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DNA Damage-Induced Reactive Oxygen Species: A Genotoxic Stress Response

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DNA Damage-Induced Reactive Oxygen Species: A Genotoxic Stress Response

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Abstract

DNA Damage-Induced Reactive Oxygen Species: A Genotoxic Stress Response

By Lori Ann Rowe

Cellular DNA is essential for life, providing the genetic information required for building and maintaining the cell. There are many factors, both endogenous and exogenous to cells that can contribute to DNA damage. Aerobic organisms face the challenge of both utilizing oxygen to survive and appropriately handling the reactive nature of oxygen. Reactive oxygen species (ROS) are known to be produced both through endogenous cellular mechanisms and as a result of exposure to exogenous agents. Increased levels of intracellular ROS are associated with several human pathologies including neurological disorders, cardiovascular disease, and cancer. Elevated levels of ROS can cause DNA damage, which can contribute to these pathological changes. The eukaryotic model system, *Saccharomyces cerevisiae*, was utilized to investigate DNA damage-induced ROS in the genotoxic stress response due to the fact that the DNA repair pathways are highly conserved between yeast and humans. We examined the intracellular levels of ROS in DNA repair-proficient (WT), repair-deficient (lacking base excision repair (BER^-), nucleotide excision repair (NER^-), or both (BER^-/NER^-)), and ROS scavenging mutant (*sod1Δ*, *sod2Δ*, *cta1Δ*, and *cttΔ*) mutants following exposure to methyl methanesulfonate (MMS) and ultraviolet light (UV-C) and found that there is a dose-dependent increase in intracellular ROS. To examine ROS as a signaling molecule in the DNA damage response, we assessed the activation of a known oxidative stress responder, Yap1. We observed that Yap1 is activated in response to DNA damage primarily repaired through BER-dependent mechanisms, but not NER. To further define the role of Yap1 as a DNA damage responder, mutation rates and chromosomal rearrangements in *yap1Δ* strains were determined. There is an increase in genomic instability in *yap1Δ* mutant cells. These results suggest that while there is an increase in intracellular ROS levels regardless of the type of DNA damage induced or the genetic background of the cell, the signaling event by ROS sub-species that occurs following DNA damage is specific to the nature of DNA damage. These studies also reveal that Yap1 is likely to function as a DNA damage responder.

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CHAPTER I

General Introduction

Aerobic organisms face the paradox of survival in an oxygen rich environment. Although oxygen is required to sustain life, it can be toxic through the formation of reactive oxygen species (ROS). ROS are highly reactive oxygen molecules that include superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$), and can be formed endogenously and following exposure to exogenous agents. Endogenous sources of ROS include mitochondria, peroxisomes, as well as other cellular metabolic products [1]. Exogenous sources of ROS include environmental agents, chemotherapeutics, ionizing radiation, and ultraviolet light (UV) [2].

Under normal growth conditions, ROS levels are maintained at relatively low levels and function in intracellular signaling to maintain normal cellular functions and metabolism [3]. An example of ROS signaling in mammalian cells is through ligand activation of membrane receptors that causes the formation of $O_2^{\bullet -}$ and H_2O_2, regulating downstream tyrosine phosphorylation [4]. While this example demonstrates the importance of ROS in cells, as the levels of ROS increase, they can damage cellular macromolecules, triggering an oxidative stress response to prevent further damage [3, 5, 6]. One component of the oxidative stress response is the activation of transcription factors such as Yap1 (*Saccharomyces cerevisiae*) [7] and AP-1 (mammalian cells) [8], which will be described in detail later.

ROS=mediated damage to proteins, lipids, RNA, and importantly DNA, is believed to lead to impaired physiological function and has been associated with several human degenerative conditions including cancer [9], neurological disease [10], cardiovascular disease [11], and the process of aging [12]. These degenerative conditions are thought to progress due to toxic or mutagenic modification of nucleotides and gross chromosomal rearrangements leading to genomic instability.

Because DNA contains all the information necessary for proper cellular functions, maintaining the genetic integrity is important, especially in the face of constant damage from both endogenous and exogenous sources. It has been reported that between 10,000 and 20,000

oxidative lesions per cell per genome occur every day in mammalian cells [13, 14]. Fortunately, cells have developed several pathways for handling DNA damage including mismatch repair, base excision repair (BER), nucleotide excision repair (NER), recombination (REC), translesion synthesis (TLS), and direct reversal [15]. Previous work has shown that these pathways, while mostly specific in the types of DNA lesions they repair, exhibit some overlap in the repair or handing of DNA damage [16]. BER and NER are major repair pathways present in cells and relevant to the work described here.

Production of Reactive Oxygen Species

A variety of molecules derived from molecular oxygen are classified as ROS (reviewed in [17]) . Typically, ROS have one or more unpaired electrons, with one exception being H_2O_2 [18]. Molecules such as superoxide ($O_2^{\bullet -}$), singlet oxygen ($^{\bullet}O_2$), and the hydroxyl radical ($^{\bullet}OH$) are all classified as ROS. When molecular oxygen (O_2) is reduced by one electron, a superoxide molecule is produced, which is a relatively stable intermediate [18]. Most other ROS are produced from $O_2^{\bullet -}$, either through dismutation of $O_2^{\bullet -}$ to produce H_2O_2, or through the Fenton reaction to produce $^{\bullet}OH$. Dismutation of $O_2^{\bullet -}$ can occur either enzymatically and nonenzymatically within the cell [19]. The enzyme superoxide dismutase (SOD) is responsible for the reduction of $O_2^{\bullet -}$ to H_2O_2 [19]. Catalases are responsible for the enzymatic reduction of H_2O_2 [20]. The $^{\bullet}OH$ radical can also be produced through the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$) or the Haber-Weiss reaction ($O_2^{\bullet -} + H_2O_2 \rightarrow O_2 + {}^{\bullet}OH + OH^-$) [21]. The reduction of H_2O_2 also occurs nonenzymatically within the cell.

The generation of ROS can occur in several locations. The primary site of ROS production in the cell is the mitochondria, through the leakage of electrons from the mitochondrial respiratory chain [22]. In mitochondria, $O_2^{\bullet -}$ is generated either during the reduction of NADPH to $NADP^+$ or during the transfer of electrons from the *bc1* complex through

cytochrome *c* to the COX complex [1]. Other ROS can also be produced in the mitochondria via the mechanisms mentioned above.

ROS, primarily H_2O_2, can also be produced in the peroxisomes by several enzymes (reviewed in [23]). One family of enzymes consists of acyl-CoA oxidases, which participate in fatty acid degradation in the beta-oxidation pathway. Additional sources of H_2O_2 in peroxisomes are the D-amino acid oxidases, which are FAD-containing flavoenzymes that catalyze the oxidative deamination of D-isomers of neutral and polar amino acids. To counteract this ROS production, peroxisomes also contain catalases that catalyze the conversion of H_2O_2 to H_2O and O_2 [20, 24].

Several other enzymes are capable of producing ROS in cells as a normal byproduct of their catalyzed reaction. For example, xanthine oxidase catalyzes the oxidation of xanthine to uric acid and can produce $O_2^{\bullet-}$, H_2O_2, and $^{\bullet}OH$ [25]. Also, NADPH oxidases are multi-subunit enzyme that catalyze $O_2^{\bullet-}$ production by a one electron reduction of O_2 using NADPH or NADH as the electron donor [26]. While mitochondria are believed to be the major source of ROS production, there are many other cellular mechanisms that can produce ROS.

Saccharomyces cerevisiae: A Powerful Model System for Studying DNA Repair and Genetic Instability

Ideally, to study human processes and diseases we would directly study humans, but because of the ethical and moral issues of experimenting on humans, we must rely on model systems to gain an understanding of human processes and disease. One useful model system is the budding yeast *Saccharomyces cerevisiae* because many cellular mechanisms and pathways are highly conserved between humans and yeast.

S. cerevisiae is a single celled organism that is easily grown in the laboratory with a generation time of 1-2 hours versus 24 hours for cultured mammalian cells. Budding yeast are a

good model system for studying DNA repair due to the fact that the DNA repair pathways are highly conserved between yeast and humans. Yeast cells are also easier to genetically manipulate that cultured mammalian cells allowing for the use of multiple mutant phenotypes. These factors make yeast ideal for studying processes such as DNA repair and genomic stability, which can lead to disease in humans if malfunctioning.

DNA Repair Pathways in *S. cerevisiae* and Mammals

Base Excision Repair

Base excision repair (BER) (reviewed in [27]) is probably the most commonly used DNA repair process in cells [15]. BER is primarily responsible for the removal of small helix non-distorting DNA lesions [28]. Lesions repaired by BER include, but are not limited to: deamination products; apurinic/apyrimidinic/abasic (AP) sites; and oxidative and alkylating DNA damage [15]. BER is a multistep process that involves many proteins, from initial recognition to the final step of religating the repaired DNA strand (Figure 1).

The initial step of recognizing the damaged base is carried out by a class of enzymes called DNA N-glycosylases. A DNA N-glycosylase recognizes the damaged base and catalyzes its release by cleaving the N-glycosyl bond, leading to the formation of an AP site. In *S. cerevisiae*, there are several DNA glycosylases, including Ung1, Ogg1, Ntg1, and Ntg2, and each is able to recognize specific types of DNA damage. Following removal of the damaged base, the resulting AP site can be further processed by an AP lyase (a second activity possessed by some DNA N-glycosylases) or an AP endonuclease [28, 29].

There are three main AP lyases in *S. cerevisiae*, Ntg1, Ntg2, and Ogg1. All three of these enzymes are bifunctional having both DNA N-glycosylase and AP lyase activity. Ntg1 and Ntg2 contain strong AP lyase activity, while Ogg1 only contains weak AP lyase activity. An AP lyase cleaves the DNA backbone 3′ to the AP site resulting in a 3′α, β-unsaturated aldehyde end

or a 3′ phosphate depending on whether DNA strand scission takes place via β-elimination or β-δ-elimination [15]. The 3′ ends must undergo further processing in order for repair to proceed. Removal of the 3′ blocking group is accomplished by a 3′ phosphodiesterase or 3′ phosphatase.

There are two main AP endonucleases in *S. cerevisiae*, Apn1 and Apn2. Apn1 is the primary enzyme accounting for greater than 97% of the hydrolytic AP endonuclease in yeast [29]. AP endonucleases cleave at the 5′ side of the AP site resulting in a 3′ hydroxyl group and a 5′ deoxyribose phosphate (5′ dRP) that can be processed by a 5′ dRPase. Alternatively, this 5′ blocking group can be removed as part of a flap formed by strand displacement [29]. With ends compatible for DNA polymerases, repair can be completed through the action of DNA polymerase (DNA pol ε or δ) and DNA ligase.

There are two possible pathways through which BER can proceed, short patch or long patch repair. Which repair pathway to take is partially determined by the DNA glycosylase and the resulting AP site [30]. In short patch repair, one nucleotide is replaced by DNA polymerase and the nick sealed by DNA ligase. In long patch repair, two to thirteen nucleotides are replaced. The stretch of nucleotides is displaced by DNA polymerase forming a flap that is then removed by a flap endonuclease, such as Rad27 (*S. cerevisiae)*, and then DNA ligase seals the nick [27].

The BER pathway is highly conserved between yeast and humans. In humans, BER follows the same general pathway as described above for yeast (reviewed in [31, 32]). The proteins that are involved in mammalian BER differ mostly in name, not in function, and are listed in Table 1.

Nucleotide Excision Repair

Nucleotide excision repair (NER) is primarily responsible for the repair of bulky, helix distorting lesions, such as 6-4 photoproducts (6-4 PPs) and cyclobutane pyrimidine dimers (CPDs) produced by ultraviolet light (UV) [33]. There are two subpathways of NER that differ

from each other in their recognition of the DNA damage. Transcription coupled repair (TCR) is selective for lesions that are present in the transcribed strand of expressed genes, while global genome repair (GGR) acts over the rest of the genome, in transcriptionally silent regions, as well as the non-transcribed strand of expressed genes [15, 34]. In TCR repair, proteins are recruited to the site of DNA damage due to a stalled RNA polymerase and not through direct recognition of the DNA damage by recognition proteins, as with GGR [34].

The process of NER (reviewed in [15, 28]) requires many proteins that function in multiple complexes (Figure 2). In *S. cerevisiae*, the genes associated with NER have been divided into three epistasis groups: *RAD3*, *RAD52*, and *RAD6*. Inactivation of individual genes in the *RAD3* epistasis group results in increased sensitivity to UV, and several of the genes, if mutated, result in complete NER deficiency; these include *RAD1*, *RAD2*, *RAD 10*, and *RAD14*.

GGR is initiated following detection of distortions in the DNA by the Rad4-Rad23 complex. Following detection of the distorted DNA, Rad14, TFIIH, in complex with several other proteins, are then recruited to the site of damage, unwinding the DNA helix. Following the unwinding of the DNA, another complex including Rad2 and Rad1-Rad10, which is responsible for cleavage of the DNA, is recruited to the site of damage. Rad2 is transcriptionally upregulated in response to DNA damage and contains endonuclease activity which cleaves the DNA 3′ to the damaged site leaving a 3′ OH and a 5′ phosphate group. Rad1 and Rad10 form a complex in the absence of DNA damage. Individually, neither is active; however, the complex possesses endonuclease activity, cleaving 5′ to the damaged DNA, leaving a 3′ OH and a 5′ phosphate group. To complete the repair process, DNA synthesis by DNA polymerase ε or δ and ligation by DNA ligase must occur.

As with BER, the NER pathway is highly conserved between yeast and humans. In humans the NER pathway also has two sub-pathways, which function in the same manner as in yeast. Again the proteins in NER mostly differ in name and not in function. The NER proteins

present in humans are listed in Table 2. Because the NER pathway is highly conserved between species, it is useful to utilize yeast as a model system to study DNA repair.

Compromising DNA Repair Pathways

A useful tool for studying DNA repair mechanisms is to examine cells deficient in one or more repair pathways. A previous study in our laboratory examined DNA repair mutants and determined the spontaneous recombination and mutation rates and the sensitivity of these mutants to various DNA damaging agents [16]. Several DNA damage repair mutants were constructed that compromised either BER or NER. Ntg1 and Ntg2 are N-glycosylases with associated AP-lyase activity, and Apn1 is the major AP-endonuclease for the BER pathway [15]. Any single, double, or triple mutant of these genes did not exhibit an increase in sensitivity to H_2O_2, menadione, or ionizing radiation. There were small increases in the recombination and mutation rates in *apn1Δ* strains; however, there was no increase in the *ntg1Δ ntg2Δ* strains. In contrast, when all three genes were simultaneously deleted (*ntg1Δ ntg2Δ apn1Δ*) there was a significant increase in both recombination and mutation rates. Thus, in order to severely compromise the BER pathway, *NTG1*, *NTG2*, and *APN1* needed to be eliminated in combination[16].

The NER pathway was also examined. Yeast cells were made deficient in NER by deleting *RAD1*. Rad1 is part of the Rad1-Rad10 complex responsible for cleaving the DNA 5' to the damaged base [15]. Rad1 mutants did not show an increase in spontaneous recombination or mutation rates nor was there increased sensitivity to H_2O_2 or menadione [16].

To investigate cells that were deficient in both BER and NER pathways, *NTG1*, *NTG2*, *APN1*, and *RAD1* (BER^-/NER^- deficient) were deleted. When *RAD1* was deleted in conjunction with *NTG1*, *NTG2*, and *APN1* there was an increase in sensitivity to H_2O_2 and menadione [16]. There was also a synergistic increase in spontaneous recombination and mutation rates in BER^-/NER^- deficient cells revealing that there is an overlap in the repair of oxidative DNA damage by

both the BER and NER pathways [16]. While BER is the primary repair pathway for oxidative DNA damage [15], when the BER pathway is compromised, the NER pathway is able to repair a subset of the oxidative DNA damage present in the cell [16]. When both BER and NER pathways are compromised, then the cell must rely on other DNA damage handling pathways such as homologous recombination and translesion synthesis [15].

Previous studies by our group examined repair proficient (WT) and deficient (BER^-, NER^-, and BER^-/NER^-) strains for the levels of genomic oxidative DNA damage and levels of intracellular ROS [35, 36]. One important observation of these earlier studies was a dose dependent increase in the intracellular levels of ROS in wild type (WT) and BER^- deficient strains, following exposure to methyl methanesulfonate (MMS) (0-55mM) [36]. This increase in ROS levels was associated with an increase in the number of oxidative DNA lesions per genome [36]. In addition, control BER^-/NER^- deficient strains not exposed to DNA damaging agents possessed significantly increased levels of intracellular ROS compared to WT and also contained a significant elevation in spontaneous oxidative DNA lesions [35]. These findings suggest that the increase in ROS levels is indicative of an increase in the levels of oxidative DNA damage [36, 37]. From these studies an intriguing idea arose: part of the cells response to DNA damage is to increase the intracellular levels of ROS. Is the increase in intracellular ROS a general response to all types of DNA damage? What is the biological role of the increased intracellular ROS? And what are the cellular oxidases that produce the DNA damage-induced ROS? In the present studies we begin to examine some of the question.

DNA Damaging Agents

There are numerous DNA damaging agents present in a cell's surrounding environment including environmental toxins, UV light, ionizing radiation, alkylating agents, and oxidizing agents. Each DNA damaging agent produces a unique spectrum of lesions that are handled by

various DNA repair pathways. In this project, three classes of DNA damaging agents were employed.

