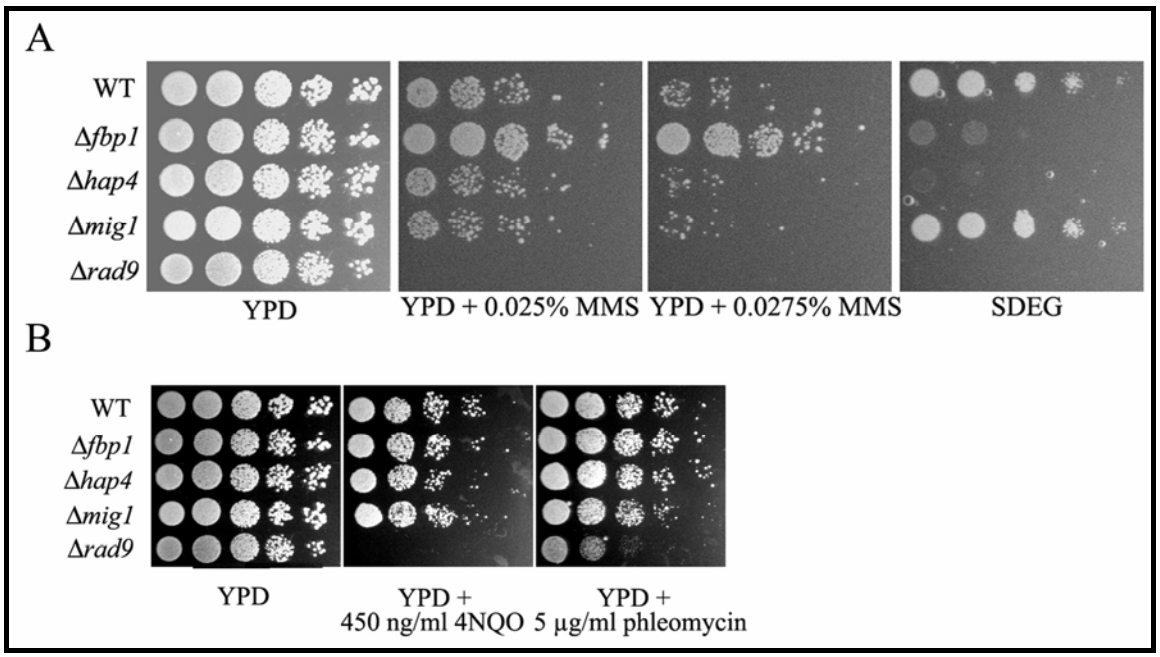

Figure 15. Deletion of *FBP1* influences cellular sensitivity to MMS, but does not have any effect on sensitivity to 4-NQO or phleomycin. (A) MMS sensitivity on full medium (YPD) and viability on non-fermentable carbon sources (SDEG). Wild-type strain (WT) and deletion strains *Δfbp1*, *Δhap4*, *Δmig1* and *Δrad9* were grown in YPD until mid-log phase at 30°C. 5-fold dilutions (starting OD_{600} = 0.5) were spotted onto plates with YPD, YPD + MMS or SDEG (EG indicates ethanol and glycerol) and incubated for 48h at 30°C. (B) Sensitivity of deletion strains to 4-NQO and phleomycin. Cells were grown and diluted as described above for Fig. 15A.

On non-fermentable carbon sources, both *Δfbp1* and *Δhap4* mutants as well as *Δrad9* mutant display impaired growth, while the growth of wild-type and *Δmig1* was not changed (Fig.15A). Low viability of the *Δrad9* mutant strain on non-fermentable carbon sources can be explained by inability of the strain to slow down proliferation and adapt to conditions with limited nutrients. Deletion of *FBP1* has already been characterised to yield *petite* or *pet* mutants (Dimmer *et al.*, 2002; Steinmetz *et al.*, 2002). The reason for such mutant phenotype is the fact that growth of yeast cells on non-carbohydrate substrates as sole carbon sources necessitates the synthesis of sugars which are required for macromolecular biosynthesis. As expected, *Δrad9* mutant cells did not survive any MMS or 4-NQO treatment and had a very low viability on plates with phleomycin (Fig.15A, 15B). In response to increasing MMS

concentrations, the *Δfbp1* mutant showed much lower sensitivity to MMS than the wild-type strain. However, in the treatment with the two other DNA-damaging agents, – 4-NQO and phleomycin, the *Δfbp1* mutant did not show a comparable reduction of sensitivity (Fig. 15A, 15B). 4-NQO is considered to be a UV-mimetic agent that produces single strand DNA breaks and pyrimidine dimers (Mirzayans *et al.*, 1999), while phleomycin reacts with deoxyribose to cleave phosphodiester bonds, generates 3´-phosphoglycolate and oxidizes AP sites what block DNA polymerase and cause cleavage of DNA leading to double strand DNA breaks (Bennett, 1999). Thus, reduced sensitivity to MMS in absence of functional Fbp1p appears to be specific for this genotoxic agent. Remarkably, *Δmig1* was more sensitive to MMS and only slightly more sensitive to phleomycin (Fig. 15A, 15B). Deletion of *HAP4* also caused higher sensitivity to the MMS (Fig. 15A) what is in consistence with previous reports that increased antioxidant status after respiratory adaptation contributes to an increased oxidative-stress tolerance (reviewed in Moradas-Ferreira *et al.*, 1996).

The reduced sensitivity of cells lacking Fbp1p to MMS could be a result of direct influences of mutation on cellular damage and accumulation of dead cells or an impact on the proliferation potential of damaged cells. To address this question we scored the number of viable cells and screened for colony formation ability after both short- and-long term treatment with MMS. MMS was used in the concentration of 0.03% that on plates caused no colony formation in wild-type and large number of colonies produced in the *Δfbp1* mutant. Viability staining of wild-type and the *Δfbp1* mutant cells was performed by fluorescein diacetate (FDA) and propidium iodide (PI) staining during a 48h time-course of MMS treatment. The results revealed that after a short-term treatment with 0.03% of MMS (after 2h, 4h of treatment) there are still more than 95% of metabolically active cells in both strains, while this number is reduced to app. 80% after long-term treatment (24h and 48h of treatment) (Fig.16). The ratio of dead versus viable cells was determined by flow cytometry

analysis. There were no differences in the amount of PI positive cells in wild-type and the *Δfbp1* cells over 48h of MMS treatment (data not shown).

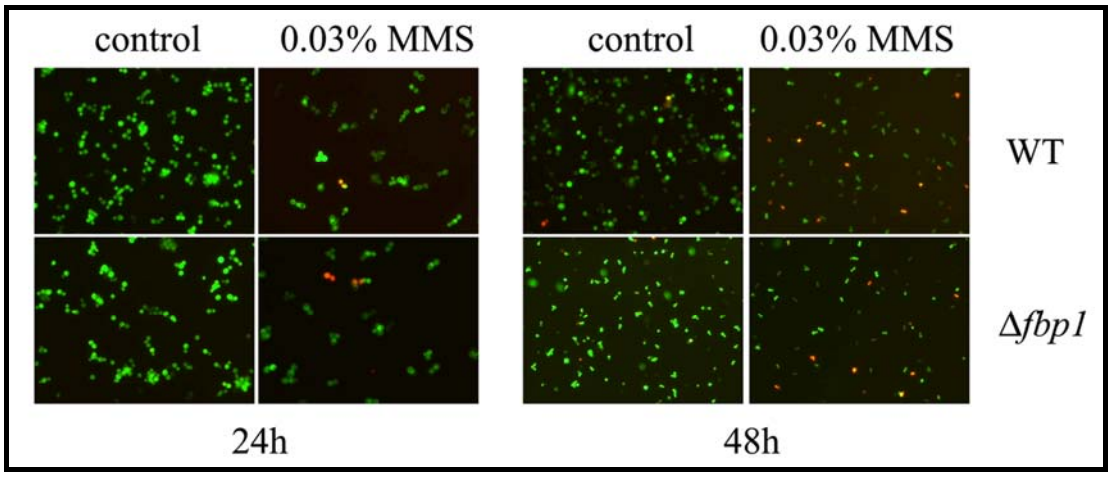

Figure 16. Cell viability after the long-term treatment with MMS. Wild-type (WT) and *Δfbp1* mutant cells were grown in YPD until mid-log phase. The cultures were divided, one part was used as control (untreated sample) and the other part was treated with 0.03% MMS for 24h or 48h. Samples from indicated time-points were stained in parallel with PI and FDA and cells were visualised with fluorescence microscope.

In contrast, when we analysed the ability to proliferate and form colonies we found remarkable differences between wild-type and the *Δfbp1* mutant. A 2h MMS treatment reduced the number of cells that were able to proliferate for approximately 43% in wild-type and 70% in the *Δfbp1* strain. (Fig.17). After 4h of treatment the number of wild-type colonies further reduced to only 14% of control one, while in *Δfbp1* further decrease was not observed (36% of proliferating cells). The fast decrease in the percentage of MMS treated cells able to form colonies is in consistence with previous results obtained after treatment with lower concentrations of this agent. The results show that treatment with MMS reduces cellular proliferation which probably gives enough time to cells to repair damage. Interestingly, after long term treatment with MMS (24h and 48h) we observed a significant recovery of the *Δfbp1* mutant, but not of wild-type. Also, the number of non-treated cells from the 24h culture that are able to form colonies was significantly higher in the *Δfbp1* mutant.