Methyl Methanesulfonate (MMS)

MMS is an alkylating agent (Figure 3). Alkylating agents are typically electrophilic compounds that react with a nucleophilic center in organic macromolecules (reviewed in [15, 38]). When MMS reacts with DNA, the most common modifications are on N^7-deoxyguanine (82%) and N^3-deoxyadenine (11%) (Figure 3). MMS can also alkylate on N^1-deoxyadenine, N^7-deoxyadenine, N^3-deoxyguanine, and O^6-deoxyguanine (7% for all others) (Figure 3). Methylating the N^3 or N^7 position of purines causes the N-glycosidic bond to be unstable making these bases more susceptible to hydrolysis and subsequent generation of abasic sites, which have mutagenic and toxic consequences [15]. Additionally, N^3-MeA blocks replication [39, 40].

Hydrogen Peroxide (H_2O_2)

H_2O_2 is a nonradical form of ROS that can cause oxidative DNA damage (reviewed in [15]). Compared to other ROS, H_2O_2 is relatively non-reactive and is capable of freely diffusing through the cell. It is believed that H_2O_2, itself, does not cause damage to DNA, but the conversion of H_2O_2 to $^{\bullet}OH$ through the Fenton reaction creates the major reactive species [15]. $^{\bullet}OH$ is highly reactive and can diffuse only short distances in the cell before it reacts with other cellular component [15]. The conversion of H_2O_2 to $^{\bullet}OH$ is believed to occur at or near DNA, following free diffusion of H_2O_2 and subsequent reaction with Fe^{2+} [14]. There are numerous DNA lesions that can be introduced into DNA following exposure to H_2O_2, and over 100 following reaction with $^{\bullet}OH$ [14]. Some examples are thymine glycol, 5-hydroxycytocine, 5-hydroxyuracil, 8-oxoguanine, 8-oxoadenine, abasic sites, and single strand breaks (Figure 4) [13, 15, 41]. Some of these lesions block the replication machinery (i.e. abasic sites), while others

result in mutagenic bypass by DNA polymerase (i.e. 8-oxoguanine), and others do not block the replication machinery and are not mutagenic (i.e. 8-oxoadenine) [41]. All of these lesions are primarily repaired by BER [15]. H_2O_2 is a unique DNA damaging agent in that it also directly activates the oxidative stress response [15, 42].

Ultraviolet light (UV)

UV light can be divided into three categories: 1) UV-A with a wavelength of 400nm – 320nm, 2) UV-B with a wavelength of 320nm – 280nm, and 3) UV-C with a wavelength of 280nm – 100nm (reviewed in [43-45]). UV is a component of sunlight; however, UV-C is efficiently absorbed by ozone in the atmosphere, UV-B is substantially absorbed by ozone in the atmosphere, and UV-A is the only component of UV light to reach the earth's surface in an appreciable amount. Each category of UV light produces distinct, but overlapping, classes of DNA damage (reviewed in [45]). The majority of DNA damage caused by UV-A is through indirect mechanisms of photoactivation of endogenous photosensitizers (i.e. prophyrins, riboflavins, and quinones) that lead to oxidative stress. Therefore, the major DNA lesions produced by UV-A are oxidative DNA damage (i.e. 8-oxoguanine) [46], as described above. The major DNA lesions produced by UV-C are cyclobutyl pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts (6-4PPs) [47] (Figure 5). These are bulky helix-distorting lesions that are repaired primarily by NER. UV-B produces a mixture of lesions that are observed with UV-A (oxidative damage) and UV-C (bulky helix-distorting lesions) [46]. For these studies, UV-C was employed to minimize the formation of indirect, UV-mediated oxidative DNA damage on cells, and to be able to specifically induce DNA damage (CPDs and 6-4 PPs) that is primarily repaired by NER.

Reactive Oxygen Species Scavenging

Oxidative stress has been historically defined as the imbalance of prooxidants and antioxidants [48, 49]. Maintaining the redox balance in cells is essential for proper cellular function and survival. To facilitate this balance several ROS scavenging enzymes (e.g. catalase, superoxide dismutase, and glutathione peroxidase) and small molecules (e.g. glutathione and thioredoxins) have evolved to process excess ROS present in cells [42]. These pathways are highly conserved between yeast and mammalian cells.

Superoxide Dismutase (SOD)

Superoxide dismutases (reviewed in [19]) are proteins with metal ion cofactors, such as copper and zinc, or manganese, iron, or nickel, and are capable of scavenging $O_2^{\bullet-}$ present in cells. SODs remove $O_2^{\bullet-}$ by catalyzing a dismutation reaction involving oxidation of two molecules of $O_2^{\bullet-}$ to one molecule of O_2 and H_2O_2 [17]. In *S. cerevisiae* there are two SODs, Sod1 and Sod2. Sod1 is a Cu/ZnSOD and is located in the cytoplasm. Sod2 is a MnSOD and is located in the mitochondria. In human cells, there are three SODs, SOD1, SOD2, and SOD3 [50]. SOD1 is a Cu/ZnSODs and is found in the cytoplasm, nucleus, and intermembrane space of mitochondria. SOD2 is a MnSOD and is found in mitochondria. SOD3 is a Cu/ZnSOD and is found in the extracellular matrix [51]. While these enzymes are capable of scavenging the reactive $O_2^{\bullet-}$ molecule, in the process they produce another type of ROS, H_2O_2, which can still be harmful to cells [19].

Catalase (CAT)

There are three unrelated families of genes that encode catalases (reviewed in [20, 24]). Manganese-catalases have been found only in prokaryotes. Catalase peroxidases have dual function as both catalases and peroxidases and are widely distributed in prokaryotes, but have

also been found in eukaryotes [52]. Classical catalases are homotetrameric, heme-containing enzymes capable of degrading H_2O_2 by dismutation to H_2O and O_2 in a two step process [53]. In the first step of a classical catalase reaction, one H_2O_2 molecule is reduced to water and the $Fe^{3+}$of the catalase is converted to $Fe^{5+}O$. In the second step of the reaction, , along with $Fe^{5+}O$, a second H_2O_2 molecule is reduced to a second molecule of H_2O and O_2 and $Fe^{5+}O$ is converted back to Fe^{3+} [20, 53]. The family of "classical" catalases is found throughout eukaryotes, but are also found in prokaryotes [20]. In *S. cerevisiae* there are two catalases that belong to the classical family of catalases, Cta1 and Ctt1. Cta1 is localized to the mitochondria and peroxisomes [54], while Ctt1 is localized to the cytoplasm [55]. In humans, there is only one classical catalase, CAT [56].

Other ROS Scavenging Molecules

There are several other enzymes and small molecules that are capable of scavenging ROS (reviewed in [42]). Small molecule, non-enzymatic ROS scavengers include glutathiones, ascorbic acid, thioredoxins, and glutaredoxins. Enzymatic scavengers of ROS include glutathione reductase, glutathione peroxidase, thioredoxin peroxidase, and thioredoxin reductase. Many of these proteins are involved in the thiol redox pathway in yeast, which consists of the thioredoxin and glutathione pathways [57].

Yeast Activator Protein - 1 (Yap1)

Yap1 belongs to a family of proteins that contain a conserved bZIP DNA-binding domain, consisting of a leucine zipper that mediates dimerization, and an adjacent basic region that specifically interacts with DNA sequences [58] (Figure 6B). Yap1 is a homolog of the mammalian activator protein-1 (AP-1) [59]. In mammalian cells, there are a number of AP-1 transcription factors that include Jun, Fos, and ATF [8]. AP-1 transcription factors form homo- and heterodimers, and the formation of each seems to have a distinct function [8]. AP-1 binds to

DNA regions that contain an AP-1 response element (ARE) via the basic region. The ARE consensus sequence in mammalian cells is TGACTCA [60]. AP-1 factors are involved in the upregulation of genes in response to a number of stress situations [8].

The yeast AP-1 family (Yap) consists of eight members: Yap1 through Yap8 [58]. They differ from their mammalian counterparts in that some of the active site residues that are highly conserved in mammalian cells are different in yeast, and their preferred DNA binding site is TTACTAA [58]. The functional domains of Yap1 include a nuclear export sequence (NES), nuclear localization sequence (NLS), bZIP domain, and two cysteine rich domains: nCRD and cCRD [61]. Yap1, the most extensively studied of the yeast AP-1 family of proteins, is activated (via translocation to the nucleus) in response to oxidative stress [7, 62-65].

Under normal growth conditions, Yap1 is actively transported out of the nucleus by the nuclear export factor Crm1 [66] (Figure 7A). Crm1 is a member of the β-karypherin (β-importins) family of proteins and binds to substrates only in the presence of Ran-GTP [66, 67]. The Yap1 NES is embedded within the cCRD [68]. Once in the cytoplasm the Crm1/Ran-GTP is converted to Crm1/Ran-GDP which is enhanced by Ran–GTPase-activating protein (RanGAP) and Crm1 dissociates from Ran-GDP and its cargo, Yap1 [69]. Therefore, under basal conditions, Yap1 is rendered inactive by its rapid export from the nucleus to the cytoplasm by Crm1 [66].

Under oxidative stress condition Yap1 is no longer exported from the nucleus by Crm1due to the formation of intramolecular disulfide bonds [61] (Figure 7B). Yap1 contains six cysteine residues: three are in the cCRD, and three are in the nCRD [70] (Figure 6). These cysteine residues can form both intradomain and interdomain disulfide bonds [70]. In a study by Okazaki, *et. al*., the formation of the disulfide bonds, and the order in which they appear in response to H_2O_2 was determined [70] (Figure 6B). Initially, an interdomain disulfide bond forms that is likely to block the NES, therefore inhibiting Crm1 binding, and sequestering Yap1 in the nucleus. For Yap1 to be fully functional as a transcription factor, two additional interdomain

disulfide bonds are formed [70]. The oxidation of cysteine bonds in Yap1is not always direct, but can be facilitated by a thiol peroxidase, Gpx3 [64] (Figure 7B). Gpx3 and Yap1 form a transient intermolecular bond that aids in the oxidation and activation of Yap1 [64]. The redox state of Yap1 is also coupled to the thioredoxin pathway in that Yap1 is reduced by thioredoxin to return its fully reduced state [63].

Yap1 upregulates numerous genes in response to oxidative stress, including *SOD1*, *CTT1*, and thioredoxin (*TRX2*) [71]. These genes are directly involved in the scavenging of ROS. Furthermore there is indirect evidence through genome-wide studies that Yap1 can also upregulate genes that are involved in DNA repair (*NTG1* and *MAG1*) and in DNA check point control (*POL1*, *MEC1*, and *POL3*) [72, 73].

Summary of Project Objectives

This research project examines the involvement of ROS in the DNA damage response. We employed the *S. cerevisiae* model system and utilized several repair deficient mutants (BER^-, NER^-, and BER^-/NER^-), as well as several ROS scavenging mutants (*sod1Δ*, *sod2Δ*, *cta1Δ*, and *ctt1Δ*), to determine the levels, sub-cellular localization, and subspecies of ROS produced in response to DNA damaging agents that produce damage repaired by either BER (MMS) or NER (UV-C). We determined that an increase in intracellular ROS is a general DNA damage response; however, aspects of this response are unique to the type of DNA damage produced. Following exposure to MMS, there is a dose-dependent increase in $O_2^{\bullet-}$ levels that are localized to the cytoplasm; however, following exposure to UV-C, while there is a dose-dependent increase in $O_2^{\bullet-}$ levels, it is localized to the mitochondria. The difference in localization could be due to the type of DNA damage produced, or to the cellular toxicity of the damage itself. Our findings support the hypothesis that the increase in intracellular ROS levels is biologically relevant and involved in cellular signaling processes.

Yap1 was utilized as a biological sensor of the intracellular redox state in cells following exposure to DNA damaging agents (MMS and UV-C). We examined the intracellular localization of Yap1 in response to DNA damaging agents, and observed that Yap1 relocalizes to the nucleus following exposure to MMS, but not UV-C. A novel finding in this study was that Yap1 is involved in the maintenance of genomic integrity. Increased mutation rates and large scale chromosomal rearrangements (chromosomal instability) were observed in *yap1Δ* strains compared to WT cells. These studies provide insight into the consequences of genotoxic stress in cells and a potential new responder to DNA damage.

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Figure 1. Base Excision Repair. Base excision repair recognizes small helix non-distorting lesions (*). These lesions are first recognized by a DNA glycosylase that removes the damaged base leaving an AP site. The AP site is then processed either by an AP lyase (right sub-pathway) or an AP endonuclease (left sub-pathway). An AP lyase cleaves 3′ to the AP site leaving a 3′ phosphate group (P) or a 3′ unsaturated aldehyde (UA). A 3′ phosphodiesterase or 3′ phosphatase is able to remove the blocking group left by an AP lyase. Alternatively, an AP endonuclease cleaves 5′ to the AP site leaving a 5′ dRP and a 3′ OH. A dRPase is able to remove the blocking group. After processing of the AP site, the remaining gap is filled in by a DNA polymerase and the nick is sealed by a DNA ligase. Depending partially on the DNA glycosylase and the resulting AP site, and on the stage of the cell cycle, the process may proceed through long patch repair. In long patch repair, the 5′ DNA blocking group is displaced by a DNA polymerase creating a flap. A flap endonuclease then cleaves the flap and DNA ligase seals the nick. A list of proteins that function in yeast and human BER are listed in Table 1.

Base Excision Repair

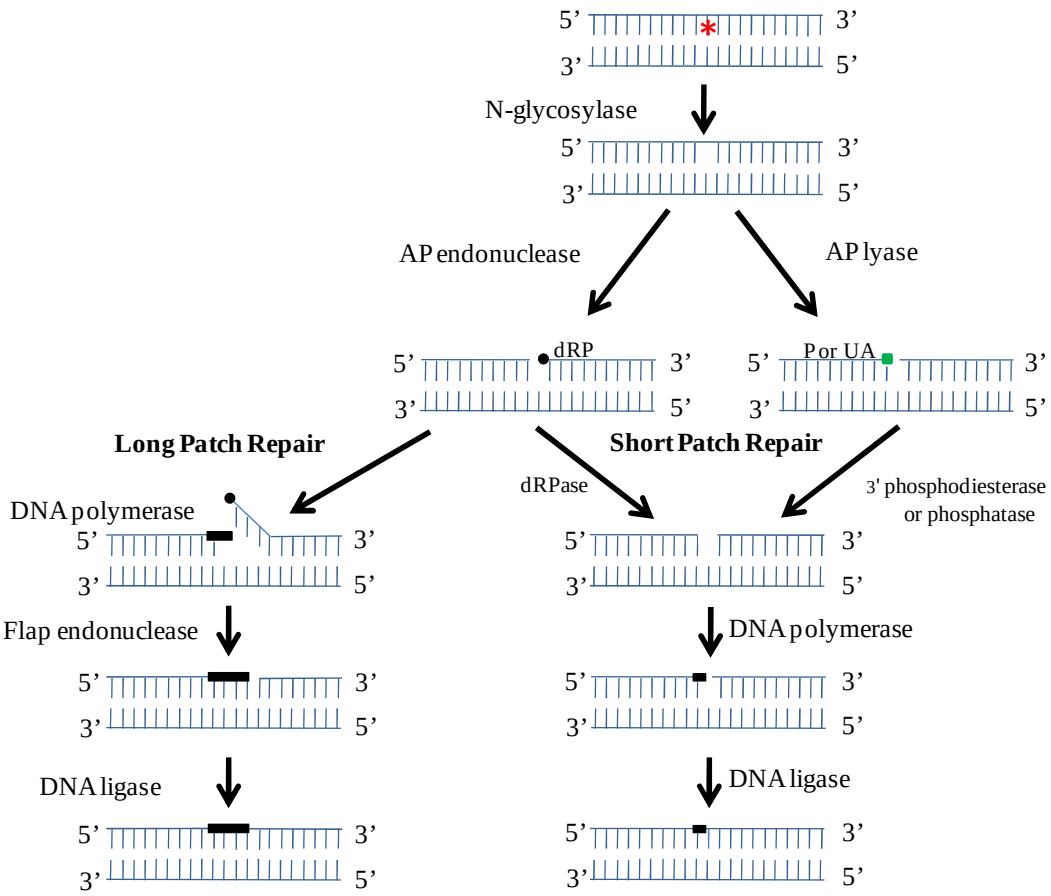


Figure 1. Base Excision Repair. Adapted from [31].