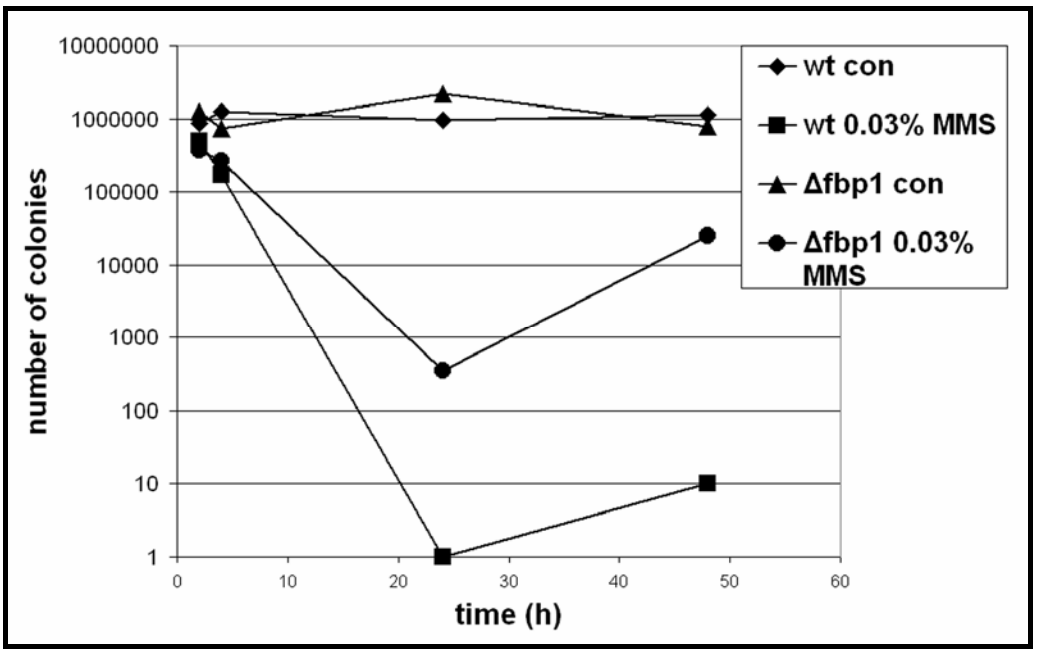

Figure 17. The *Δfbp1* mutant cells are able to proliferate after release from long-term treatment with MMS. Wild-type (wt) and *Δfbp1* mutant cells were grown in YPD until mid-log phase. The cultures were divided, one part was used as control (untreated sample; con) and the other part was treated with 0.03% MMS. Samples were taken at indicated time-points, plated in triplicate on YPD and incubated at 30°C. After 48h numbers of colonies were counted. The results are presented as logarithmic mean values ± SEM.

Therefore, deletion of *FBP1* seems to have impact on the ability of cells to recover after MMS treatment which could be a consequence of reduced toxic effect of this agent or more efficient repair mechanism.

3.6. Overproduction of Fbp1p has a toxic effect on cells

In order to prove that the observed results are caused by the absence of the *FBP1* gene, and not by secondary mutations in the deletion strain, we overexpressed *FBP1* in wild-type and in *Δfbp1*. All transformants were tested for viability on non-fermentable carbon sources (ethanol and glycerol) and for MMS-sensitivity (Fig.18).

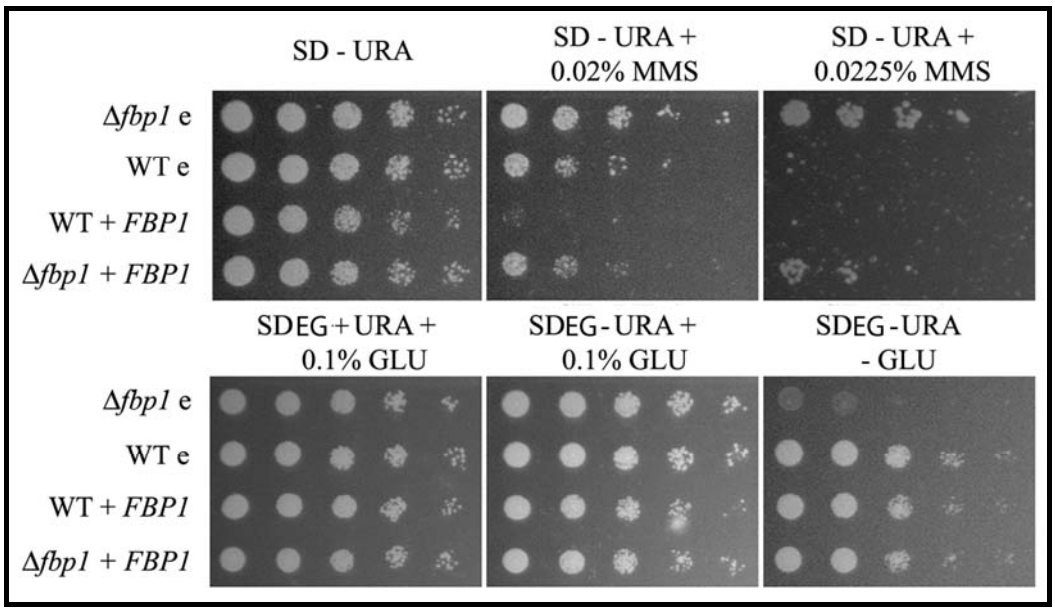

Figure 18. Sensitivity of strains overexpressing *FBP1* to MMS and their viability on selective medium (SD-URA) and on non-fermentable carbon sources (SDEG). Wild-type (WT) and *Δfbp1* were transformed with the empty plasmid pRS426 (e), or with the plasmid pRS426 containing the *FBP1* cassette under the control of the GPD promoter (+ FBP1). Cells were grown in SD-URA until mid-log phase at 30°C. 5-fold dilutions (starting OD_{600} = 0.5) were spotted onto indicated media and incubated for 48h at 30°C. SDEG +URA or SDEG –URA represent selective or non-selective ethanol/glycerol medium, correspondingly and +0.1% GLU or -GLU indicate plates with or without glucose.

Viability of the strains on non-fermentable carbon sources was tested under non-selective or plasmid selective conditions in presence or absence of limited amounts of glucose. Results confirmed that the *Δfbp1* mutant strain is not viable on medium without glucose while limited amounts of glucose (0.1%) are sufficient to support growth. Overexpression of *FBP1* completely restored the mutant to wild-type phenotype on non-fermentable carbon sources and restored wild-type sensitivity to MMS (Fig.18). In wild-type overexpression of *FBP1* increased sensitivity to MMS leading to lower viability of this strain. *FBP1* overexpression slowed down the growth rate resulting in production of smaller colonies in both, wild-type and *Δfbp1*. These results confirm that sensitivity to DNA-damage caused by MMS depends, at least in part, on *FBP1* expression. Also, the increased sensitivity of

wild-type cells overexpressing *FBP1* to MMS implicated that the high intracellular level of this protein could be actually toxic for the cells.

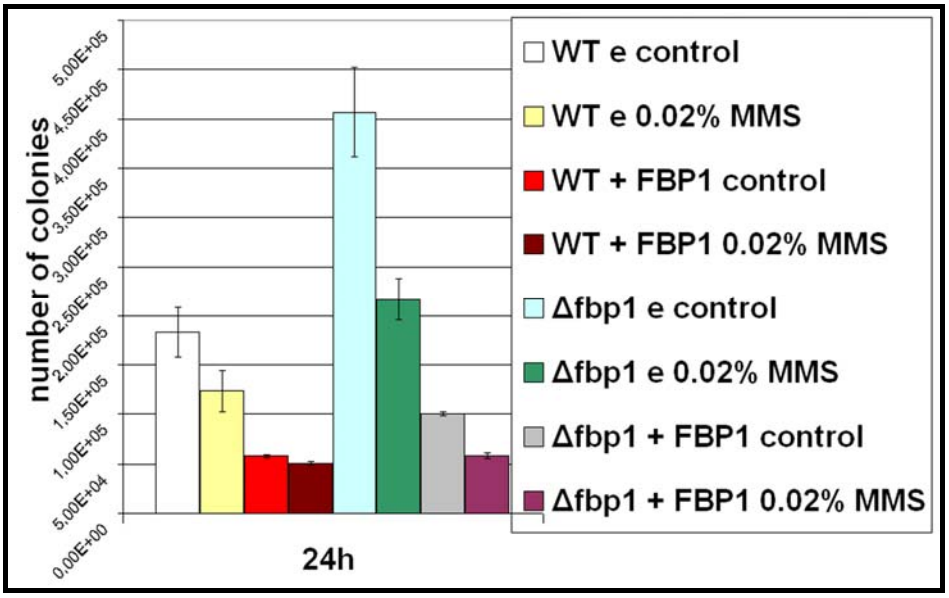

Figure 19. Effect of *FBP1* overexpression on cell sensitivity to MMS. Survival test of cells lacking or overexpressing *FBP1* after 24h treatment with 0.02% MMS. Cells carrying control plasmid (e) or plasmid overexpressing *FBP1* (+*FBP1*) were mock-treated (control) or treated with 0.02% MMS for 24h and plated in triplicate on SD-URA medium. Data represents mean value ± SEM.

Similar results were obtained when we analysed the ability of colony formation of wild-type and the *Δfbp1* mutant overexpressing *FBP1* after 2h and 24h of treatment with MMS. This time, we used a lower MMS concentration (0.02%) that in the survival test on solid medium with MMS showed only slight cytotoxicity (Fig.7). Results showed that in both wild-type and *Δfbp1* mutant cells the overexpression of *FBP1* reduced the amount of cells able to form colonies even in untreated cells (Fig.19). This effect was visible only in 24h old stationary phase cultures while in young cells from mid-log phase there was no effect of *FBP1* overexpression on cellular ability to form colonies on solid medium (data not shown). Interestingly, 24h non-treated stationary phase cultures of the *Δfbp1* mutant again showed that many more cells were able to continue proliferation and form colonies, although during exponential growth in full and minimal media, similar growth rate in both wild-type and

Δfbp1 strain were found (Fig.20). Total biomass yield after 24h of culturing was only slightly lower (88.3 % of wild-type density) for the *Δfbp1* strain. Therefore, better viability of mutant cells in stationary culture and more colonies produced can not be explained by faster growing of the *Δfbp1* strain.