Table 1. Proteins involved in Base Excision Repair

			Activity
Organism	*S. cerevisiae*	Human	
DNA Glycosylases			major altered base released
	UNG1	*UNG*	U
		SMUG1	U
		MBD4 (MED1)	Uor T opposite G at CpG sequences
	OGG1	*OGG1*	8-oxo-G opposite C
		MYH	A opposite 8-oxo-G
	NTG1, NTG2	*NTHL1 (NTH1)*	Ring saturated or fragmented pyrimidines
	MAG1	*MPG (MAG, AAG)*	3-meA,ethenoA, hypoxanthine
		NEIL1	removes thymine glycol
		NEIL2	removes oxidative products of C,U
		NEIL3	removes fragmented/oxidized pyrimidines
AP endonucleases			
	APN1	--	endonuclease
	APN2	*APEX1 (HAP1. APE1, REF1)*	endonuclease
		APEX2 (APE2)	endonuclease
Other BER Factors			
		XRCC1	accessory factor for DNA Lig III
	RAD 27	*FEN1*	flap endonuclease
		ADPRT	Poly(ADP-ribose)polymerase (PARP)
	DNA Pol δ or ε	*DNA Pol* δ or ε	DNA polymerase
		DNA Pol β	DNA polymerase
	DNA Lig I	*DNA Lig I*	DNA ligase
		DNA Lig III	DNA ligase

Figure 2. Nucleotide Excision Repair. Nucleotide excision repair recognizes bulky helix distorting lesions. Transcription coupled repair (TCR) is selective for lesions that are present in the transcribed strand of expressed genes, while global genome repair (GGR) acts over the rest of the genome in transcriptionally silent regions as well as the non-transcribed strand of expressed genes. In TCR (not depicted in this figure), proteins are recruited to the site of DNA damage due to a stalled RNA polymerase, and not through recognition of the DNA damage by recognition proteins as with GGR. In GGR, the DNA damage is recognized by proteins that detect distortions in the DNA. Once the DNA is detected, a complex of proteins is recruited to the site of DNA damage. This first complex contains DNA helicases that unwind the DNA allowing room for the second complex to bind. Once the DNA is unwound, the second complex binds to the DNA that contains two endonucleases, one that cleaves 3′, and one that cleaves 5′ to the DNA damage. Following cleavage of the DNA backbone, DNA polymerase resynthesizes a new strand, and DNA ligase seals the nick. A list of proteins that function in yeast and human NER are listed in Table 2.

Nucleotide Excision Repair

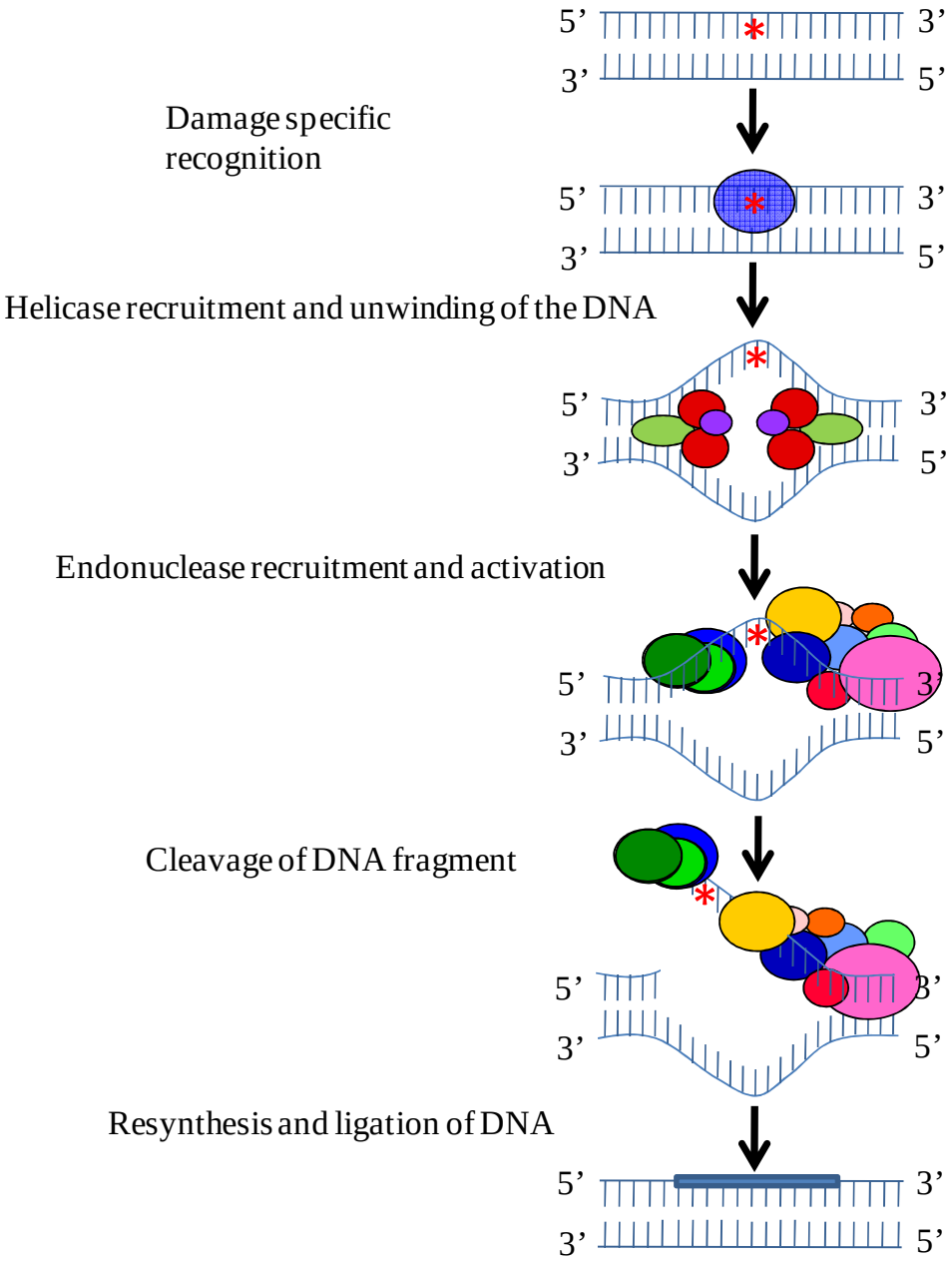


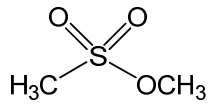
Figure 2. Nucleotide Excision Repair. Adapted from [74]

Table 2. Proteins involved in Nucleotide Excision Repair

			Activity
Organism	*S. cerevisiae*	Human	
DNA damage recognition proteins	*RAD4*	*XPC*	Binds distorted DNA as a complex
	RAD23	*RAD23B*	
		RAD23A	RAD23 paralog
	RAD14	*XPA*	Binds DNA and proteins in a preincision complex
TFIIH subunits			
	RAD25	*XPB*	3′ to 5′ DNA helicase
	RAD3	*XPD*	5′ to 3′ DNA helicase
NER nucleases			
	RAD2	*XPG*	3′ incision nuclease
	RAD10	*XRCC1*	5′ incision nuclease
	RAD1	*XPF*	
Other NER Factors			
	CDC9	*LIGI*	DNA end joining
	RFA1	*RPA1*	Binds ssDNA intermediates in recombination, NER and gap-filling pathways
	RFA2	*RPA2*	
	RFA3	*RPA3*	
	RAD28	*CSA*	Needed for TCR
	RAD26	*CSB*	Needed for TCR

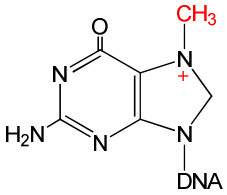
Figure 3. Methyl methanesulfonate induced alkylation base damages. A. Structure of methyl methanesulfonate. **B.** The most common types of DNA damage are N^7-methylguanine (82%) and N^3-methyladenine (11%). However, to a lesser extent MMS also induces formation of N^1-methyladenine, N^7- methyladenine, N^3-methylguanine, and O^6-methylguanine (7% for all others).

A.

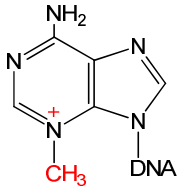


Methyl Methanesulfonate

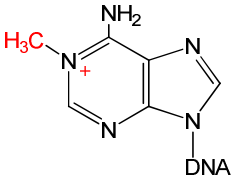
B.



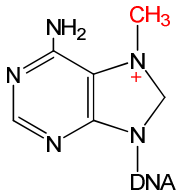
N^7 methylguanine



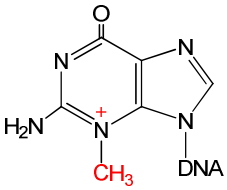
N^3 methyladenine



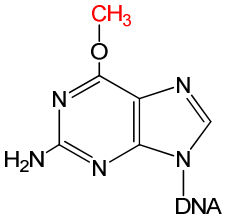
N^1 methyladenine



N^7 methyladenine



N^3 methylguanine

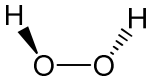


O^7 methylguanine

Figure 3. Methyl methanesulfonate induced alkylation base damage products.

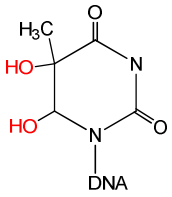
Figure 4. Hydrogen peroxide induced oxidative base damage products. A. Structure of H_2O_2. **B.** Numerous DNA lesions can be produced following exposure to H_2O_2. Some examples are thymine glycol, 5-hydroxycytosine, 5-hydroxyuracil, 8-oxoguanine, 8-oxoadenine, abasic sites (AP), and single strand breaks

A.

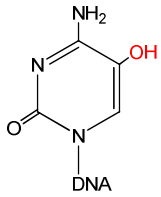


Hydrogen Peroxide

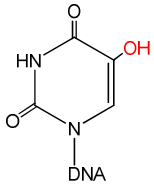
B.



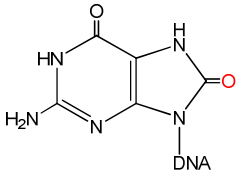
Thymine Glycol



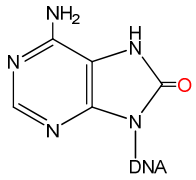
5-hydroxycytocine



5-hydroxyuracil



8-oxoguanine



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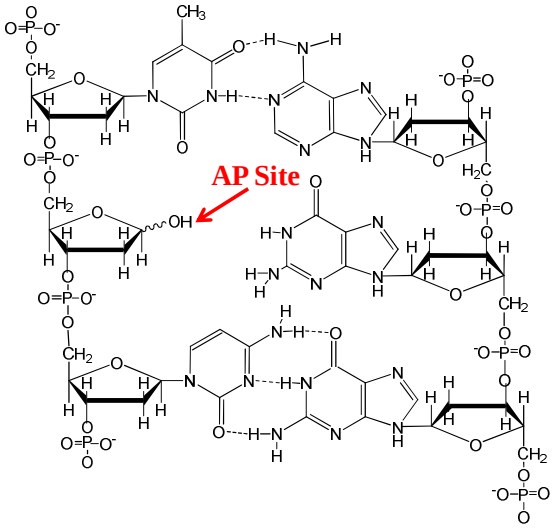
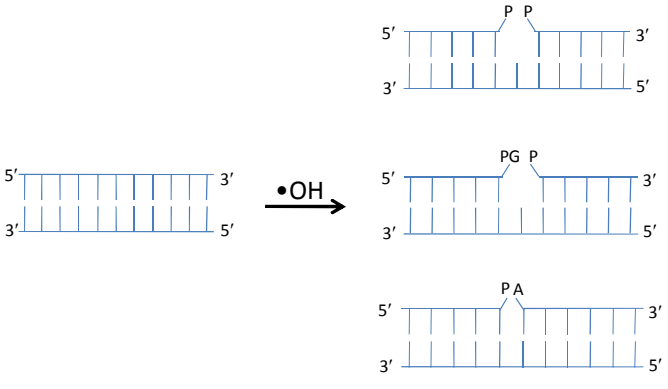


Abasic Site



Single Strand Break

Figure 4. Hydrogen peroxide induced DNA damage.

Figure 5. UV-C induced base damages. A. UV light is a component of sunlight and can be divided into three components: 1) UV-A (400nm – 320nm) reaches the earth's surface 2) UV-B (320nm – 280nm) is significantly absorbed by the ozone layer of the earth's atmosphere and 3) UV-C (280nm – 100nm) is completely absorbed by the earth's upper atmosphere and does not reach the earth's surface. **B.** The major DNA lesions produced by UV-C are cyclobutyl pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts (6-4PPs). UV-A does not produce these lesions to any appreciable amount. UV-B produces CPDs and 6-4PPs, as well as other oxidative DNA damages of the types produced by UV-A.

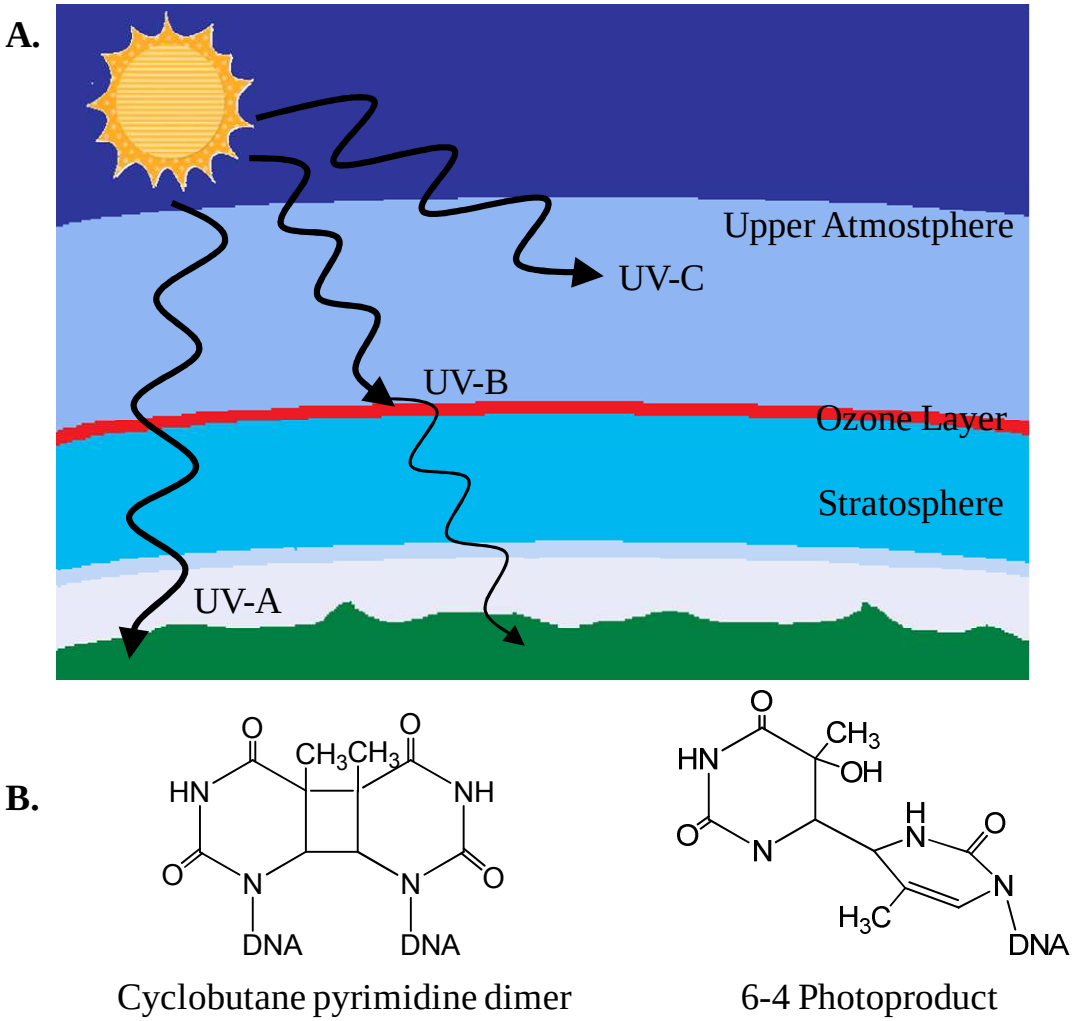


Figure 5. UV-C induced base damages.

Figure 6. Schematic of Yap1 domains and binding to Crm1. A. Yap1 domains. There are two cysteine rich domains (CRD) located in Yap1: nCRD and cCRD. Yap1 contains three conserved regions; the NLS is the nuclear localization sequence (red), the bZIP is the DNA binding domain (blue), and the NES is the nuclear export sequence (green), which is located within the cCRD. **B.** The binding of Yap1 to the nuclear export protein Crm1 is dependent on the oxidation of cysteine residues within the cCRD and nCRD domains. Under normoxic environments Yap1 is in the reduced state, allowing for recognition and binding to Crm1. Under oxidative stress up to three disulfide bonds can form, blocking the binding of Crm1 and resulting in Yap1 sequestration in the nucleus.

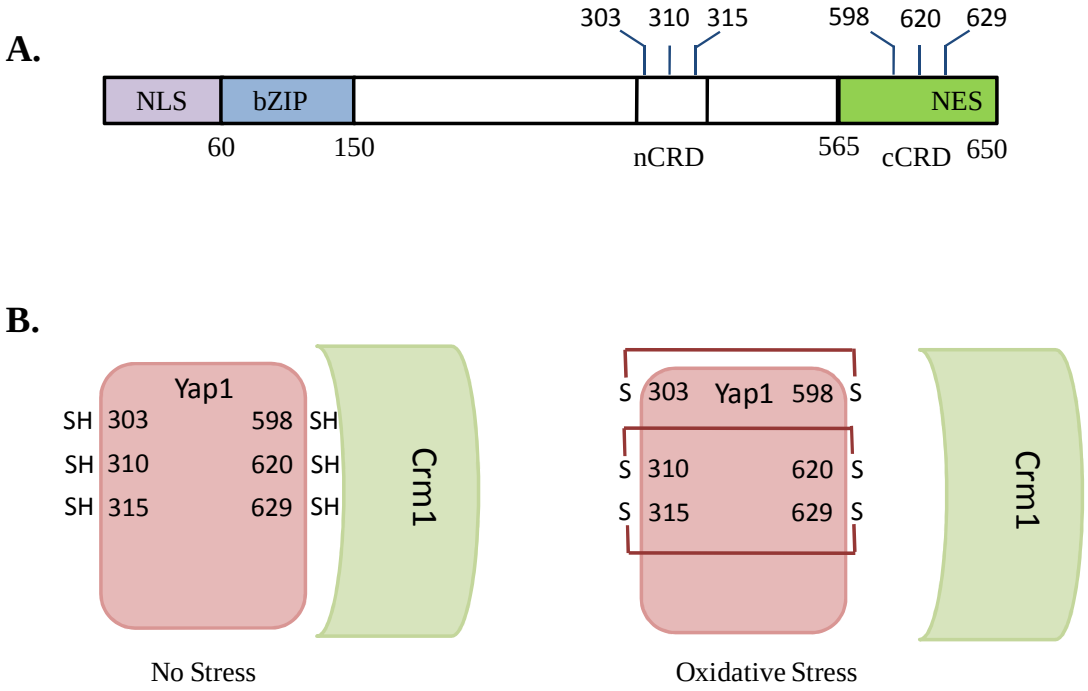


Figure 6. Schematic of Yap1 domains and binding to Crm1.

Figure 7. Model of Yap1 localization and activation. A. Under normal, non-stressed condition Yap1 is rapidly exported from the nucleus by the nuclear export factor Crm1. Crm1 binds to its cargo (Yap1) in the presence of RanGTP. After exportation from the nucleus RanGTP is reduced to RanGDP and Crm1 releases both RanGDP and its cargo (Yap1). Yap1 can freely diffuse into the nucleus, however the rate of export is faster than the diffusion of Yap1 into the nucleus and therefore Yap1 accumulates in the cytoplasm. **B.** Under oxidative stress conditions Yap1 either through direct oxidation of cysteines or indirect oxidation of cysteines via Gpx3 can forms up to three interdomain disulfide bonds that block the binding of Crm1. With Crm1 no longer able to bind to Yap1 and export it from the nucleus, Yap1 accumulates in the nucleus where it functions as a transcription factor. The formation of one disulfide bind is sufficient to block the binding of Crm1; however, two or three disulfide bonds are necessary for full activation of Yap1 as a transcription factor.

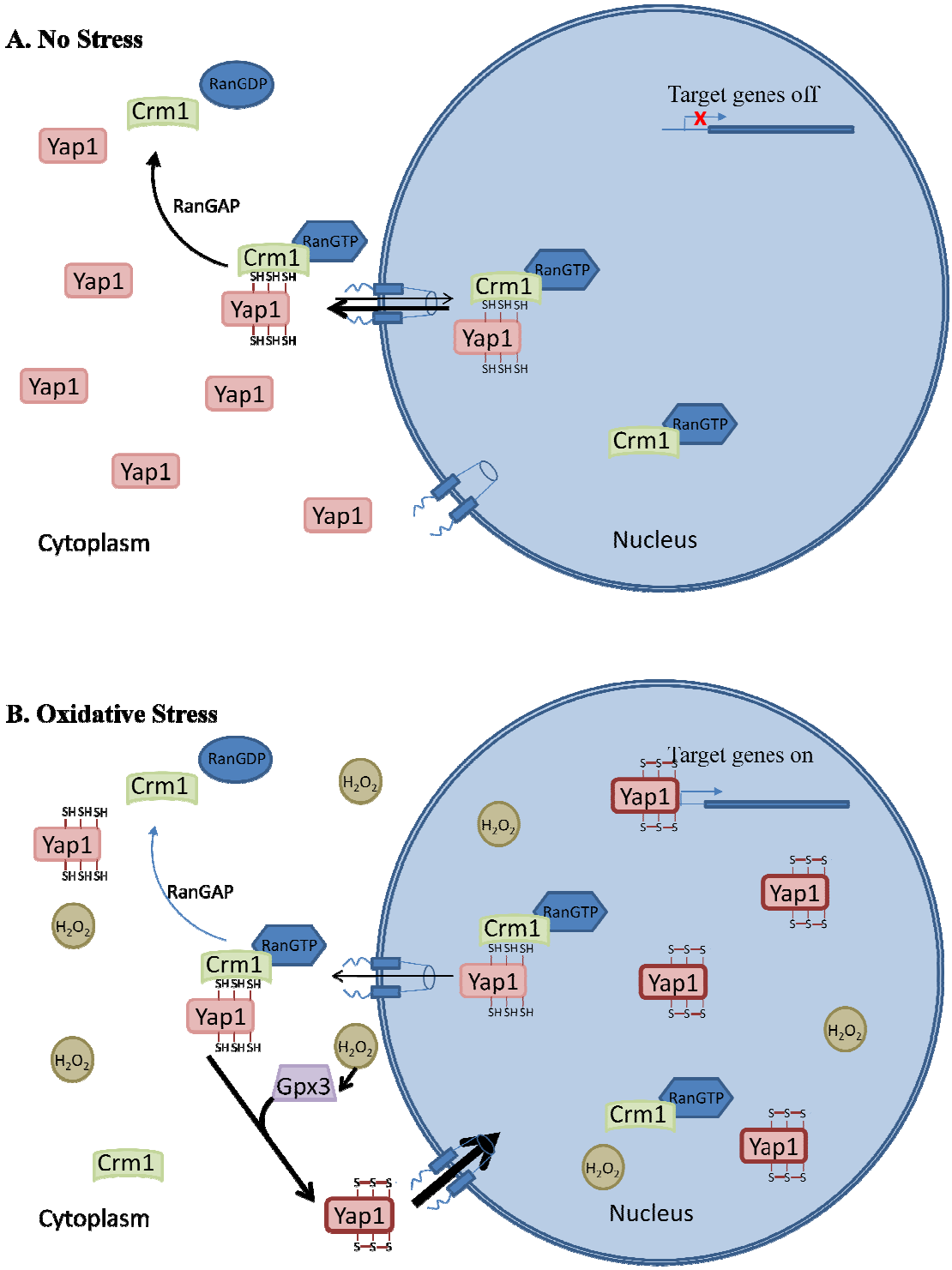


Figure 7. Model of Yap1 localization and activation

CHAPTER II

DNA Damage-induced Reactive Oxygen Species (ROS) Stress Response in *Saccharomyces cerevisiae*

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All experiments were performed by Lori Rowe

Abstract

Cells are exposed to both endogenous and exogenous sources of reactive oxygen species (ROS). At high levels, ROS can lead to impaired physiological function through cellular damage of DNA, proteins, lipids, and other macromolecules, which can lead to certain human pathologies including cancers, neurodegenerative disorders, and cardiovascular disease, as well as aging. We have employed *Saccharomyces cerevisiae* as a model system to examine the levels and types of ROS that are produced in response to DNA damage in isogenic strains with different DNA repair capacities. We find that when DNA damage is introduced into cells from exogenous or endogenous sources there is an increase in the amount of intracellular ROS which is not directly related to cell death. We have examined the spectrum of ROS in order to elucidate its role in the cellular response to DNA damage. As an independent verification of the DNA damage-induced ROS response, we show that a major activator of the oxidative stress response, Yap1, re-localizes to the nucleus following exposure to the DNA alkylating agent methyl methanesulfonate. Our results indicate that the DNA damage-induced increase in intracellular ROS levels is a generalized stress response that is likely to function in various signaling pathways.

Introduction

Cells are continuously exposed to numerous exogenous and endogenous agents that damage DNA. DNA damage alters replication and transcription, causes cell death and can lead to mutations and neoplastic transformation in many organisms. Reactive oxygen species (ROS)-mediated deleterious effects are thought to contribute to human degenerative conditions including neurological disorders [1], cardiac dysfunction [2], and cancer [3], as well as the process of aging [4]. While ROS have been shown to be deleterious to cells, they also can function as stress-induced signaling molecules [5-8]. Recent reports indicate that DNA damage alone results in increased levels of intracellular reactive oxygen species (ROS) [9, 10]. In response to oxidative stress, cells activate both the DNA repair processes and transcription factors. These factors in turn, modulate levels of expression of ROS-scavenging and processing enzymes [11, 12]. For example, increased levels of intracellular ROS cause post-translational modifications of one such transcription factor, Yap1, resulting in induction of numerous genes [13-17].

In order to maintain genomic stability under ROS-induced stress, cells have evolved a number of pathways to repair or respond to the presence of DNA damage. In *Saccharomyces cerevisiae*, these pathways include direct reversal, base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), translesion synthesis (TLS), and recombination (REC) [18]. While some of these pathways mediate the repair of the damaged DNA (direct reversal, BER, NER, and MMR) others function to bypass the damage such that the DNA lesion is tolerated and replication can occur (TLS and REC). All of these pathways are individually important to the cell, however there is overlap in the types of DNA damage handled by each pathway [10, 18]. BER is primarily responsible for the repair of small, non-bulky base lesions and abasic sites, such as those caused by oxidizing and alkylating agents [19]. NER is primarily responsible for the repair of bulky, DNA helix distorting lesions such as UV light-induced cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4 PPs) [19].

We have previously reported that various types of spontaneous and chemically induced DNA damage cause increases in intracellular ROS in repair-proficient (wild type, WT) and repair-deficient *S. cerevisiae* strains [20, 21]. Cells deficient in both BER and NER (BER^-/NER^-) spontaneously accumulate approximately 800-fold more oxidative DNA damage than WT cells that correlated with a substantial increase in intracellular ROS in repair-deficient cells [10]. In a separate study, Salmon *et. al.* examined the levels of ROS in WT and repair deficient yeast strains (BER^-, BER^-/REC^-, and NER^-/REC^- deficient strains) following exposure to the oxidative DNA damaging agent hydrogen peroxide (H_2O_2) or the DNA alkylating agent methyl methanesulfonate (MMS) [21]. These studies revealed a dose-dependent increase in intracellular levels of ROS in all four isogenic strains after exposure to either H_2O_2 or MMS, suggesting that DNA damage alone is capable of causing an increase in intracellular ROS.

A major issue emerging from the above observations is whether the increase in ROS is a response to specific types of DNA modifications or a general DNA damage-induced stress response. Another important issue concerns the nature of the subspecies of ROS, such as superoxide ($O_2^{\bullet-}$), H_2O_2, and hydroxyl radical ($^{\bullet}OH$), produced in response to DNA damage. A major goal of this study was to further define the nature of DNA damage capable of inducing ROS and to characterize the subspecies of ROS that were produced in response to such damage. Such information is crucial for delineation of the biological role of ROS and the mechanisms leading to their production in a putative DNA damage-induced ROS stress response.

As an independent method for verifying the production of DNA damage-induced intracellular ROS, we also examined the cellular response of a ROS sensor and transcription factor, Yap1. Yap1 is important in the oxidative stress response in *S. cerevisiae* [11, 16, 17]. Oxidative stress within a cell can be viewed as an imbalance between ROS production and ROS scavenging/metabolizing capacity. When an imbalance leading to oxidative stress occurs, Yap1 is activated to rapidly upregulate gene expression of enzymes (e.g. catalase, superoxide dismutase, and glutathione peroxidase) and small molecules (e.g. glutathione and thioredoxins)

capable of scavenging ROS [22]. H_2O_2 activates Yap1 by inducing disulfide bond formation between C303 and C598, causing the release of the nuclear export protein Crm1p and resulting in nuclear accumulation of Yap1 [14, 23]. Once in the nucleus, Yap1 functions as a transcription factor activating numerous genes that are involved in the oxidative stress response [16, 17, 24, 25]. Thus, Yap1 serves as a biological sensor for elevated ROS and also as a key mediator of the ROS-activated signaling pathway.

In this study, we have defined the levels and types of ROS induced by DNA damage that are influenced by two major DNA excision repair pathways (BER and NER) and find that there is a dose-dependent increase in ROS in all cell types following exposure to the DNA alkylating agent MMS or short wavelength ultraviolet light (UV-C). We also find that there is a dose-dependent increase in several types of ROS ($O_2^{\bullet-}$, H_2O_2, and $^{\bullet}OH$) that were evaluated. However, the specific patterns and magnitudes of the observed ROS increases varied, depending on the nature of the DNA damaging agent and the cellular DNA repair background. We examined the role of Yap1 as a potential mediator of the DNA damage response by monitoring its subcellular localization following exposure to MMS in DNA repair proficient cells. We found that Yap1 relocalizes to the nucleus in response to MMS exposure and that this effect is similar to that seen with direct exposure to H_2O_2. The activation of Yap1 in response to MMS further supports the idea that the increase in ROS in response to DNA damage may function in signaling processes.

Experimental Procedures

Strains, Media, and GrowthConditions

The set of isogenic *S. cerevisiae* strains used in this study was derived from wild type (WT) SJR751 (*MATα ade2-101oc his3Δ200 ura3ΔNco lys2ΔBgl leu2-R*). The genotypes derived from SJR751 are BER$^-$ deficient strain SJR867 (*MATα ade2-101oc his3Δ200 ura3ΔNco lys2ΔBgl leu2-R ntg1Δ::LEU2 ntg2Δ::hisG apn1D1::HIS3*), NER$^-$ deficient strain SJR868 (*MATα ade2-101oc his3Δ200 ura3ΔNco lys2ΔBgl leu2-R rad1Δ::hisG*), and BER$^-$/NER$^-$ deficient strain SJR1101 (*MATα ade2-101oc his3Δ200 ura3ΔNco lys2ΔBgl leu2-R ntg1Δ::LEU2 ntg2Δ::hisG apn1D1::HIS3 rad1Δ::hisG*). All of the SJR derived strains were constructed as previously reported [26]. Yeast strains were grown on YPD media (1% yeast extract, 2% peptone, 2% dextrose and 2% agar for plates). All YPD media were supplemented with 0.5% adenine sulfate. For selection of strains containing the Yap1-GFP plasmid, the strains were grown on SD-minimal –URA media (0.5% ammonium sulfate, 0.17% yeast nitrogen base without amino acids, 2% dextrose, 0.14% minimal Ura drop out mix, and 2.5% agar for plates) [27].

Cell Growth and Viability

Liquid YPD media was inoculated with yeast cells and grown at 30 °C for ~24 hrs to a density greater than 7 x 10^7 cells/mL. The density of the cells was determined by counting on a hemacytometer. Fifty milliliters YPD cultures were inoculated with an appropriate amount of cells so that the culture would reach a density of 2 x 10^7 after 12 hrs of growth at 30 °C. Cell viability was determined by plating on YPD after exposure to MMS or UV-C. Cultures were diluted to a density that would yield approximately 100-200 colonies per plate.

YAP1-GFP Plasmid Construction

For studies of the subcellular localization of Yap1, WT strains were transformed with pLR1 plasmid. The pLR1 plasmid is a centromeric vector containing a *YAP1::GFP* fusion protein and a *URA3* marker. The plasmid was constructed using the "Drag&Drop" method of cloning [28]. The original pLDB419 plasmid is a *YAP1::GFP LEU2* 2μ plasmid [23]. We amplified by PCR the *YAP1::GFP* DNA fragment from pLDB419 using the following primers. 5'-CACTATAGGGCGAATTGGAGCTCCACCGCGGTGGCGGCCGCTCTAATGACCA TGATTACGAATTCGAGCT-3' and the reverse primer was 5'-GGGAACAAAAGCTGGGTA CCGGGCCCCCCCTCGAGGTCGACGGTCCAAGCTTGCATGCCTGCAGGTCGT-3'. The sequences of these primers are homologous to the flanking sequences of *XbaI-XhoI* digested plasmid pRS306 [29]. Co-transformation of the WT strain with the *YAP1::GFP* containing PCR fragment and *XbaI-XhoI* digested plasmid pRS306 and following rescue of the plasmid yielded pLR1.

Measurement of Reactive Oxygen Species Levels

ROS subspecies ($O_2^{\bullet-}$, H_2O_2, and $^{\bullet}OH$) were detected using a panel of fluorescent probes (Table 1). Cells were grown to mid-log (~2 x 10^7 cells/mL) in YPD at 30 °C overnight. Cells were counted (hemacytometer), washed twice in H_2O and then adjusted to 2 x 10^7 cells/mL in H_2O. Cells were then exposed to various doses of either UV-C or MMS. For experiments involving UV irradiation, cells (15 mL) were placed in a 15 mm petri dish and exposed to a range of UV-C doses (0-50 J/m^2). Immediately after UV exposure, cells were placed in the dark to prevent photoreactivation. For experiments involving MMS, cells (3 mL) were placed in the dark and exposed to a range of MMS doses (0-55 mM) for 30 min at 30 °C. Following exposure to MMS or UV-C, 1 mL aliquots were plated for survival measurements and 2 mL were incubated with various fluorescent probes (Dihydrorhodamine (DHR), 25 μg/mL; Dihydroethidium

(DHEt), 50 μg/mL; N-can-Acetyl-3,7dihydroxy-phenoxazine (Amplex Red (APR)), 12 μg/mL; and 2-[6-(4'-Hydroxy)phenoxy-3*H*-xanthen-3-on-9-yl] benzoic acid (HPF), 20 μg/mL) for ROS measurements. Immediately following fluorescent probe addition, cells were held in the dark and incubated at 30 °C for 2 hrs. Cells were subsequently washed twice with H_2O and then resuspended in 2 mL phosphate buffered saline (PBS) and assessed for fluorescence intensity employing a BD™ LSR II flow cytometer (BD Biosciences).