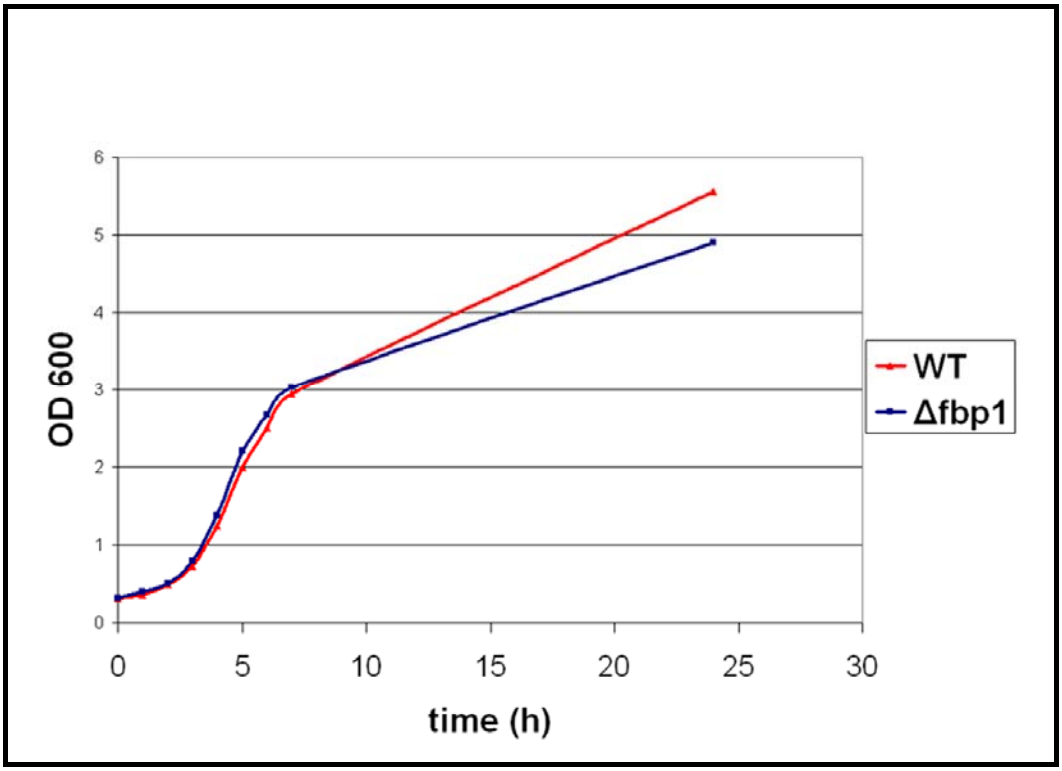

Figure 20. Growth curve of wild-type (WT) and *Δfbp1* mutant in YPD medium. Cultures were prepared in YPD medium by inoculation with pre-cultures, and grown for 24h. Optical density of the cultures (OD_{600}) was measured after 1h, 2h, 3h, 4h, 5h, 6h, 7h and 24h. Experiment was repeated three times showing similar results at every repetition.

When we transformed cells with *FBP1* fused with yEGFP to its C-terminus Fbp1-GFPp showed mostly a vacuolar localisation of the protein in control and MMS-treated cells in glucose rich conditions (Fig.21). This is in accordance with the observation that under glucose-rich conditions Fbp1p is transported from the cytosol to the vacuole for degradation (Shieh and Chiang, 1998). The test for growth on ethanol and glycerol without glucose proved that the C-terminally GFP-tagged Fbp1p restored the lack of Fbp1p in the *Δfbp1* strain. In contrast to this, it was not possible to restore wild-type MMS-sensitivity in *Δfbp1* cells with

this fusion protein, although slightly higher sensitivity of the *Δfbp1* cells overexpressing *FBP1-GFP* in comparison to the *Δfbp1* strain could be observed (Fig.22). This indicates that the function of FBPase in cellular growth on media with alternative carbon sources (e.g. ethanol and glycerol) could be independent, at least in part, from its role in response to MMS treatment.

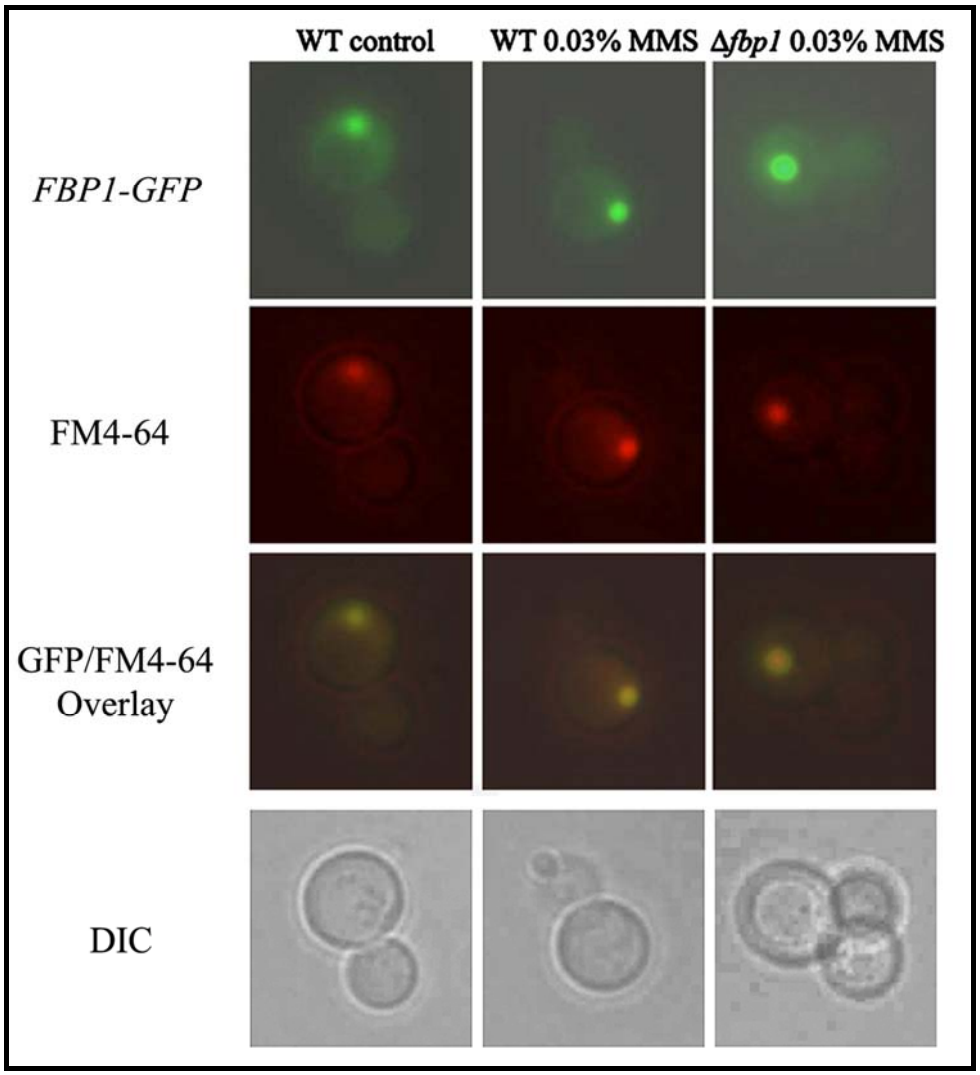

Figure 21. Fbp1p fused with GFP on its C-terminus is targeted to the vacuole. Representative images of wild-type (WT) and *Δfbp1* mutant cells transformed with plasmid overexpressing *FBP1-GFP*. For control an aliquot of each sample was stained with the vacuolar marker FM 4-64, a dye specific for vacuolar membranes. DIC: differential interference contrast microscopy.

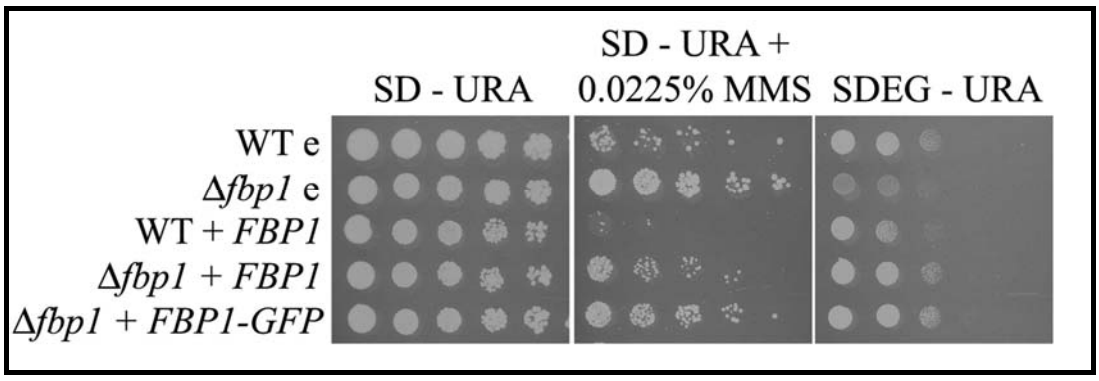

Figure 22. Overexpression of Fbp1-GFPp complements the lack of Fbp1p in growing on non-fermentable carbon sources, but its function in conferring MMS-sensitivity is impaired. MMS sensitivity and viability on ethanol-glycerol medium (SDEG) of the wild-type (WT) transformed with empty plasmid pRS426 (e), or with the plasmid pRS426 containing the *FBP1* cassette under the control of the GPD promoter (+*FBP1*) and *Δfbp1* mutant transformed with empty pRS426 (e), pRS426 containing *FBP1* cassette (+*FBP1*), or plasmid overexpressing *FBP1-GFP* under control of actin promoter (+*FBP1-GFP*). Cells were grown in SD-URA until mid-log phase at 30°C. 5-fold dilutions (starting OD_{600} = 0.5) were spotted onto indicated media and incubated for 48h at 30°C.