Yap1 Cellular Localization Studies

Cells were grown to mid-log phase (~2 x 10^7 cells/mL) in YPD at 30 °C overnight, counted, and washed twice with H_2O, and then adjusted in H_2O to a density of 2 x 10^7 cells/mL. Cells were then stained with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen) to visualize DNA in nuclei and mitochondria. Cells were incubated with 1 μL 100 nM DAPI/mL of cells for 5 min, washed once with H_2O, and then resuspended to the original volume in H_2O. Cells were then exposed to either H_2O_2 or MMS as described above. Cells were subjected to fluorescent confocal microscopy (Zeiss LSM510 META) and images were analyzed using the Carl Zeiss LSM Image Browser software.

Results

In the present study, we used the budding yeast *S. cerevisiae*, as a model system to address whether cells exhibiting compromised DNA damage repair contain increased levels of ROS. Previously, we reported that unrepaired, endogenously generated oxidative DNA damage accumulates in cells deficient in both BER$^-$ (*ntg1Δ ntg2Δ apn1Δ* triple mutant) and NER$^-$ (*rad1Δ* mutants) (BER$^-$/NER$^-$) and is accompanied by an increase in intracellular ROS [10]. Despite the fact that Rad1p functions in homologous recombination, it mediates the relatively minor role of removing of the heterologies during strand invasion and single strand annealing (reviewed in [30]). These studies suggested that even non-toxic or moderately toxic DNA damage might be capable of mediating the ROS response. However, the specific types of ROS involved were not identified due to the utilization of DHR, a fluorescent probe that detects multiple ROS species including H_2O_2, $^{\bullet}OH$, and $NO^{\bullet}$ [31, 32] (Table 1).

Two major objectives of the present study were (i) to determine whether the ROS response could be elicited by different classes of DNA damaging agents, thus implicating a role for ROS in a general genotoxic stress response and (ii) to define the nature of the stress response with respect to individual types of ROS induced by different classes of DNA damage. Taking into account that cell death could cause the release of ROS [33, 34], we first defined the cytotoxicity profiles for MMS and UV-C in cells defective in different repair pathways.

Cytotoxicity of MMS and UV-C in Yeast Strains with Different DNA Repair Backgrounds

To delineate the potential relationship between DNA damage-induced cell death and intracellular ROS levels, dose dependent cytotoxicity profiles were determined for isogenic strains with different DNA repair deficiencies (WT, BER$^-$, NER$^-$, and BER$^-$/NER$^-$) exposed to MMS or UV-C. MMS induces several different alkylation base damage products that are primarily repaired by BER [35]. Thus, it could be expected that BER$^-$ deficient cells should

exhibit the greatest cytotoxicity in response to MMS exposure. Cells were exposed to a range of doses (0-55 mM) of MMS and the cytotoxicity was determined. Severely repair-deficient cells (BER^-/NER^-) exhibit a moderate (35%) decrease in survival upon exposure to low doses (0.5 mM) of MMS and exhibited extreme sensitivity to higher doses (Fig. 1 A). In contrast, for repair-proficient strains (WT) and single pathway-compromised (BER^- deficient and NER^- deficient) strains, significant decreases in survival are observed only with exposure to doses above 5 mM MMS. At such higher MMS doses, the single pathway-deficient mutants (BER^- or NER^-) displayed greater sensitivity than repair-proficient cells (WT), with BER-deficient strains exhibiting greater sensitivity compared to NER^- deficient strains. Thus, as might be expected, MMS sensitivity is variable depending on the DNA repair capacity of the cells, with cells deficient in BER alone or in combination with NER displaying the greatest sensitivity.

Short wavelength ultraviolet radiation (UV-C) induces toxic, mutagenic bipyrimidine photoproducts that are repaired primarily by the NER pathway [36]. Cells were exposed to a range of doses (0-50 J/m^2) of UV-C and the cytotoxicity was determined. As expected, repair proficient (WT) and BER^- deficient cells exhibited similar sensitivities to UV-C with NER^- and BER^-/NER^- deficient strains displaying extreme sensitivity (less than 1% survival) at lower doses (5 J/m^2) (Fig. 1 B). These experiments established a cytotoxicity profile for MMS and UV-C for each isogenic strain harboring DNA damages primarily repaired by BER or NER, respectively. Such profiles were then utilized to determine whether DNA damage-induced cytotoxicity was directly related to DNA damage-induced increases in intracellular ROS levels.

Endogenous DNA Damage and Intracellular ROS Levels in DNA Repair Compromised Strains

To determine the levels of individual types of ROS, WT, BER^-, NER^-, and BER^-/NER^- deficient strains were analyzed using a panel of florescent probes including DHR, DHEt, HPF, and APR. These probes detect different, specific subspecies of ROS (Table 1). As discussed above, DHR is a relatively non-specific probe as it will detect a variety of ROS subspecies [31,

32]. In contrast, DHEt is specific for $O_2^{\bullet-}$ [37, 38], APR is specific for H_2O_2 [39, 40], and HPF is specific for $^{\bullet}OH$ [41]. Cells treated with DHR (Fig. 2 A) revealed that ROS levels (multiple species) were significantly increased (approximately 30%) in the BER$^-$/NER$^-$ deficient cells compared to repair proficient cells (WT). However, with this probe there was no increase observed in the strains deficient in either BER or NER pathways alone. A similar result was obtained when cells were incubated with the $^{\bullet}OH$-specific probe HPF (Fig. 2 C). When the levels of $O_2^{\bullet-}$ were determined with DHEt (Fig. 2 B), small to moderate increases were observed in both BER$^-$ deficient (~33% increase) and NER$^-$ deficient (~4% increase) cells, and a substantial (~80% increase) increase was observed in BER$^-$/NER$^-$ deficient cells. These findings are consistent with previous studies employing DHR [10] but also reveal the nature of the specific types of ROS elevated in response to endogenously produced DNA damage in the absence of exposure to exogenous agents (Experimental Procedures). Only BER$^-$/NER$^-$ deficient cells showed any significant increase in H_2O_2 when probed with APR (Fig. 2 D), and interestingly, a 30% decrease in H_2O_2 was observed in the NER$^-$ deficient strain. In general, when cells are severely repair-deficient and therefore accumulating endogenous DNA damage, there is a substantial increase in ROS levels. We conclude from these experiments that cells harboring elevated levels of endogenous DNA damage (BER$^-$/NER$^-$) have increased intracellular levels of $O_2^{\bullet-}$, H_2O_2, and $^{\bullet}OH$.

The finding that cells deficient in the repair of endogenous DNA damage (particularly BER$^-$/NER$^-$) contained elevated levels of oxidative damage that correlate with an elevation of ROS suggested that DNA damage induced by exogenous chemical and physical agents might be capable of causing a similar ROS response [10]. We addressed whether different classes of DNA damage, repaired by different DNA repair pathways were capable of eliciting an increase in ROS by examining the response to MMS, which produces DNA damage primarily repaired by BER and UV-C, which produces DNA damage primarily repaired by NER [19].

MMS Induced DNA Damage Causes an Increase in Intracellular ROS

First, we examined the spectrum of ROS produced in the repair deficient strains in response to MMS. All four strains (WT, BER^-, NER^-, and BER^-/NER^-) were exposed to a range (non-toxic to toxic) of MMS doses (0-55 mM) for 30 min and then assesed for ROS using the fluorescent probes described in Experimental Procedures (Table 1). When cells were exposed to MMS and then treated with DHR (Fig. 3B) or DHEt (Fig. 3C), an increase in ROS is observed that is related to the exposure dose. There is a significant increase in the levels of ROS in response to MMS (25 mM) in all strains irrespective of the DNA repair background and the corresponding level of cytotoxicity caused by this dose. The observed increases in ROS range from 15-2500% depending on the probe employed and the strain analyzed. In general, the increases in ROS are related to an increase in DNA damage and not cytotoxicity (Fig. 3A). The cytotoxicity observed following a 0.5 mM MMS exposure dose is similar in all four strains while the levels of intracellular $OH^\bullet$ and H_2O_2 (Fig. 3 D, E) differ significantly. In WT and NER-deficient cells, there is no significant change in the levels of H_2O_2 and $^\bullet OH$ while there is a 5% and 20% increase (indicated with "$*$" in Fig. 3 D,E) in H_2O_2 and $^\bullet OH$, respectively, in BER^- deficient cells and a 30% and 50% increase (indicated with "#" in Fig. 3 D,E) in H_2O_2 and $^\bullet OH$, respectively, in BER^-/NER^- deficient cells.

When cells were probed with HPF (Fig. 3 D) or APR (Fig. 3 E), an increase in H_2O_2 and $^\bullet OH$, respectively, is observed at lower exposure doses of MMS (0.5 mM or 5 mM) followed by a decrease at the higher doses (25 mM and/or 55 mM MMS). This increase is observed at 0.5 mM MMS for BER^- deficient cells (18% and 6% increases with APR and HPF, respectively) and BER^-/NER^- deficient cells (36% and 20% increases with APR and HPF, respectively). A similar increase is not observed in WT and NER^- deficient cells until higher exposure doses (5 mM) of MMS are used. The decrease in the levels of different ROS subspecies occurs at different doses of MMS. Unlike the decrease in $^\bullet OH$ that is observed at higher MMS doses, (Fig. 3 D), the

decrease in H_2O_2 observed with APR (Fig. 3 E) was observed at a 25 mM MMS exposure dose in BER^-/NER^- deficient cells and 55 mM MMS in all other strains. The trend that the levels of ROS increase at lower doses of MMS followed by a decrease at higher doses is observed for all strains investigated.

UV Light Induced DNA Damage Causes an Increase in Intracellular ROS

Exposure of cells to MMS reveals a potential role for ROS in the cellular response to a DNA alkylating agent. As previously reported with the nonspecific probe DHR., there is an increase in ROS in several DNA repair deficient strains in response to H_2O_2 and MMS exposure [21].

An important issue in the present study was to determine whether or not the increase in ROS in response to DNA damage repaired by BER is also observed for DNA lesions primarily repaired by the other major excision repair pathway, NER. To address this possibility, we determined the levels of ROS in response to UV-C induced DNA damage. Unlike MMS and H_2O_2, UV-C produces helix-distorting bipyrimidine adducts (CPDs and 6-4 PPs) that are repaired by NER [19].

All four strains (WT, BER^-, NER^-, and BER^-/NER^-) were exposed to a range (non-toxic to toxic) of doses (0-50 J/m^2) of UV-C and then probed for ROS. A pattern of a dose-dependent increase in ROS levels similar to that obtained with MMS exposure was observed. However, the maximum relative levels of ROS detected following exposure to UV-C (Fig. 4 B,C) were lower than that observed for MMS (Fig. 3 B,C). As with MMS, the observed increase in $O_2^{\bullet-}$ is directly related to the UV exposure dose (Fig. 4 C). There is a significant increase in the levels of $O_2^{\bullet-}$ in response to 5 J/m^2 UV-C in all strains (ranging from 14-117%) as compared to the levels of ROS in unexposed cells (indicated with "$*$" in Fig. 4C). These results are similar to those observed in cells following exposure to MMS in that there is a dose- dependent increase in the levels of ROS

in response to DNA damage. Furthermore, NER$^-$ deficient strains are more sensitive to UV-C induced DNA damage at lower doses. NER$^-$ deficient cells also exhibited a significant increase in $O_2^{\bullet-}$, but at a lower dose (2 J/m^2) as compared to unexposed (indicated with "#" in Fig. 4C).

As in the case of MMS exposure, exposure to UV-C results in changes in ROS levels that are related to DNA damage and not cell death. When cells are exposed to 25 J/m^2 UV-C, there is a greater than 50% survival in both the WT and BER$^-$ deficient cells while there is less than 1% survival in NER$^-$ and BER$^-$/NER$^-$ deficient cells (Fig. 4 A). Despite substantial differences in survival, these cells display similar relative increases in the levels of $O_2^{\bullet-}$ in response to 25 J/m^2 UV-C (Fig. 4 C). The fact that these increased levels of ROS are not associated with cell death is also revealed when cells are probed with HPF. For example, BER$^-$ deficient cells exposed to 50 J/m^2 UV-C and NER$^-$ deficient cells exposed to 5 J/m^2 UV-C display similar survival yet have significantly different levels of $OH^{\bullet}$ (indicated with "‡" in Fig. 4D).

It is important to emphasize that when cells were probed with DHR (Fig. 4 B), a difference was observed between cells capable of repairing UV-C-induced damage (WT and BER$^-$ deficient), and cells that are not capable of repairing such DNA damage (NER$^-$ and BER$^-$/NER$^-$ deficient). NER$^-$ deficient cells exhibited a significant (compared to unexposed cells in the corresponding strain) increase (indicated with "†" in Fig. 4 B) in ROS at lower doses (2 and 5 J/m^2). As with MMS we observed decreases in ROS at higher, more toxic doses (25 and 50 J/m^2) for NER$^-$ deficient strains. A similar result was observed when cells were analyzed for $^{\bullet}OH$ and H_2O_2 (Fig. 4 D,E). We conclude that cells respond to DNA damage through changes in the types and levels of ROS produced. As discussed below, the biological relevancy of an apparently bimodal ROS response to DNA damage is likely to involve specific signal transduction pathways.

Yap1 Relocalization in Response to DNA Damage

Under normal cellular environments, the ROS sensor and transcription factor, Yap1 is rapidly exported from the nucleus to the cytoplasm where it resides until activated in response to oxidative stress [14, 16, 23, 24]. Upon activation, Yap1 can no longer bind to the nuclear export receptor Crm1p and thus accumulates in the nucleus where it functions as a transcription factor [24, 25, 42]. Previous reports have shown that H_2O_2 exposure causes Yap1 to rapidly relocalize to the nucleus [14]. To address the potential role of Yap1 in the ROS-mediated DNA damage response, the localization of Yap1 was determined following exposure to MMS and compared to the previously characterized effects caused by direct exposure to H_2O_2 [14, 23]. Cells were exposed to a range of non-toxic to toxic doses of H_2O_2 or MMS for 1 hr and the localization of Yap1-GFP was determined by direct fluorescence microscopy. Following exposure to H_2O_2, DNA repair proficient (WT) cells exhibited a rapid relocalization of Yap1 to the nucleus that was maintained for at least 60 min. On average, approximately 75% of the cells analyzed exhibited nuclear localization of Yap1 (Fig. 5). WT cells exposed to a range (0.5-55 mM) of MMS doses displayed a delay in relocalization of Yap1 to the nucleus compared to the relocalization pattern observed following exposure to H_2O_2 (Fig. 6). When WT cells were exposed to 0.5 mM MMS, fewer than 20% of the cells displayed nuclear localization of Yap1 throughout the time course (60 min) of the experiment (Fig. 6 B). However with higher doses (25 and 55 mM) of MMS Yap1 nuclear localization significantly increased (up to 60%), indicating a response to oxidative stress that occurs after the introduction of non-oxidative DNA damage into the genome (Fig. 6 C,D).

Discussion

By examining the levels of ROS in cells harboring DNA damage caused by endogenous or exogenous sources, insight can be gained into the nature of genotoxic stress responses. DNA damage occurs continuously in all cells. Multiple overlapping systems for handling DNA damage exist. However, deleterious consequences can result when such pathways are compromised or when their capacities to process DNA damage have been exceeded. Although increases in ROS levels are thought to be involved in numerous pathological states, it is clear that modulation of ROS is also important for normal cellular physiology [43].

The endogenous levels of several different types of ROS were determined for WT and DNA repair deficient yeast strains. These strains were previously evaluated for the levels of endogenous oxidative DNA damage [10]. An increase in oxidative DNA damage in BER^- and BER^-/NER^- deficient strains was observed and was found to be highest in the BER^-/NER^- deficient strain. The results presented in the current study, establish a relationship between the levels of accumulated oxidative DNA damage and the levels of various ROS subtypes. We showed that BER^-/NER^- deficient cells harboring increased levels of oxidative DNA damage displayed the highest levels of intracellular $O_2^{\bullet-}$ as well as increased levels of intracellular H_2O_2 and $^{\bullet}OH$. BER^- deficient cells exhibited an increase in oxidative DNA damage that is less than that observed in BER^-/NER^- deficient cells. BER^- deficient cells exhibit a correspondingly smaller increase in $O_2^{\bullet-}$ with no observed increase in either H_2O_2 or $^{\bullet}OH$ as compared to BER^-/NER^- deficient cells (Fig. 2). NER^- deficient cells show no detectable increases in the levels of oxidative DNA damage and exhibit a very small increase in the levels of $O_2^{\bullet-}$ and no increases in the other types of ROS. Thus, low levels of endogenous DNA damage causes production of $O_2^{\bullet-}$. As the levels of endogenous DNA damage increase, $O_2^{\bullet-}$ also increases with concomitant elevation in both H_2O_2 and $^{\bullet}OH$.

In this study, by utilizing two different classes of DNA damaging agents (MMS and UV-C), we were able to determine the response to damage that is primarily repaired by either BER or NER. When cells were exposed to either MMS or UV-C, an increase in three different subspecies of ROS was observed, as a general genotoxic stress response regardless of the nature of the damage.