3.7. Fbp1p overproduction influences the level of DNA damage caused by MMS

One of the main DNA damage-induced pathways goes from the transcriptional activator Mec1/Tel1 via activation of Rad9p that in turn activates both Rad53p and Chk1p pathways (Blankley and Lydall, 2004). The induction of this pathway results in activation of a large group of genes necessary for DNA repair via Rad53p and cell cycle arrest via Chk1p. One of the proteins downstream from Rad53p is Rnr2p. Induction of *RNR2* is necessary for DNA repair (Chabes *et al.*, 2003).

To check if the lack of Fbp1p alters the impact of MMS on the Mec1/Tel1 pathway we used an RNR2-GFP reporter system (Affanasiev *et al.*, 2000) to measure the induction of the *RNR2* after MMS damage in wild-type, *Δfbp1* and *Δrad9* mutant cells. Induction of the *RNR2* gene is a very good indicator of a genotoxic effect of various toxic agents (Affanasiev *et al.*, 2000). Considering that transcriptional induction of *RNR2* is Rad9p-dependent (Blankley and

Lydall, 2004) *Δrad9* mutant transformed with the reporter plasmid served as additional negative control along with wild-type cells without RNR2-GFP reporter plasmid.

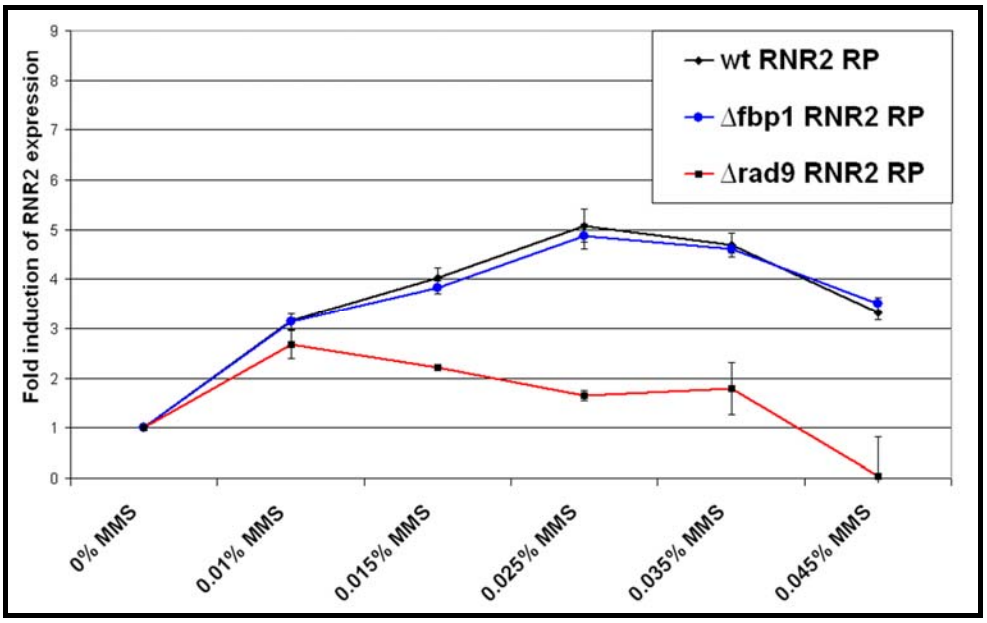

Figure 23. Deletion of *FBP1* does not influence the level of DNA damage after treatment with MMS. MMS induction of yEGFP expression from the *RNR2* promoter in wild-type (WT) and deletion strains, *Δfbp1* and *Δrad9*. Wild-type (wt), *Δfbp1* and *Δrad9* were transformed with RNR2 reporter plasmid (RNR2 RP) and intensity of GFP-fluorescence was measured after 16h of treatment with different MMS concentrations 0% - 0.045%. The *Δrad9* mutant was used as negative control. The values present fold induction of *RNR2* expression ± SEM, calculated as mean fluorescence signal for MMS treated samples, normalised for cell density and divided by mean signal for non-treated samples.

Induction of the RNR2-GFP reporter after MMS treatment, calculated as change in fluorescence intensity per cell number, showed that in the *Δfbp1*mutant induction of the *RNR2* was similar as in wild-type, whereas it was absent in the *Δrad9* mutant (Fig.23). However, overexpression of *FBP1* significantly increased fold-induction of the *RNR2* in comparison with strains transformed with a control plasmid (pRS425 e) where RNR2 induction was again similar in the wild-type and *Δfbp1* mutant (Fig.24). The facts that deletion of *FBP1* shows no effect on *RNR2* induction, while overexpression increase it suggests that the increased intracellular level of Fbp1p after DNA damage caused by MMS probably acts as a signal that mediate cellular response to this toxic agent.

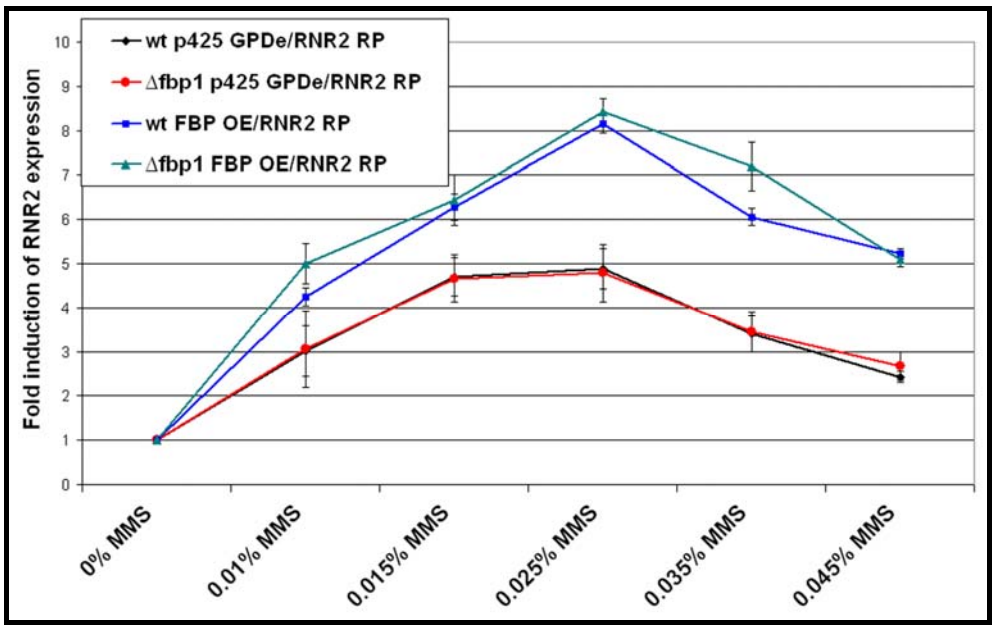

Figure 24. Overexpression of FBP1 increases the level of DNA damage after treatment with MMS. Wild-type (wt) and *Δfbp1* were transformed with two plasmids: empty plasmid pRS425 GPD (GPDe) and RNR2 reporter plasmid (RNR2 RP) or pRS425 containing the *FBP1* cassette under the control of the GPD promoter (FBP OE) and RNR2 RP. Intensity of GFP-fluorescence was measured after 16h of treatment with different MMS concentrations 0%-0.045%. The values present fold induction of *RNR2* expression ± SEM, calculated as mean fluorescence signal for MMS treated samples, normalised for cell density and divided by mean signal for non-treated samples.

3.8. Influence of Fbp1p on ROS production and aging

Salmon and colleagues (2004) showed that DNA damage can trigger an increase in ROS production suggesting that ROS may function as a signal mediating cellular response to unrepared DNA damage. Therefore, increase in ROS and oxidative stress caused by MMS treatment could be a consequence of DNA damage.

To investigate if Fbp1p influences ROS accumulation in MMS treated cells, we measured ROS production after MMS treatment in *Δfbp1* mutant cells using dihydroethidium, which can be oxidized by ROS to fluorescent ethidium. Results showed that 0.03% MMS triggered ROS production in wild-type cells after 1h of treatment (Fig.25, Fig.26). Under the same conditions in mutant cells there was no significant increase in ROS level.

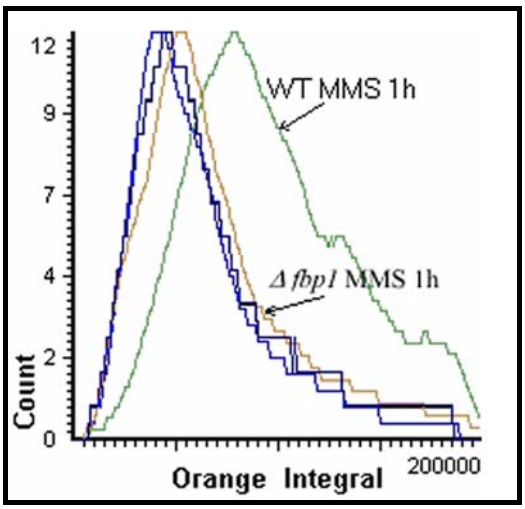

Figure 25. **Lack of Fbp1p influences generation of ROS after treatment with 0.03% MMS.** Quantification of ROS production by LSC**.** Y-axes: number of cells. X-axes: relative fluorescence intensity (orange chanel). Light blue line presents wild-type before MMS treatment, dark blue *Δfbp1* mutant before MMS treatment, green wild-type after 1h treatment with 0.03% of MMS and orange *Δfbp1* mutant after 1h treatment with 0.03% of MMS.

That the high intracellular accumulation of ROS is one of the reasons of cellular death after MMS treatment we proved with survive test on plates treated with MMS and TMPO. TMPO is an oxygen radical scavenger (Knecht and Mason, 1993) that was added on the surface of plates with MMS 15 min before dropping the cellular serial dilution. Results showed protective effect of the TMPO on cellular viability after MMS treatment. In both wild-type and *Δfbp1* mutant ROS scavenging decreased sensitivity to MMS (Fig.27). However, the effect was weaker in the *Δfbp1* mutant strain because this mutant has already very low intracellular accumulation of ROS.

High accumulation of ROS was also observed in chronologically aged yeast cells (Herker *et al*., 2004). Therefore, we investigated the production of ROS and the life span of chronologically aged cells in the wild-type and in the *Δfbp1* mutant strain. Considering that Fbp1p is induced by the diauxic shift in nutrient deprived medium (DeRisi *et al*., 1997), including minimal medium (Fig.10), chronological aging experiments were performed in full (YPD) as well as minimal medium (SD).

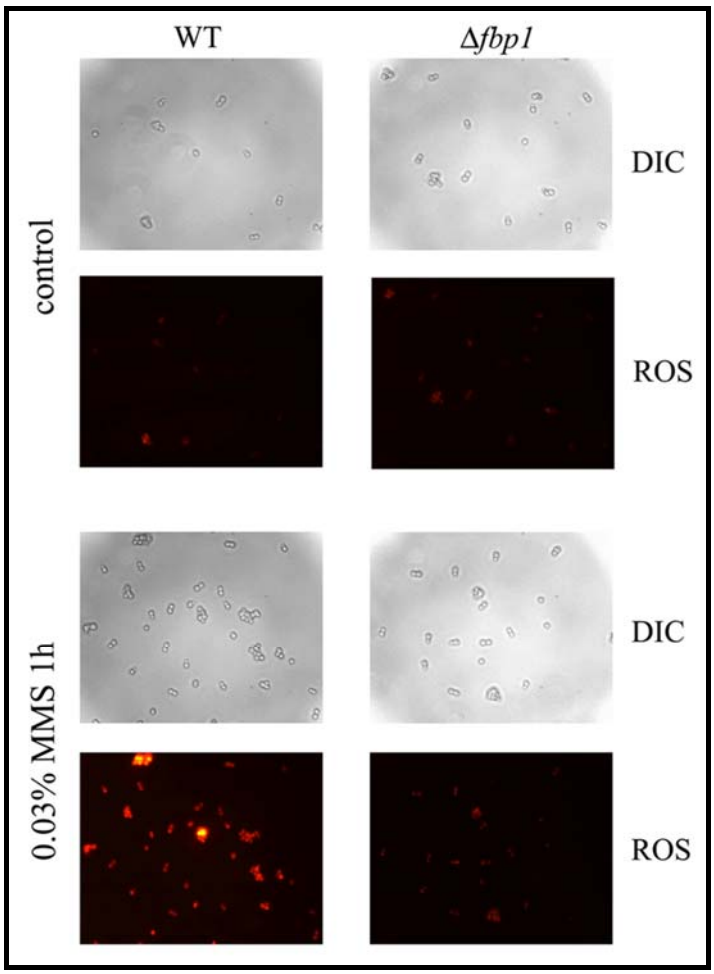

Figure 26. **Lack of Fbp1p influences generation of ROS after treatment with 0.03% MMS.** Representative images of ROS production in wild-type (WT) and *Δfbp1* cells treated for 1h with 0.03% MMS. DIC: differential interference contrast microscopy. ROS: staining with DHE.

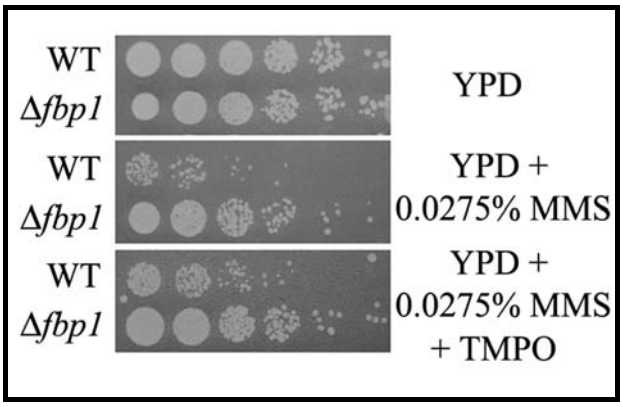

Figure 27. **Protective effect of the TMPO on cellular viability after MMS treatment.** Sensitivity of wild-type (WT) and *Δfbp1* mutant to MMS in presence and absence of ROS scavenger TMPO was tested. 5 mM TMPO was added on top of plates 15 min before cell spotting. Cells were grown in YPD until mid-log phase at 30°C. 5-fold dilutions (starting OD_{600} = 0.5) were spotted onto indicated media and incubated for 48h at 30°C.

Surprisingly, we found a significantly increased survival rate of the *Δfbp1* mutant cells during first 15 days in stationary culture in full medium (Fig.28). Looking at ROS production, in wild-type, the number of ROS-accumulating cells significantly increased with aging, showing 60% of ROS-accumulated cells already after 6 days in stationary culture (Fig.29A, Fig.29B). Aged *Δfbp1* mutant cells started to accumulate ROS after 15 days.

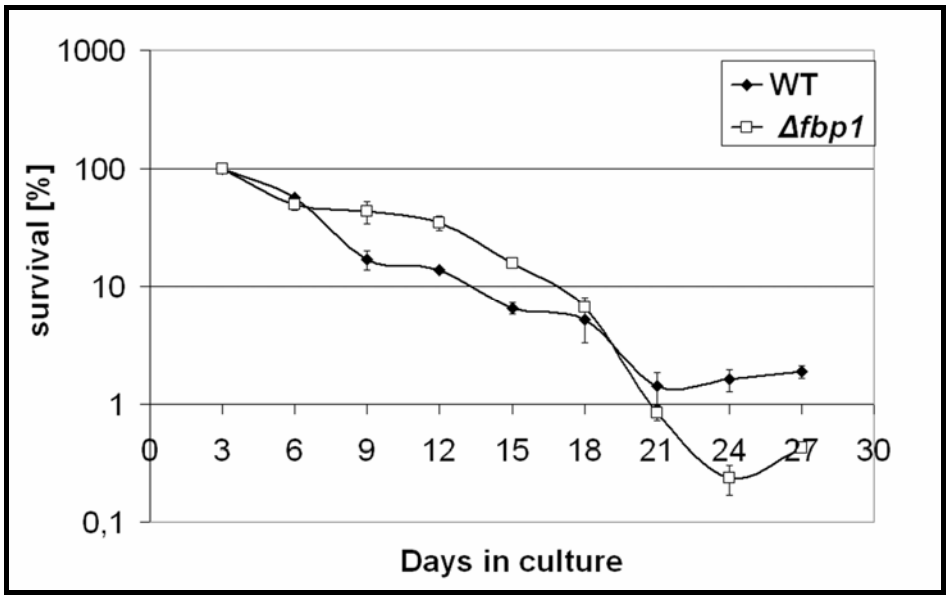

Figure 28. Chronological aged *Δfbp1* mutant cells survive better in the first 15-th days of aging in full medium. Survival of chronologically aged wild-type (WT) and *Δfbp1* mutant cells in full medium (YPD). The number of colonies on day 3 is considered to denote 100% survival. The results are presented as logarithmic mean values ± SEM.

This correlated with a fast loss of viability after 18 days. In minimal medium the lack of Fbp1p was no advantage for cellular survival. Aged *Δfbp1* mutants survived even less (Fig.30) and accumulated similar level of ROS (data not shown). Overexpression of *FBP1* caused faster aging in both wild-type and *Δfbp1* mutant cells (Fig.30), but did not have any influence on ROS accumulation (data not shown). These results show that Fbp1p directly influences aging in *S.cerevisiae*. Fbp1p seems to be an important factor that modulates ROS production in response to MMS treatment and aging. However, in the media where nutrients

become limited (minimal medium or full medium after 15 days of aging) Fbp1p is a critical factor for cellular survive and its lack is rather disadvantage.