Previous reports have suggested that increased levels of ROS are associated with cell death [33, 34]. Our results indicate that ROS increases are also associated with DNA damage that occurs following non-toxic to moderately toxic exposure to MMS or UV light. Thus elevated levels of ROS cannot be attributed solely to cell death. For example, the cytotoxicity resulting from a low dose (0.5 mM) MMS exposure is similar in all strains (70-98% survival), but the levels of intracellular H_2O_2 produced are significantly different for BER^- and BER^-/NER^- deficient cells (5% and 20% increases, respectively) while there was no change in intracellular ROS levels for WT or NER^- deficient cells (Fig. 3). DNA damage produced by MMS is primarily repaired by BER and accumulation of base damage would be expected in BER^- and BER^-/NER^- deficient strains. We have established that there is a substantial increase in the levels of H_2O_2 without significant changes in cell survival. These results confirm the notion that ROS is produced directly in response to DNA damage and is not due to cell death.

Redox homeostasis in cells is important for maintaining proper cellular functions [44]. Elevated levels of ROS can be biologically deleterious, but have also been implicated in a variety of cell signaling processes [5-8]. For example, ROS alter protein structure through direct interaction with cysteinyl sulfhydryl groups [7]. To maintain normal redox states, cells utilize several systems for buffering ROS including the thioredoxin and glutathione pathways [45]. In dividing cell populations, when DNA damage occurs, the cell must respond to maintain the integrity of the genome and eventually continue through the cell cycle. ROS-mediated signaling should enable the rapid activation of the stress response systems necessary for cell survival. To

our knowledge, this is the first report defining the relationship between DNA damage and the types and relative levels of ROS produced.

To further delineate the possible role of ROS in the DNA damage response, the localization of the ROS sensor, Yap1 in WT cells in response to two different DNA damaging agents was examined. Previously Yap1 has been shown to relocalize to the nucleus in response to H_2O_2. Yap1 localization is directly altered by the presence of H_2O_2 in the cell [13, 23], regardless of its DNA damage effect. In this study, we found that the relocalization of Yap1 in response to MMS exposure is similar to the relocalization of Yap1 as a result of direct exposure to ROS (e.g. H_2O_2). When cells are exposed to MMS, there is a time- and dose-dependent increase in the number of cells that have predominantly nuclear Yap1 localization. However, unlike the response observed, after exposure to H_2O_2, of rapid relocalization of Yap1 to the nucleus, there is a delay of approximately 25 min before a significant number of MMS-exposed cells contain nuclear Yap1 (Fig. 6). As the redox state of the cell shifts to a more reduced state through the increased production of ROS in response to DNA damage, the localization of Yap1 changes from cytoplasmic to nuclear where it functions as a transcription factor in response to cell stress. It should be emphasized that MMS, does not directly produce ROS, yet causes the accumulation of Yap1 in the nucleus as does H_2O_2. This observation provides independent verification that DNA damage per se elicits the ROS stress response. The delayed accumulation of Yap1 in the nucleus following MMS exposure suggests a signal transduction process from DNA damage to the generation of ROS. These observations provide biological confirmation of the DNA damage-induced intracellular ROS production revealed by the panel of fluorescent probes employed in these studies.

Our results are consistent with the following model for the role of ROS in the cellular genotoxic stress response (Fig. 7). Elevation of ROS in response to DNA damage appears to be associated with two modes of DNA damage levels involving $O_2^{\bullet-}$ as the primary mediator. At low to moderate levels of DNA damage, there is a moderate increase in the levels of $O_2^{\bullet-}$, H_2O_2,

and $^{\bullet}OH$ (Level 1). At Level 1, $O_2^{\bullet-}$ is a likely source for H_2O_2 and $^{\bullet}OH$ through chemical and enzymatic conversion pathways [46]. For example, $^{\bullet}OH$ can be non-enzymatically generated via the Fenton Reaction and H_2O_2 can be enzymatically produced by superoxide dismutase [46]. The redox state (Mode 1) of the cell at these levels of DNA damage leads to cell survival-related and stress response signaling processes. In response to changes in cellular redox states, there are several known signaling pathways that can be activated including stress activated kinases (SAPKs), multistep phosphorelay, and AP-1 like transcription factor sensing [47]. These pathways are conserved between yeast and other higher organisms, making these studies potentially relevant to other eukaryotes. Activating protein-1 (AP-1)-like transcription factor sensing is mediated by a family of transcription factors. AP-1-like proteins regulate a variety of cellular processes including proliferation, differentiation, apoptosis, and various stress responses [48]. In *S. cerevisiae* Yap1 is the major AP-1-like transcription factor. Yap1 is responsible for the upregulation of numerous genes that are involved in the oxidative stress response. These include the gultatione reductase (*GLR1*), thioredoxin (*TRX2*), catalase (*CTT1*), and superoxide dismutase (*SOD1*) [49]. It is possible that the DNA damage stress response in *S. cerevisiae* may be initiated by changes in the redox state of the cell that, in turn, can lead to subsequent signaling through one or more of the above pathways. Many of these pathways may contain components that sense intracellular ROS levels and then initiate events that trigger a stress response in cells. A Mode 1 response could be mediated by activated Yap1 as we have demonstrated that it accumulates in the nucleus following exposure to MMS.

At high levels of DNA damage, there is a corresponding increase in the level of $O_2^{\bullet-}$, but not in H_2O_2 or $^{\bullet}OH$ due to saturation or blockage of conversion pathways. With such decreases in H_2O_2 or $^{\bullet}OH$ the appropriate signaling response does not occur, thus leading to a decrease in cell survival. At the corresponding redox state (Mode 2), the cell is well beyond its repair capacity and reorchestrates signaling from cell survival/stress responses to cell death processes.

As the redox state of the cell continues to move towards an oxidized state due to high levels of DNA damage causing an increase in the levels of $O_2^{\bullet-}$, the cell can no longer survive because of extensive nuclear and/or mitochondrial DNA damage as well as damage to other macromolecules. Alternatively, the cell reorchestrates the internal signaling processes to initiate cell death. Several recent studies have shown that unicellular eukaryotes undergo a caspase-independent apoptotic-like cell death process [50, 51]. In yeast, many features of apoptosis have been observed including chromatin condensation, phosphatidylserine exposure on the outer membrane, and cytoplasmic shrinkage [50, 52]. Importantly, one feature of this cell death process is an increase in the intracellular levels of $O_2^{\bullet-}$ [33].

To fully understand how the DNA damage-induced stress response functions in cells, an understanding of the generators of ROS and their role in the ROS response is necessary. There are several possible $O_2^{\bullet-}$ generators that have been identified in yeast cells including the mitochondria (e.g. cytochrome C) and various oxidases. Xanthine oxidase is a interesting candidate in that it has been shown to produce $O_2^{\bullet-}$, H_2O_2, and $^{\bullet}OH$ [53]. In addition, we are currently determining the extent to which a similar DNA damage-induced ROS stress response occurs in mammalian cells. Such a response has obvious implications for several important human pathologies, including cancer.

Abbreviations used in text: Reactive oxygen species (ROS), Base excision repair (BER), Nucleotide excision respair (NER), Mismatch repair (MMR), Translesion synthesis (TLS), Recombination (REC), Cyclobutane pyrimidine dimmer (CPD), 6-4 photoproduct (6-4 PP), Hydrogen peroxide (H_2O_2), methyl methanesulfonate (MMS), Superoxide ($O_2^{\bullet -}$), Hydroxyl radical ($^{\bullet}OH$), Short wavelength ultraviolet light (UV-C), Wild type (WT), Dihydrorhodamine (DHR), Dihydroethidium (DHEt), 2-[6-(4'-Hydroxyy)phenoxy-3H-xanthen-3-on09yl] (HPF), N-can-Acetyl-3,7dihydroxyphenoxazine (Amplex Red (APR)), Phosphate buffered saline (PBS), 4',6-diamidino-2-phenylindole (DAPI), Stress activated kinases (SAPK), Mitogen-activated protein kinases (MAPK), and Activating protein-1 (AP-1).

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Table 1. ROS Fluorescent Probes

Fluorescent Probe	ROS detected	Reference
Dihydrorhodamine (DHR)	multiple species	(28,29)
Dihydroethidium (DHEt)	$O_2^{\bullet -}$	(34,35)
2-[6-(4'-Hydroxy)phenoxy-3H-xanthen-3-on-9yl] bezoic acid (HPF)	$^{\bullet}OH$	(36,37)
N-AcN-Acetyl-3,7-dihydroxyphenoxazine (Amplex Red (APR))	H_2O_2	(38)

Figure 1. MMS and UV-C cytotoxicity profiles of cells with different DNA repair capacities. Sensitivity of DNA repair deficient strains to MMS and UV-C. WT (diamonds), BER^- (squares), NER^- (triangles), and BER^-/NER^- (circles). Cells were exposed to **(A)** 0, 0.5, 5, 25, and 55 mM MMS for 30 min at 30 °C or **(B)** 0, 2, 5, 25, 50 J/m^2 UV-C. The results are an average of six different experiments. Error bars represent ± SD. Experimental details are provided in the text.

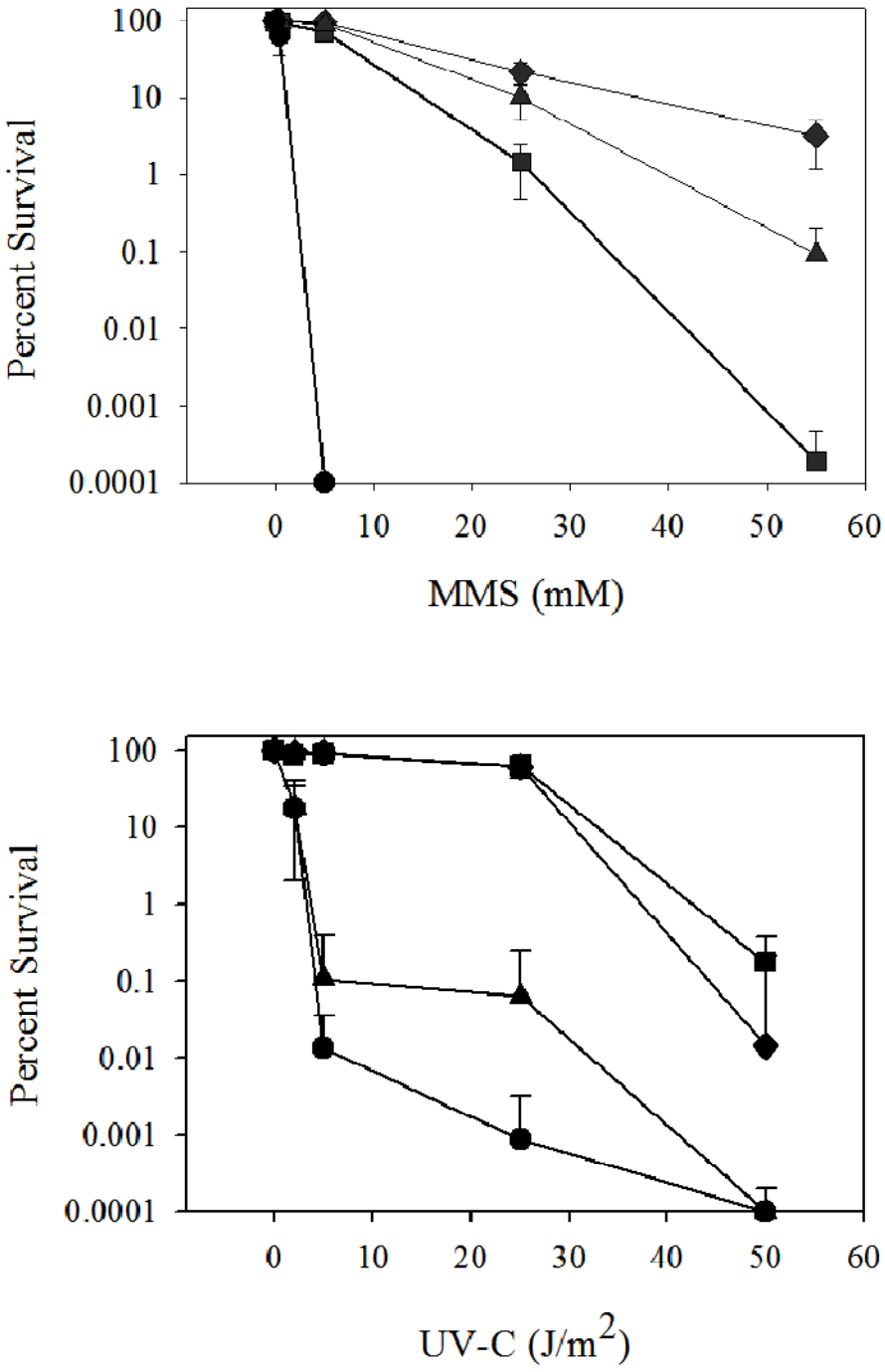

Figure 1. MMS and UV-C cytotoxicity profiles of cells with different DNA repair capacities.

Figure 2. Endogenous levels of ROS in isogenic DNA repair-proficient and -deficient strains. Isogenic WT, BER$^-$, NER$^-$, and BER$^-$/NER$^-$ strains were incubated with the indicated fluorescent probes (**A**) DHR, (**B**) DHEt, (**C**) HPF, or (**D**) APR for 2 hours as described in Experimental Procedures. Following incubation, cells were analyzed for ROS levels by flow cytometry as described in the text. Fluorescence values were obtained from measurement of the mean peak values of the cytograms. Fluorescence values for each ROS probe are reported as the fold change relative to the WT (repair proficient) strain (set to a value of 1.0). Error bars represent ± SD. Asterisks above bars indicate statistical significance of a p value <0.05 as compared to no exposure condition.

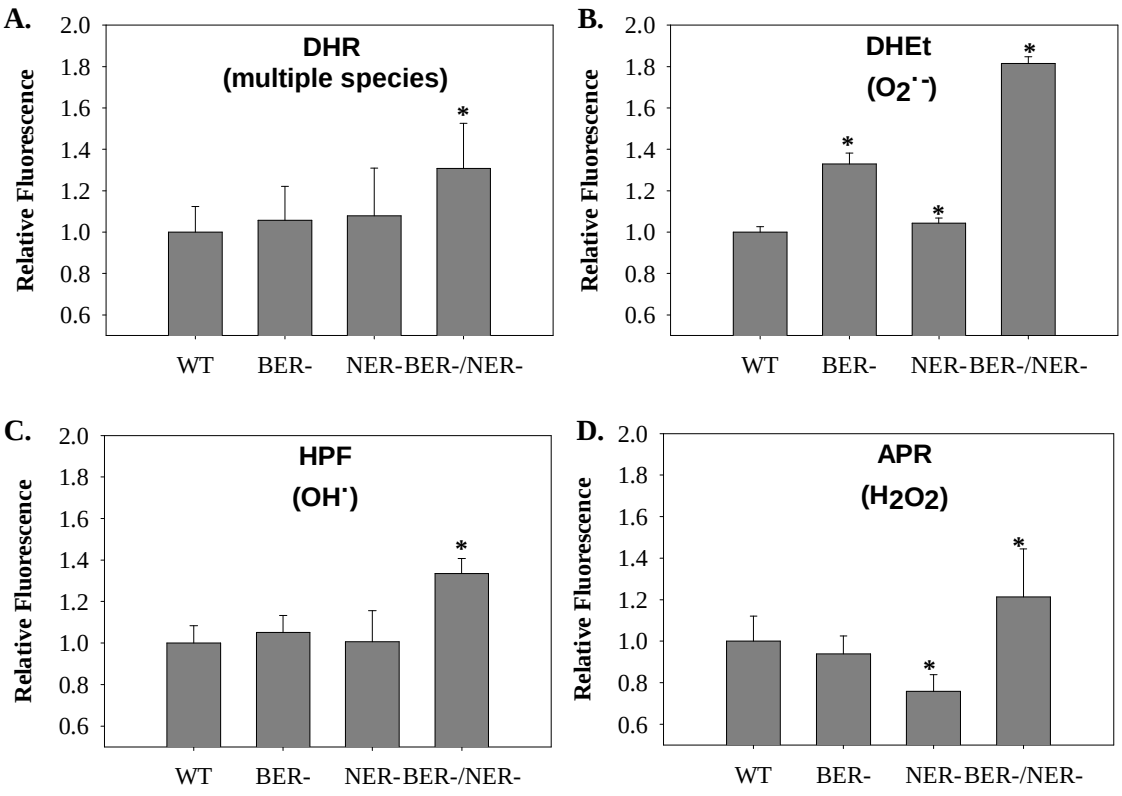

Figure 2. Endogenous levels of ROS in isogenic DNA repair-proficient and -deficient strains.

Figure 3. ROS levels in DNA repair deficient strains in response to DNA alkylation damage. **A.** MMS cytotoxicity of strains with different DNA repair backgrounds. WT (diamonds), BER^- (squares), NER^- (triangles), and BER^-/NER^- (circles). Cells were exposed to 0, 0.5, 5, 25, and 55 mM MMS for 30 min at 30 °C as described in the text. Results displayed are an average of six different experiments. **B – E.** Isogenic WT, BER^-, NER^-, and BER^-/NER^- strains were first exposed to MMS (0-55 mM) for 30 min and then incubated with the indicated fluorescent probes (**B**) DHR, (**C**) DHEt, (**D**) HPF, or (**E**) APR for 2 hours. Following incubation, cells were analyzed for ROS levels by flow cytometry as described in Experimental Procedures. Fluorescence values were obtained from measurement of the mean peak values of the cytograms. Fluorescence values for each ROS probe are reported as fold changes relative to the WT (repair proficient) strain (set to a value of 1.0). Error bars represent ± SD. "$*$" symbols above bars indicate statistically significant (p value <0.05) differences between the levels of H_2O_2 or $^{\bullet}OH$ in BER^- deficient strains at 0.5 mM MMS compared to no exposure conditions and "#" symbols above bars indicate statistically significant (p value <0.05) differences between the levels of H_2O_2 or $^{\bullet}OH$ in BER^-/NER^- deficient strains at 0.5 mM MMS compared to no exposure conditions.