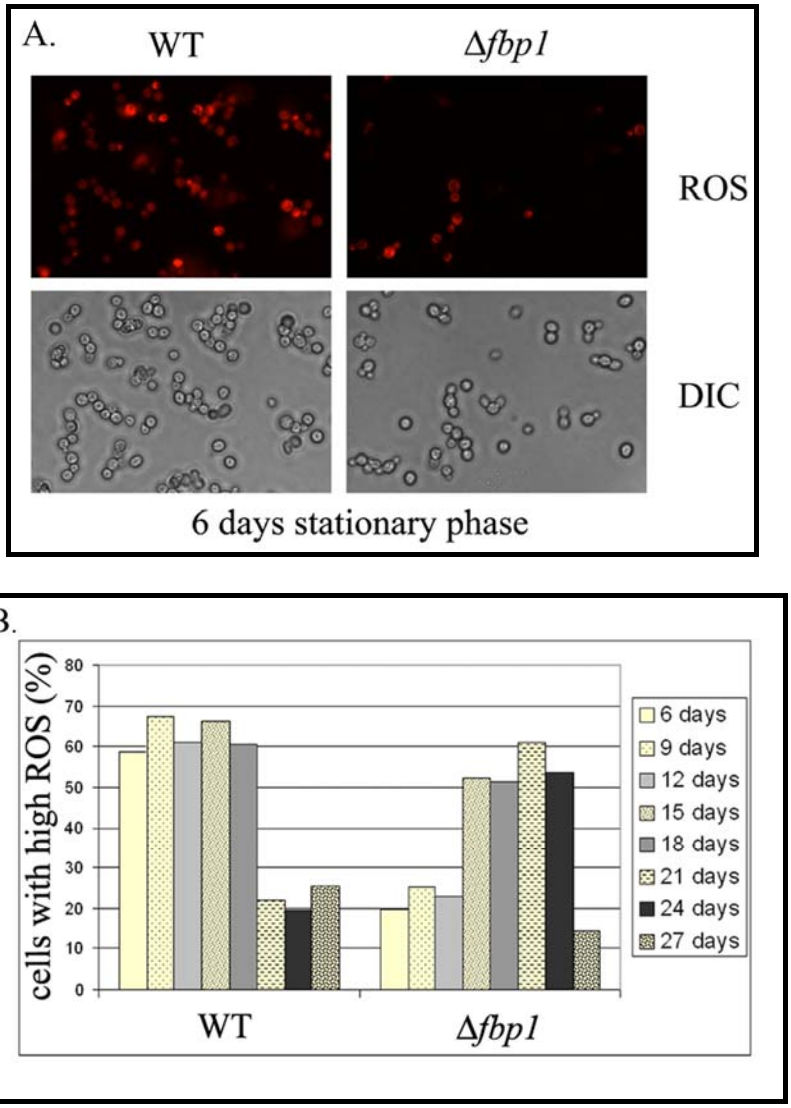

Figure 29. Chronological aged *Δfbp1* mutant cells delay ROS production. (A) Representative images of ROS production in 6 days aged cells. DIC: differential interference contrast microscopy. ROS: staining with DHE. (B) ROS production of chronologically aged wild-type (WT) and *Δfbp1* mutant cells measured by flow cytometry. Data represent the percentage of cells that showed induced ROS production. This experiment was performed three times independently with similar results.

That Fbp1p is rather involved in induction of ROS production as a cellular response to DNA damage and not a factor that directly modify sensitivity to oxidative stress could be proved with the fact that the sensitivity of the *Δfbp1* mutant to external sources of ROS, like

inorganic peroxide (hydrogen peroxide-H_2O_2), or organic peroxide (tert-butyl hydroperoxide-tBHP) did not differ from wild-type sensitivity. Both, H_2O_2 and tBHP treatment led to accumulation of ROS and a low viability of the *Δfbp1* mutant strain (data not shown).

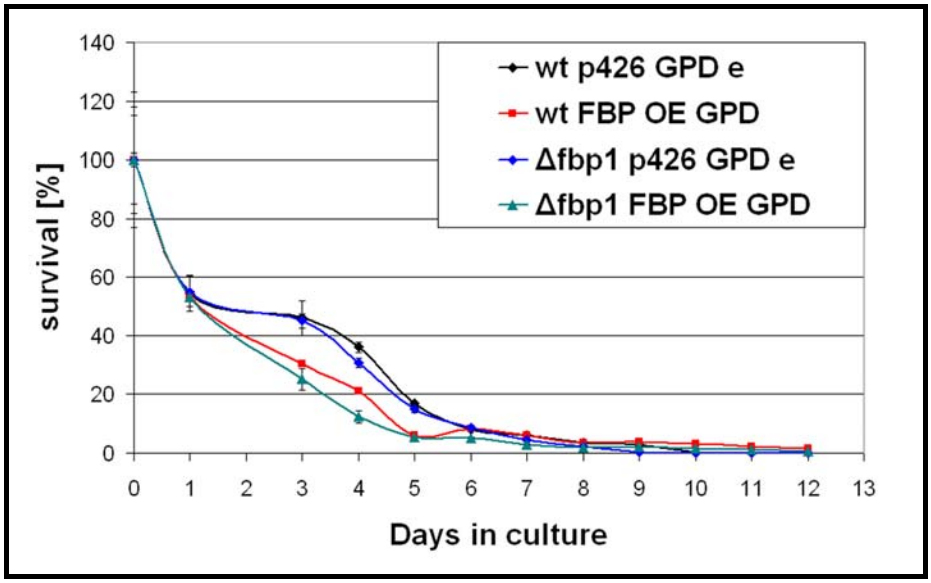

Figure 30. *FBP1* overexpression leads to faster aging. Survival of chronologically aged wild-type (WT) and *Δfbp1* mutant cells transformed with pRS426 GPD empty plasmid (p426 GPD e) or pRS426 containing the *FBP1* cassette under the control of the GPD promoter (FBP1 OE GPD). The number of colonies on day 0 is considered to denote 100% survival. The results are presented as mean values ± SEM.

The main source of ROS are mitochondria. However, the observed phenotype of the *Δfbp1* mutant in response to MMS and aging, especially the decreased ROS accumulation, is not a consequence of Fbp1p-dependent changes in mitochondrial metabolism. ATP production, mitochondrial membrane potential and mitochondria biomass (rhodamine 123 and nonyl acridine orange staining) of cells lacking or overproducing Fbp1p in mock- and MMS-treated cultures showed no significant differences from wild-type (data not shown). Therefore, it seems that *FBP1* influences the connection between DNA damage, aging and oxidative stress through either direct signalling or an intricate adaptation in energy metabolism, but has no detectable impact on mitochondrial metabolism.

4. Discussion

4.1. Cellular sensitivity to MMS – strain background differences

Cellular sensitivity to treatment with MMS strongly depends on the strain background. Treatment with the same MMS concentrations had a much stronger cytotoxic effect in the BY4742 strain than in the FF18984 strain. Measured as sensitivity on plates with MMS or as optical density in liquid cultures treated with MMS, the BY4742 strain in both cases showed higher reduction in biomass yield. When the number of cells capable to proliferate was measured at different time points of MMS treatment, results revealed reduced colony formation in the FF18984 strain already after 2h. The first drop in number of cells able to form colonies in the BY4742 strain was observed just after 24h of treatment. It should be underlined that vitality staining of the cells treated with MMS revealed that more than 95% of cells were still detected as metabolically active even after 24h of treatment (data not shown). Thus, reduced ability of cells to form colonies after treatment with MMS is the result of cell cycle arrest that prevents reproduction of damaged or mutated cells. Slow response of the BY4742 strain and persistent proliferation at the first hours of MMS treatment, most probably leads to a higher accumulation of mutations, conversion of primary lesions to DSB and thus a higher sensitivity to the toxic agent. In long-term MMS treatment reduced number of cells forming colonies due to the effect of toxic agent is combined with reproductive aging of cells when cells reach finite number of division, stop proliferation and enter chronological aging what results in the fast drop in vitality after 24h of treatment in both strains.

4.2. First line response to low doses of DNA damaging agent MMS – influence of growth conditions

Transcriptional response of cells treated with low doses of MMS showed that the magnitude of cellular response is proportional to the dose of the toxic agent applied and

nutrient availability. Significant differences in the response of cells grown in restricted nutrient conditions were observed. With already elevated expression of many ESR genes these cells could respond to MMS treatment much faster and with induction of more specific genes directly involved in DNA damage response. However, this response in F1 medium occurred only in the treatment with the higher MMS dose. This was particularly the case for genes encoding proteins involved in DNA damage repair, stress response/detoxification, cell cycle control and mitochondrial function. For example, *MAG2* encoding a protein involved in DNA dealkylation, *GSH1* encoding gamma glutamylcysteine synthetase involved in glutathione biosynthesis, or *YBP1* required for oxidation of specific cysteine residues of transcription factor Yap1 were up-regulated only in F1 medium. Transcriptional response of cells cultivated in nutrient rich medium, was however, characterised with an induction of general, stereotypical genes involved in ESR. ESR was first reported by Gasch and colleagues (2000) as the orchestrated machinery that protects critical functions within the cell during times of stress and includes the proteins involved in carbohydrate metabolism, protein folding and degradation, cytoskeletal reorganization, amino acid metabolism, protein biosynthesis, RNA metabolism and processing, cell wall organisation and membrane transport. In YPD medium ESR characterised the response to MMS treatment with both concentrations after 1h, while no changes in transcription were observed after 30 min.

In many cases genes from the same functional groups up-regulated in YPD medium had already elevated basal transcription in F1 medium. This observation could be especially important for genes encoding proteins involved in cellular stress response and membrane transport required for transport of drugs from the cells. Indeed, basal transcriptions of two transcription factors *HAC1* and *IKI1*, many chaperones like *HSC82*, *HSP82*, *SSA1* and *SSA2*, or membrane transporters *CTR2*, *PMA1* and *PMA2*, were higher in cells cultivated in F1 medium. Moreover, in many functional groups that belong to ESR, up-regulation of additional genes was detected in F1 medium. The observation that the transcription of large

number of yeast genes in minimal medium is higher than in complex medium is consistent with previous observation by Wodicka *et al.* (1997). These results, therefore, indicate that induction of ESR could be the most important prerequisite for the proper and fast cellular response to the DNA damage and stress conditions. If the ESR machinery is active, cells are able to regulate transcription of more specific genes necessary for DNA damage repair, cell cycle arrest or detoxification. In a case of treatment with lower MMS concentration ESR seems to be only important factor in cellular defence. In the F1 medium this requirement is already fulfilled and the large transcription alteration is omitted.