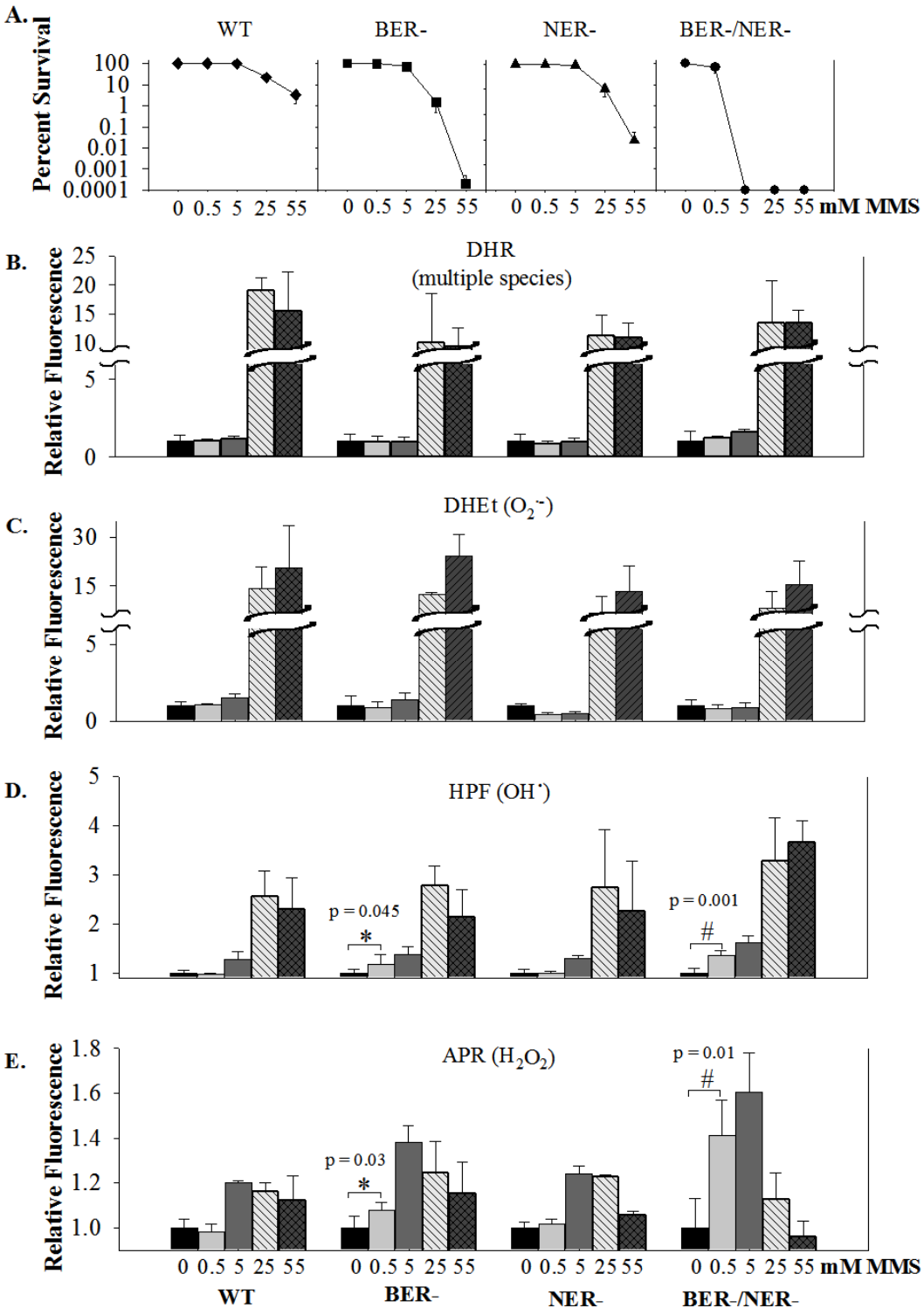

Figure 3. ROS levels in DNA repair deficient strains in response to DNA alkylation damage.

Figure 4. ROS levels in DNA repair-deficient strains in response to UV-C induced DNA damage. A. UV-C cytotoxicity of strains with different DNA repair backgrounds. WT (diamonds), BER^- (squares), NER^- (triangles), and BER^-/NER^- (circles). Cells were exposed to 0, 2, 5, 25, and 50 J/m^2 UV-C as described in the text. Results displayed are an average of six different experiments. **B - E.** Isogenic WT, BER^-, NER^-, and BER^-/NER^- strains were first exposed to UV-C (0-50 J/m^2) and then incubated with the indicated fluorescent probes (**B**) DHR, (**C**) DHEt, (**D**) HPF, or (**E**) APR for 2 hours. Following incubation, cells were analyzed for ROS levels by flow cytometry as described in Experimental Procedures. Fluorescence values were obtained from measurement of the mean peak values of the cytograms. Fluorescence values for each ROS probe are reported as fold changes relative to the WT (repair proficient) strain (set to a value of 1.0). "$*$"symbols above bars indicate statistically significant (p value <0.05) differences between the levels of $O_2^{\bullet-}$ in all strains at 5 J/m^2 UV-C compared to no exposure conditions. "#" symbols above bars indicate statistically significant (p value <0.05) differences between the levels of $O_2^{\bullet-}$ in NER^- deficient strain at 0.5 J/m^2 UV-C compared to no exposure conditions. "†"symbols above bars indicate statistically significant (p value <0.05) differences between the levels of ROS in NER^- and BER^-/NER^- deficient strains at 0.5 J/m^2 UV-C compared to no exposure conditions. "‡" symbols above bars indicate statistically significant (p value <0.05) differences between the levels of $^{\bullet}OH$ at the 5 J/m^2 UV-C exposure in NER^- deficient cells as compared to 55 J/m^2 UV-C exposure in BER^- deficient cells.

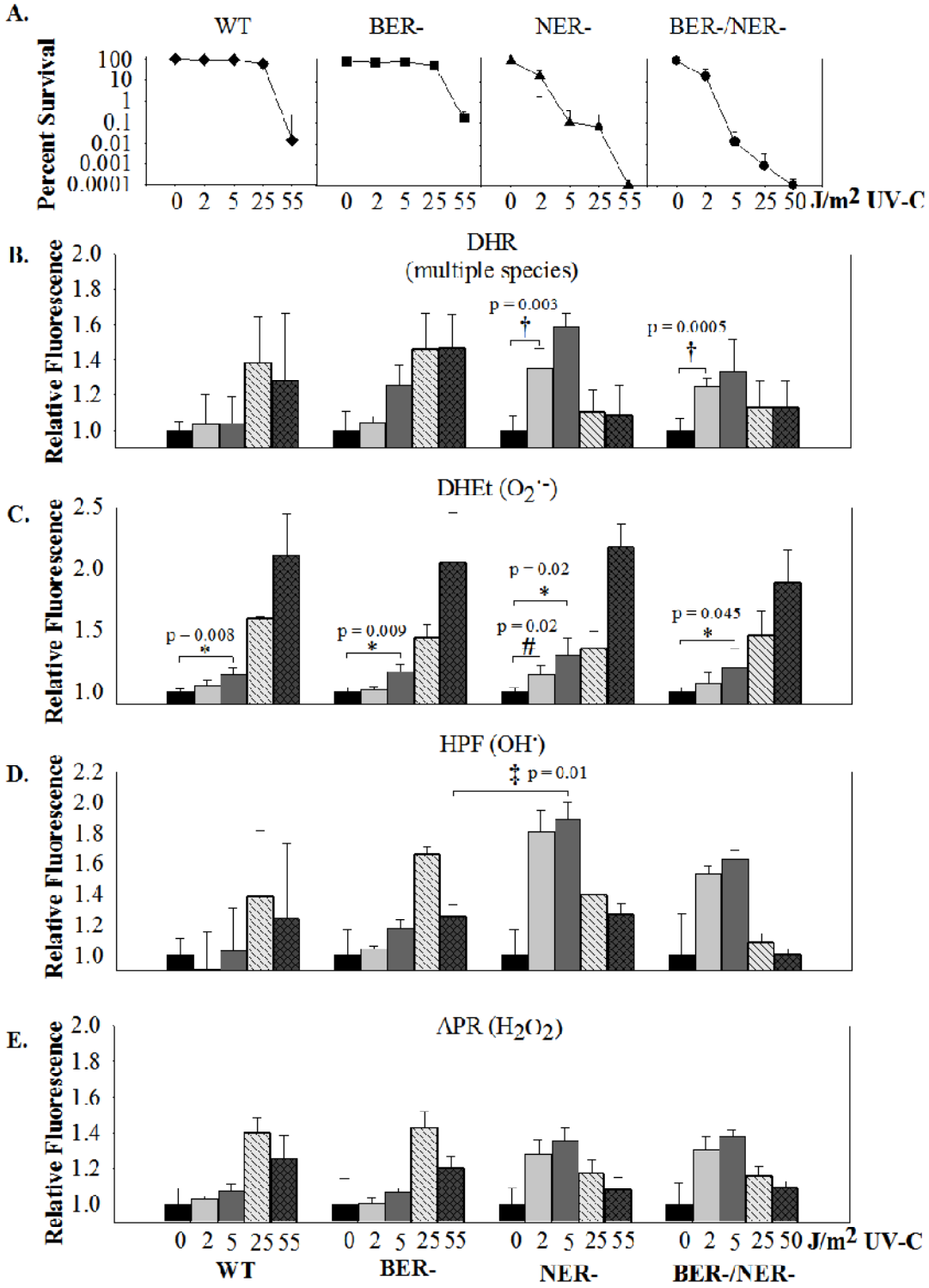

Figure 4. ROS levels in DNA repair-deficient strains in response to UV-C induced DNA damage.

Figure 5. Yap1 localization in WT cells after exposure to H_2O_2. A. Yap1-GFP was visualized by direct fluorescence microscopy in WT cells either untreated (Control) or treated with 0.5 mM H_2O_2 for 5 min. Cells were stained with DAPI to visualize the position of the chromatin within the nuclei. The Merge image indicates the overlap (yellow) between the Yap1-GFP signal (green) and DAPI (red) staining of nuclei. Corresponding DIC images are also shown. **B-E**. Graphical representation of fluorescence microscopy analysis assessing Yap1-GFP localization. Cells were exposed to (**B**) 0.5 mM, (**C**) 5 mM, (**D**) 25 mM, or (**E**) 55 mM H_2O_2 and fluorescence images were obtained. Cells were counted and evaluated in 10 min interval groups. Percentages of cells scored as showing no nuclear localization (gray bars) or nuclear localization alone or nuclear plus cytoplasmic localization for Yap1 (black bars) for every 10 minute interval group for the duration of the experiment (60 min) are indicated.

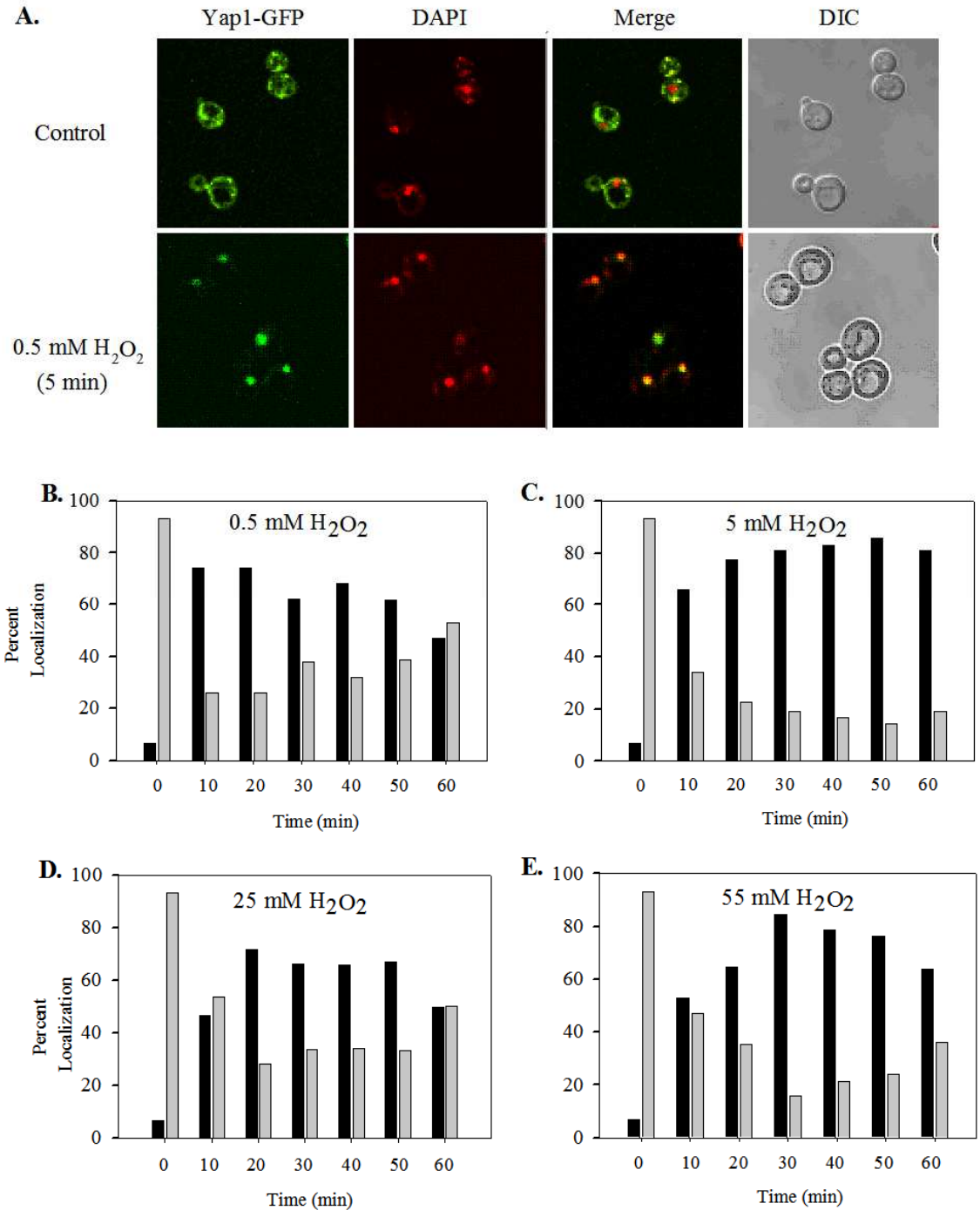

Figure 5. Yap1 localization in WT cells after exposure to H_2O_2.

Figure 6. Yap1 localization in WT cells after exposure to MMS. A. Yap1-GFP was visualized by direct fluorescence microscopy in WT cells either untreated (Control) or treated with 25 mM MMS for 25 min. Cells were stained with DAPI to visualize the position of the chromatin within the nuclei. The Merge image indicates the overlap (yellow) between the Yap1-GFP signal (green) and DAPI (red) staining of nuclei. Corresponding DIC images are also shown. **B-D**. Graphical representation of fluorescence microscopy analysis assessing Yap1-GFP localization. Cells were exposed to (**B**) 0.5 mM, (**C**) 25 mM, or (**D**) 55 mM MMS and fluorescence images were obtained. Cells were counted and presented in 10 min interval groups. Cells were scored as showing no nuclear localization (gray bars) or nuclear localization alone or nuclear plus cytoplasmic localization for Yap1 (black bars) for every 10 min interval group for the duration of the experiment (60 min) are indicated.