4.3. Differences in transcriptional response between strains

Expression profiling of the BY4742 strain after 1h treatment with 0.0125% MMS suggested that sensitivity to MMS is directly correlated with the extent of the transcriptional response. This strain is more sensitive to MMS than FF18984 and shows a much lower induction of ESR genes. Except for the groups of chromosome maintenance, protein degradation and protein sorting, other functional groups did not show up-regulation of more than three genes. Especially the large group of carbohydrate metabolism was notably absent from the response in the BY4742 strain. Even if the same groups were involved in the response of both strains, they were represented with different genes. What could be even more important, the response of the BY4742 strain did not include significant decrease in the transcription of many genes, particularly those involved in protein biosynthesis, mRNA processing and regulation of transcription. Taking into consideration that in survival experiments BY4742 also did not show significant reduction in number of cells able to form colonies after short-term MMS treatment, these results contribute to the theory that fast and strong induction of the ESR machinery is the first factor that ensures accurate cellular defence against many cytotoxic and genotoxyc substances. The first role of ESR seems to be transcription modulation of metabolic and stress response pathways in order to preserve

energy and redirect transcription into “stress program”. This was seen in the response of cells grown in YPD medium to the lower MMS concentration. If the level of DNA and cellular damage is elevated by higher doses of the toxic substance used or extended time of treatment, the first line response is followed with induction of additional ESR genes whose products contribute to the basic cellular defence and detoxification: degradation and replacement of damaged proteins, regulation of cellular redox potential, export of toxic substances from the cell and reorganisation of cell wall in order to make it less porous. Finally, when the “stress program” is switched on, as seen in the F1 medium, cells can further respond with a more specific program involved in the repair of damaged DNA, control of cell cycle progression and elimination of the toxic agent. However, this response did not include transcription induction of many genes involved in DNA damage repair, especially those that belong to the Mec1/Tel1 pathway. Birrell and colleagues (2002) showed that many genes encoding proteins involved in protection against DNA damage are actually not regulated by DNA damage. These genes are rather constantly expressed at a relatively high level, so there is always sufficient amount of proteins required for an initial fast activation of the repair process. The results obtained with BY4742 point to the conclusion that this strain is not able to activate this “stress program” in the first hours of treatment. Because more time is needed for the similar response in the BY4742 strain (which should be exanimated in the future) it simply may be too late to cope with existing DNA and protein damage.

4.4. Influence of nutrient availability and metabolic adaptation on cellular sensitivity to MMS

The modulation in energy metabolism, as a part of ESR, seems to play an important role in cellular response to DNA damage. Previous gene expression studies of other or similar stress conditions reported - or contained in their supplementary data sets - similar regulations of genes involved in glycolysis/gluconeogenesis (DeRisi *et al.*, 1997; Godon *et al.*, 1998;

Dumond *et al.*, 2000; Jelinsky *et al.*, 2000; Causton *et al.*, 2001; Gasch *et al.*, 2001). A potential effect of these changes could be a shift from glycolysis to the pentose-phosphate pathway, as suggested by Dumond and colleagues (2000), what could lead to a generation of reducing equivalents (NADH, NADPH) required for cellular antioxidant systems. As already mentioned in the introduction, growth arrest and efficient utilization of nutrients could save ATP and NADPH/NADH for macromolecular stabilization and repair processes which require energy and reducing equivalents (Kültz, 2005). We observed that nutrient deprivation mediated pre-induction of ESR and metabolic adaptation by changes in nutrient availability helps to cope better with the toxic effect of genotoxic agents applied later such as MMS. The lowest sensitivity to MMS was found in cells cultivated in respiratory induced medium. Slightly higher was the sensitivity of cells cultivated in minimal medium. The most sensitive were cells cultivated in full medium. The effect was visible in log phase as well as in early and late stationary phase. The effect is more pronounced in case of BY4742 and weaker in FF18984. An explanation could be that the MMS triggered transcriptional response is faster and stronger in FF18984, activating many more genes involved in ESR, so the response triggered by nutrient lack does not play such an important role as observed for BY4742. The strain BY4742 responds to MMS more slowly and the adaptive response, induced by nutrient depleted media, protects these cells against subsequent challenge with genotoxic agent like MMS.

We obtained the best cellular resistance to MMS just by changing the carbon source in the medium, e.g. reducing the glucose concentration. Moreover, in F1 medium we could also prove higher basal expression of genes involved in gluconeogenesis and respiratory metabolism. Therefore, seems that the major stimulus that triggers the adaptive response and induction of ESR genes is actually alteration in glucose utilization. It was shown earlier that yeast cells grown on non-fermentable substrates (e.g. ethanol and glycerol) express higher levels of antioxidant activity and display higher oxidative stress tolerances (reviewed in

Moradas-Ferreira *et al.,* 1996). Many of primary antioxidant defence genes are repressed by glucose and their derepression occurs with respiratory adaptation that follows glucose exhaustion (Krems *et al.,* 1995). This transcriptional adaptation that occurs in diauxic shift is an important factor that contributes to the increased oxidative-stress tolerance of stationary-phase yeast cells (Jamieson *et al.,* 1994; Steels *et al.,* 1994). In our experiments this primary antioxidant program was induced by MMS even in the cells grown in medium with high glucose concentration showing that the modulation of major pathways of energy metabolism is inevitable part of minimal stress response. Presumably, energy metabolism and antioxidant defence genes are under control of the same transcriptional mechanisms involving glucose, transcription factors or secondary messengers. There are ample evidences showing that changes in cAMP levels are important in regulation of growth arrest at the diauxic shift (Russell *et al.,* 1993). A reduction in cAMP level is essential for traversing the diauxic shift while transcription of many genes involved in this process is directly or indirectly controlled by the cAMP level (Boy-Marcotte *et al.,* 1996). Other evidences showed that STRE-regulated genes are also under negative regulation by cAMP (Marchler *et al.,* 1993). Therefore, energy metabolism and stress proteome are inseparably intertwined in cellular response to environmental changes, nutrient depletion or DNA damage.

Taken together, yeast cells seem to utilize a common response to cope with diverse stress conditions, nutrient starvation or DNA damage which includes: modulation in basic metabolic pathways, induction of environmental, especially oxidative stress response (ESR) and energy conservation. Those cells that are able to induce alterations in cellular energy homeostasis and adjust to calorie restriction are also better prepared to survive treatment with toxic agents. Our results show an important correlation between metabolic pathways and the ability of living organisms to cope with adverse conditions.

4.5. The role of Fbp1p in cellular response to DNA damage

The importance of respiratory metabolism and gluconeogenesis for proper response to DNA damage was also supported by results obtained from the sensitivity assays of various yeast mutants. Deletion of the *HAP4*, encoding a subunit of the heme-activated, glucose-repressed Hap2p/3p/4p/5p CCAAT-binding complex which serves as a transcriptional activator and global regulator of respiratory gene expression (Lascaris *et al.*, 2002), slightly increased cellular sensitivity to MMS. The sensitivity of this mutant to phleomycin or 4-NQO did not differ from wild-type one. Although the effect is not so striking, this result supports the conclusion that induction of respiratory metabolism is not just a consequence of having the similar regulatory elements, as found in stress response genes, but is an active counterpart in cellular defence to DNA damage. On the other hand, the deletion of the key enzyme in gluconeogenesis, Fbp1, or its transcriptional repressor, Mig1, showed some surprising results. Lack of Fbp1p in the cells drastically increased cellular resistance to MMS, while the *Δmig1* mutant had higher sensitivity to MMS and phleomycin. The reduced sensitivity of the *Δfbp1* mutant was specific for MMS treatment. Deletion of *FBP1* did not confer resistance to other DNA damaging agents such as 4-NQO or phleomycin. Even 4-NQO, which reacts with DNA and forms stable quinoline-purine monoadducts (Galiegue-Zouitina *et al.*, 1986), and which is known to undergo redox cycling and produce substantial amounts of ROS in the cell (Biaglow *et al.*, 1977), did not have the same effect as MMS on the *Δfbp1* mutant. These results suggest that the gluconeogenesis pathway and its key regulator Fbp1p must have some additional role in DNA damage and stress response other than just energy conservation and higher production of glucose-6-phosphate, the key substrate for the pentose phosphate pathway. NADPH synthesis in this pathway is an important factor for reduction of oxidized glutathione. In this case *FBP1* deletion could lead to an increased MMS-sensitivity due to reduced NADPH production.

That the lack of Fbp1p is responsible for increased resistance to MMS was proved by reintroduction of *FBP1* into *Δfbp1* mutant cells. The wild-type MMS sensitivity was restored. Moreover, overexpression of *FBP1* in wild-type led to a slightly higher sensitivity to MMS in comparison with the *Δfbp1* mutant cells overexpressing *FBP1*. How can this be explained considering that induction of genomic *FBP1* by MMS should only contribute marginally due to the higher copy number of the transformed gene? One explanation could be that additional control mechanisms post-transcriptional and on protein level, such as degradation and vacuolar targeting, significantly contribute to the regulation of protein activity. Thus, upon stress caused by MMS, a marginal increase in transcription without adaptation of degradation could lead to a significant higher level of active Fbp1p, which could mediate the higher sensitivity to MMS in wild-type overexpressing *FBP1*. In addition, we showed that *FBP1* is induced not only in response to glucose deprivation, but also by MMS treatment and under limited nutrient conditions in the presence of glucose, like in F1 medium. Induction of *FBP1* can also be found in the results of genome wide gene expression studies in response to H_2O_2 (0.32 mM; Gasch *et al.,* 2000); to oligomycin (Epstein *et al.,* 2001); to a temperature shift from 30°C to 25°C (Causton *et al.,* 2001) and after treatment with griseofulvin, an antifungal agent that disrupts mitotic spindle structure leading to metaphase arrest (Savoie *et a.,* 2003). In the work from Schaus and colleagues (2001) *FBP1* is listed among the genes transcriptionally controlled by phosphorylated Sip4p. Sip4p mediates the response to neocarzinostatin, which has an antiproliferative effect (Schaus *et al.,* 2001). Therefore, under many stress conditions *FBP1* expression could be induced even in the medium with the high glucose concentration. Considering that *MIG1* deletion caused an opposite effect to this seen with lack of *FBP1,* it seems that Mig1p plays an important role in transcription control of *FBP1* in such conditions.

The role of Fbp1p in DNA damage response mechanisms seems to be connected with the better recovery of this strain after a long-term treatment with MMS. In the *Δfbp1* mutant

prolonged treatment with MMS, e.g. 24h or 48h strongly reduced the number of cells able to form colonies after plating them on medium without the toxic agent. The first drop was observed in both strains already after 2h of treatment while a maximal reduction occurred after 24h. The *Δfbp1* mutant, however, was able to recover and continue proliferation, what was detected as an increased colony formation unit starting from 24h of MMS treatment. On the other hand, the same experiment with cells that overexpress *FBP1* led to exactly the opposite effect. This time high level of *FBP1* expression decreased the number of proliferating cells even in the non-treated culture. The results obtained from measuring the induction of the *RNR2* after DNA damage revealed that increased transcription of *FBP1* led to stronger induction of this essential gene in DNA repair. The effect, of course, could be connected with a stronger activation of the pentose-phosphate pathway and subsequently a more efficient production of nucleotides through redirection of energy metabolism into gluconeogenesis. In this case, a similar effect of *FBP1* deletion should be observed if any other DNA damaging agent is applied. Hence, Fbp1p must have an additional role in response to DNA damage, most probably by mediating the DNA damage response between damage sensors and effectors. Consistent with this assumption is the observation that C-terminally GFP-tagged Fbp1p restores the lack of this protein in utilization of non-fermentable carbon sources, but has impaired function in response to MMS.

4.6. Fbp1p delays the onset of ROS production in DNA damage or aged cells

Looking at ROS production we found that cells lacking Fbp1p did not generate substantial amounts of ROS after MMS treatment or in chronologically aged cells, both treated in full medium. Both the stationary phase cultures as well as MMS treated cultures are characterised by an accumulation of endogenously produced intracellular ROS in response to DNA damage or aging. In contrast, in case of 4-NQO, H_2O_2 or tBHP treatment, the strong oxidative stress and high ROS accumulation, which is not influenced by the deletion of *FBP1*,

is generated by these agents directly and is not the result of induced ROS production. Therefore, we conclude that Fbp1p does not modify sensitivity to oxidative stress, but rather participates in or modulates the production of ROS induced by DNA damage or in aged cells. Thus, rather than influencing MMS toxicity directly, Fbp1p plays a role in connecting DNA damage with ROS production.

Various evidences suggest that some factors that influence aging are linked to oxidative damage of DNA (reviewed in von Zglinicki *et al.*, 2001). As yeast cells grow older they cease dividing and enter a postdiauxic, hypometabolic state, where they can remain viable for weeks (Bitterman *et al.,* 2003). Maclean and colleagues (2003) showed that the longest *S.cerevisiae* life spans are obtained by adaptation of cells to efficient respiratory maintenance, achieved by growth to stationary phase on a respiratory carbon source. Moreover, they proved the vital importance of base excision repair (BER) in the prevention of mutation accumulation and the attainment of a full yeast chronological life span. Aged wild-type cells or cells treated with MMS for 24 h display metabolic activity and are negative for PI staining, but their ability to replicate and form colonies is impaired. These cells show a very high level of intracellular ROS accumulation. Without *FBP1* the amount of ROS produced after MMS treatment or in aged cells is much lower, and the cells maintain their reproduction capability and continue proliferation. In addition, treatment with a ROS scavenger resulted in a similar increase in the number of wild-type cells able to form colonies after MMS treatment as was obtained by deletion of *FBP1*. This shows that ROS contributes to the reduced vitality seen in MMS treated or aged cells which can be compensated by preventing ROS accumulation. The link between aging process and *FBP1* expression is also confirmed by overexpression of *FBP1*. Cells that produce high amounts of this protein age faster. This is an interesting point, showing that high level of Fbp1p present in the cell is cytotoxic. Therefore, precise metabolic regulation, especially tight control of the key metabolic proteins level, seems to be very important for the aging process and cellular

viability after DNA damage. Taken together, our results lead to the hypothesis that Fbp1p could serve as a signalling molecule involved in mediating the cellular response to such conditions.

What is the role of Fbp1p in those processes? A shift toward increased energy metabolism and gluconeogenesis in aged cells or in response to DNA damage leads to higher oxidative stress resistance and more efficient DNA damage repair, but also causes shorter lifespan and faster maturation (Lin *et al.*, 2001). These two, at the first glance opposite effects, may be explained by the recently published work of Herker and colleagues (2004). They found that chronological aging leads to apoptosis in yeast. It is suggested that in a monoclonal population of cells it can be evolutionarily advantageous to spare most of the dwindling resources for healthier cells. Looking for ROS production in aged cell populations we found that deletion of *FBP1* significantly delays the onset of ROS production. Thus, induction of *FBP1* could contribute to mediate the apoptotic signal in aged cells, selectively enabling younger cells to survive in adverse environmental conditions. Taken together, our results clearly show that *FBP1* influences the connection between DNA damage, aging and oxidative stress through either direct signalling or an intricate adaptation in energy metabolism. As was already observed by Lin and colleagues (2001), enhanced gluconeogenesis and increased energy storage are hallmarks of aging in *S.cerevisiae.* In consequence, the tight regulation of FBP1 expression and age-associated changes in glucose metabolism are not only crucial for the control of gluconeogenesis, but also for an appropriate response to aging and DNA damage.

5. Conclusions

1. Induction of ESR is the most important prerequisite for the proper and fast cellular response to DNA damage and stress conditions. This response is induced under many stress conditions and serves to protect the basic cellular functions and integrity of intracellular structures. If the ESR machinery is already active, cells are able to regulate transcription of more specific genes necessary for DNA damage repair, cell cycle arrest or detoxification. Fast induction of the ESR program contributes to the higher resistance of the FF18984 strain to MMS treatment.
2. The most important genes directly involved in protection against DNA damage are constantly expressed at the high level. Therefore, there is always sufficient amount of proteins required for an initial fast activation of the repair process.
3. Nutrient availability can modify transcription profile of the cells and induce activation of the cellular “stress program”. The elevated transcription of many genes involved in this program is the molecular base of cross-tolerance of cells to the other stress conditions or agents applied later. In both FF18984 and BY4742 strains pre-induction of ESR by minimal or glucose depleted media increases cellular resistance to MMS and allows more cells to continue proliferation.
4. The modulation in energy metabolism, as a part of ESR, seems to play an important role in cellular response to DNA damage. The major stimulus that triggers the adaptive response and induction of ESR genes is alteration in glucose utilization. Being under control of the same transcriptional mechanisms, energy metabolism and stress proteome are inseparably intertwined in cellular response to environmental changes, nutrient depletion or DNA damage.
5. The key enzyme in gluconeogenesis fructose-1,6-bisphosphatase plays an important role in mediating cellular responses to DNA damage and aging in yeast cells. Its

transcription is clearly up-regulated upon MMS treatment, while deletion of this gene confers resistance to MMS by increasing the number of cells able to proliferate after the treatment and prolong life-span in nutrient rich medium.

6. Overproduction of Fbp1p has a toxic effect on cells. High level of Fbp1p in the cells increases cellular sensitivity to MMS, reduces the number of cells able to proliferate even in non-treated cultures, increases *RNR2* induction upon MMS treatment and causes faster aging.
7. Both in MMS treated or chronologically aged cells, deletion of *FBP1* significantly delays the onset of ROS production. A similar effect is obtained in wild-type cells by treatment with the ROS scavenger, TMPO, which confirms that ROS contributes to the reduced vitality seen in MMS-treated or aged cells.
8. Induction of *FBP1* could contribute to mediate the apoptotic signal in aged and seriously damaged cells, selectively enabling younger and better adapted cells to survive in adverse environmental conditions

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7. Appendix

Abbreviations (without gene names)

4-NQO	4-nitroquinoline 1-oxide
ALL	acute lymphoblastic leukaemia
AP	apurinic/apyrimidinic sites
A–T	Ataxia–telangiectasia
ATP	adenosine 5'triphosphate
BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea
BER	base excision repair
BSA	bovine serum albumin
cDNA	complementary DNA
CML	chronic myelogenous leukemia or
DHE	dihydroethidium
DMSO	dimethylsulfoxide
dNTP	deoxynucleotide triphosphate
DR	direct repair
DSB	double strand DNA brake
dsDNA	double strand DNA
DTT	dithiothreitol
EDTA	ethylenediamine-tetraacetic acid
ESR	environmental stress response
EtBr	ethidium bromide
FACS	fluorescent activated cell sorter
$FADH^-$	Flavin-Adenin-Dinucleotid
FDA	fluorescein diacetate
GSH	glutathione (reduced glutathione)
HR	homologous recombination
IR	ionizing radiation
LSC	laser-scanning cytometer
MMS	methyl methanesulfonate
MNNG	*N*-methyl-*N´*-nitro-*N*-nitrosoguanidine
MR	mismatch repair
NADH	reduced nicotinamide adenine dinucleotide

NADPH	reduced nicotinamide adenine dinucleotide phosphate
n.d.	non-defined
NER	nucleotide excision repair
NHEJ	nonhomologous end-joining mechanisms
OD	optical density
ORF	open reading frames
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PI	propidium iodide
RNS	reactive nitrogen species
ROS	reactive oxygen species
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodiumdodecylsulfate
SSA	single-strand annealing repair mechanism
ssDNA	single strand DNA
TBS	translation bypass synthesis
TMPO	3,3,5,5,-tetramethyl-pyrroline *N*-oxide
UV	ultraviolet radiation
XP	Xeroderma pigmentosum

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