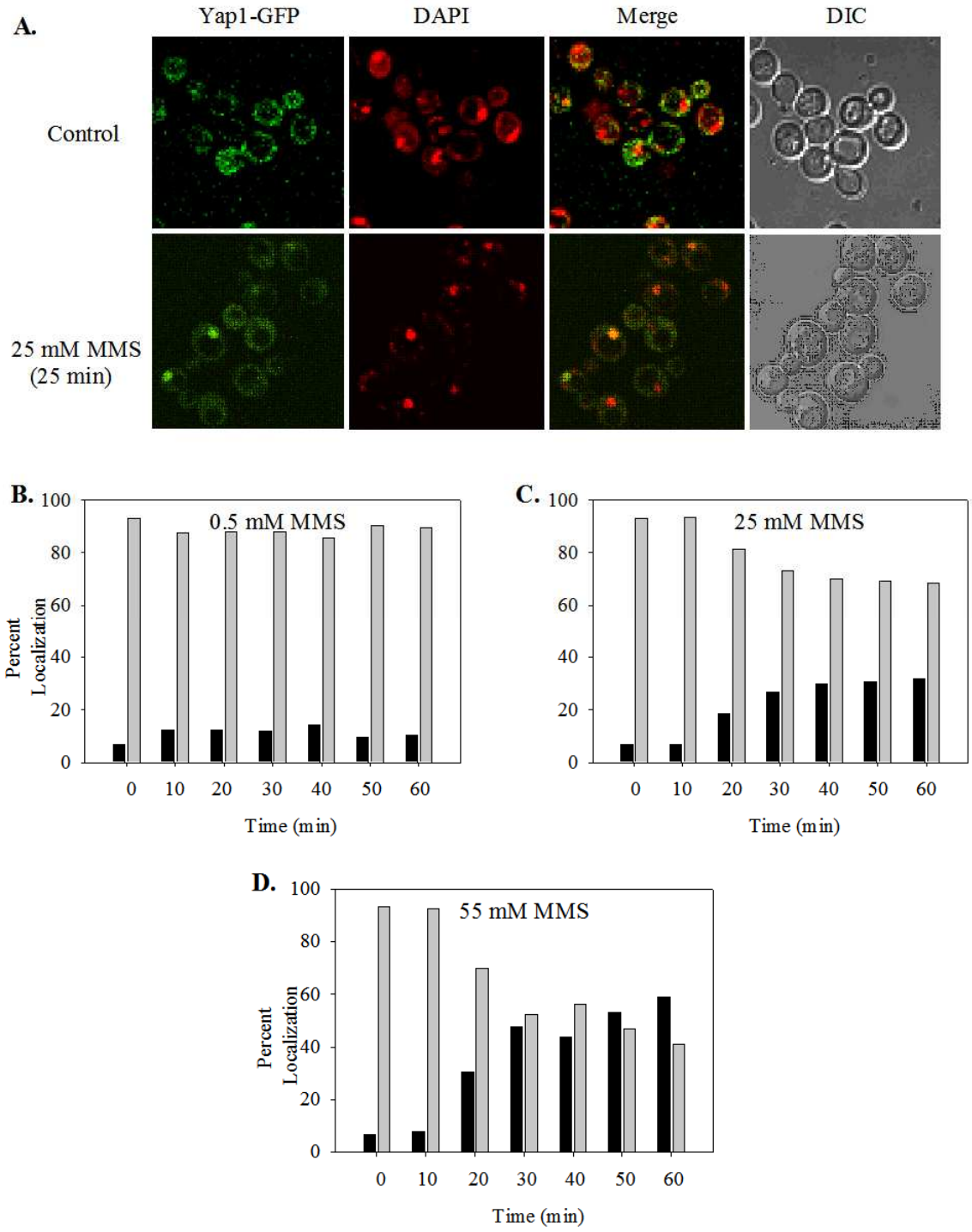

Figure 6. Yap1 localization in WT cells after exposure to MMS.

Figure 7. Model for the role of ROS in the genotoxic stress response. Under normal (no stress) growth conditions, in the absence of genomic DNA damage, the redox state of the cell is normal (Mode 0). Upon genomic DNA damage, intracellular levels of ROS increase. At low to moderate levels of DNA damage, cellular $O_2^{\bullet-}$, H_2O_2, and $^{\bullet}OH$ levels increase (Level 1). The increased levels of H_2O_2 and $^{\bullet}OH$ could be caused by direct production in response to DNA damage or through conversion from $O_2^{\bullet-}$ via non-enzymatic and enzymatic pathways [46]. $^{\bullet}OH$ and H_2O_2 function as signaling molecules to activate the genotoxic stress response including pathways involving SAPKs, multiphosphorelay and AP-1 like transcription factors. Each of these pathways is activated in response to changes in the redox state of the cell [47]. At higher levels of DNA damage, $O_2^{\bullet-}$ continues to increase (Level 2), while the production of H_2O_2 and $^{\bullet}OH$ declines. The decrease in H_2O_2 and $^{\bullet}OH$ levels could be due to a decrease in direct production or blocked conversion from $O_2^{\bullet-}$. The redox state of the cells (Mode 2) is primarily due to substantial increases in the $O_2^{\bullet-}$ levels that subsequently mediate the cell death response. The relative levels of H_2O_2 and $^{\bullet}OH$ are represented by the green shaded area and the relative levels of $O_2^{\bullet-}$ are represented by the purple shaded area.

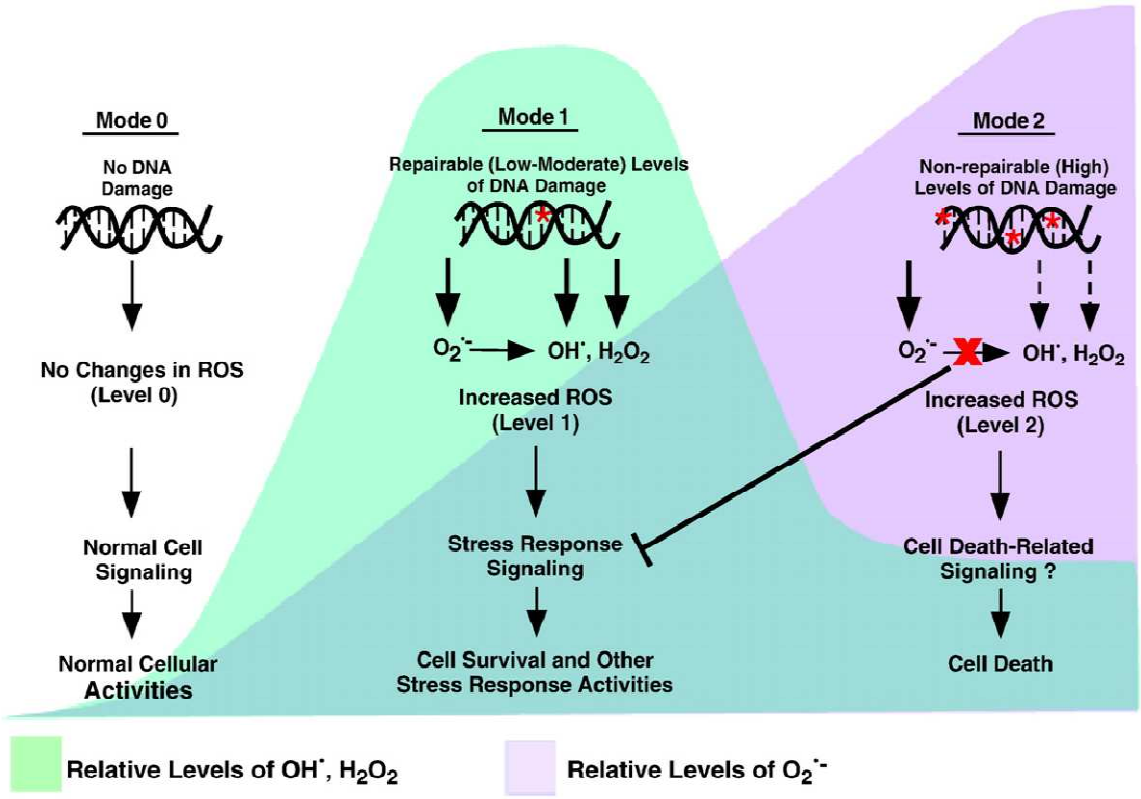

Figure 7. Model for the role of ROS in the genotoxic stress response.

CHAPTER III

The Role of the ROS Scavengers in the DNA Damage Stress Response

Abstract

Reactive oxygen species (ROS) play a dual role in biological systems. They are deleterious to cells causing damage to macromolecules such as DNA, proteins, and lipids leading to many human degenerative conditions including cancer, arthritis, neurodegenerative diseases, as well as the process of aging. However, ROS are also known to be important for normal cellular functions. For example, ROS are involved in defense against infectious agents and in cellular signaling. Previous studies by our group have shown that increased levels of DNA damage can lead to increased intracellular levels of ROS. In this investigation, we examine the role of ROS scavenging enzymes (superoxide dismutase -Sod1and Sod2, and catalases - Cta1, and Ctt1) in the DNA-damage-induced ROS response. Utilizing a set of *Saccharomyces cerevisiae* isogenic ROS scavenging mutants (WT, *sod1Δ*, *sod2Δ*, *cta1Δ*, and *ctt1Δ*), we determined the cytotoxicity and intracellular levels of ROS produced in response to two DNA damaging agents, methylmethane sulfonate (MMS) and ultraviolet light (UV-C). The deletion of either of the SODs or the CATs had no effect on cytotoxicity following exposure to MMS. Deletion of *SOD2*, *CTA1*, and *CTT1* increased the cytotoxic effects of UV-C. A dose-dependent increase in the intracellular levels of superoxide ($O_2^{\bullet-}$) occurred for all strains following exposure to both MMS and UV-C. A significant increase in intracellular $O_2^{\bullet-}$ levels was observed at high doses of MMS (25-55mM) in *sod1Δ* cells compared to WT. A significant increase in $O_2^{\bullet-}$ levels was also observed following high doses of UV-C (25-50 J/m^2) in *sod2Δ*, *cta1Δ*, and *ctt1Δ* cells. These data suggest that the DNA damage-induced ROS response is dependent on the type of DNA damage induced and that the subcellular localization of ROS production/localization influences the cells response to DNA damage.

Introduction

ROS is capable of mediating either beneficial or deleterious effects on cells. High intracellular ROS levels can damage macromolecules such as DNA, proteins, and lipids, and have been associated with many human degenerative conditions including cancer [1], neurological diseases [2], as well as the process of aging [3, 4]. However, ROS are involved in the defense against infectious agents [5] as well as in cellular stress signaling processes [6-9].

Exogenous sources of ROS include ionizing radiation, chemical agents, and various xenobiotics, and UV radiation [10]. Endogenous sources of ROS include those produced within mitochondria [11] and peroxisomes, as well as inflammatory cell activation [5]. While the production of ROS in the cell can be beneficial, homeostasis must be maintained in order to limit the deleterious effects of either increased or decreased levels of ROS. Redox balance is maintained through non-enzymatic and enzymatic antioxidant defenses. Non-enzymatic small molecules such as vitamins C and E, glutathiones, and thioredoxins possess intrinsic antioxidant properties [12]. Enzymes capable of directly scavenging ROS and converting it to less toxic moieties include superoxide dismutases (SOD), catalases (CAT) and peroxidases [13].

In *Saccharomyces cerevisiae*, there are two SODs, Sod1 and Sod2 (Figure 1) [12]. Sod1 is located in the cytoplasm while Sod2 is located in the mitochondria [14]. Both SODs are capable of scavenging superoxide ($O_2^{\bullet-}$) and converting it into the less reactive (but still toxic) hydrogen peroxide (H_2O_2) and non-toxic molecular oxygen (O_2) [15]. While the reduction of $O_2^{\bullet-}$ is beneficial, the production of H_2O_2 can be deleterious to the cell. H_2O_2 can react with Fe^{2+} via the Fenton reaction, forming the highly reactive hydroxyl radical ($^{\bullet}OH$) [15]. CATs convert H_2O_2 into H_2O and O_2 (Figure 1) [16]. There are two CATs present in *S. cerevisiae*, Cta1 and Ctt1 (Figure 1) [12]. Ctt1 is present in the cytoplasm [17] and Cta1 is present in mitochondria and peroxisomes [18]. These enzymes are important for maintaining the proper redox balance in cells.

Previous studies from our group have shown that increased DNA damage can lead to increased intracellular levels of ROS [19, 20]. In cells that are severely repair compromised (deficient in both base excision repair and nucleotide excision repair (BER^-/NER^-)), there are increased levels of oxidative DNA damage (1400 lesions/genome) [21] that are associated with increased levels of ROS, specifically $O_2^{\bullet-}$ [19, 20]. A small increase in ROS is believed to be involved in signaling leading to DNA repair, while a greater increase in ROS is associated with stress-related cell death [19]. In the present study we examined the potential role of ROS scavenging enzymes in the DNA damage-induced ROS response in order to gain insight into the regulation of ROS signaling in the response to DNA damage. The role of ROS in the DNA damage response was determined by examining 1) DNA damage-induced cytotoxicity profiles and 2) intracellular ROS levels in a set of isogenic ROS scavenging mutants (WT, *sod1Δ*, *sod2Δ*, *cta1Δ*, and *ctt1Δ*).

We determined the cytotoxicity profiles of the ROS scavenging mutants following exposure to two DNA damaging agents, methylmethane sulfonate (MMS) and ultraviolet light (UV-C). After exposure to the DNA alkylating agent, MMS, a dose-dependent decrease in survival was determined for all strains; however, ROS scavenging mutants (*sod1Δ*, *sod2Δ*, *cta1Δ*, and *ctt1Δ*) were not more sensitive to MMS than WT cells. Following exposure to UV-C, which produces bulky, helix distorting DNA lesions, a dose-dependent decrease in survival was observed for all strains. Increased cytotoxicity was observed in *sod2Δ*, *cta1Δ*, and *ctt1Δ* strains following exposure to 25 J/m^2 that was not observed in WT cells, suggesting a role for Sod2, Cta1, and Ctt1 in protection against the cytotoxic effects of UV-C. We also examined the intracellular levels of specific types of ROS ($O_2^{\bullet-}$, H_2O_2, and $^{\bullet}OH$) using a panel of fluorescent probes following exposure to MMS and UV-C. An increase in intracellular $O_2^{\bullet-}$ levels was observed in all strains following exposure to both MMS and UV-C. Interestingly, the intracellular levels of $O_2^{\bullet-}$ were significantly elevated in the *sod1Δ* strain following exposure to

MMS as compared to WT cells. A significant increase in $O_2^{\bullet-}$ levels was also observed following exposure to high doses of UV-C (25-50 J/m^2) in *sod2Δ*, *cta1Δ*, and *ctt1Δ* strains. However, the levels of $^{\bullet}OH$ and H_2O_2 varied depending on the DNA damaging agent, dose, and strain. These results reveal important aspects of the role of ROS scavengers in the DNA damage-induced ROS response. Our findings suggest that the cellular response to DNA damage is not uniform with respect to the production of ROS and that the type of DNA damage that is induced produces a specific ROS response pattern.

Experimental Procedures

Strains, Media, and Growth Conditions

The set of isogenic *S. cerevisiae* strains used in this study was derived from the Open Biosystems deletion collection (Open Biosystems, USA). The genotypes used were WT strain BY4741 (*MAT A his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*), *sod1Δ* strain (*MAT A his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sod1Δkan*), *sod2Δ* strain (*MAT A his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sod2Δkan*), *cta1Δ* strain (*MAT A his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cta1Δkan*), and *ctt1Δ* strain (*MAT A his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ctt1Δkan*). The Open Biosystems strains were constructed by inserting the KanMX cassette into the appropriate ROS-related gene using homologous recombination as previously described [22-24] .Yeast strains were grown on YPD media (1% yeast extract, 2% peptone, 2% dextrose, 0.005% adenine sulfate, and 2% agar for plates).

Cell Growth and Viability

Liquid YPD media was inoculated with yeast cells and grown at 30 °C for ~24 hrs to saturation (greater than 7 x 10^7 cells/mL). Fifty milliliters of YPD cultures were inoculated with an appropriate amount of cells so that the culture would reach a density of 2 x 10^7 cells/mL (mid-log phase) after 12 hrs of growth at 30 °C. Cell viability was determined by plating on YPD after exposure to MMS (0-55 mM for 30 min at 30°C) or UV-C (0-50 J/m^2). Cultures were diluted to a density that would yield approximately 100-200 colonies per plate.

Measurement of Reactive Oxygen Species Levels

ROS subspecies ($O_2^{\bullet-}$, H_2O_2, and $^{\bullet}OH$) were detected using a panel of fluorescent probes (Table 1). Cells were grown to mid-log phase (~2 x 10^7 cells/mL) in YPD at 30 °C overnight. Cells were washed twice in H_2O and then adjusted to 2 x 10^7 cells/mL in H_2O and then exposed to various doses of either MMS or UV-C. For experiments involving UV irradiation, cells (15

mL) were placed in a 15 mm petri dish and exposed to a range of UV-C doses (0-50 J/m^2). Immediately following UV exposure, cells were placed in the dark to prevent photoreactivation. For experiments involving MMS, cells (3 mL) were placed in the dark and exposed to a range of MMS doses (0-55 mM) for 30 min at 30 °C. Following exposure to MMS or UV-C, 1 mL aliquots were plated for survival measurements and 2 mL were incubated with various fluorescent probes (dihydrorhodamine (DHR), 25 μg/mL; dihydroethidium (DHEt), 50 μg/mL; N-can-Acetyl-3,7dihydroxy-phenoxazine (Amplex Red (APR)), 12 μg/mL; and 2-[6-(4'-hydroxy)phenoxy-3*H*-xanthen-3-on-9-yl] benzoic acid (HPF), 20 μg/mL) for ROS measurements. Immediately following fluorescent probe addition, cells were held in the dark and incubated at 30 °C for 2 hrs. Cells were subsequently washed twice with H_2O and then resuspended in 2 mL phosphate buffered saline (PBS) and assessed for fluorescence intensity employing a BD™ LSR II flow cytometer (BD Biosciences).

Results

Previous studies established that intracellular ROS increases in response to DNA damage [19, 20]. Severely repair compromised yeast mutants (base excision repair deficient (BER^-) and nucleotide excision repair deficient (NER^-) deficient) harbor high levels of oxidative DNA damage (approximately 1400 lesions/genome, present in mid-log growth phase cells) [21] that is associated with increased intracellular ROS [19, 20]. An increase in ROS levels was also observed in WT, BER^-, NER^-, and BER^-/NER^- deficient cells following exposure to both MMS and UV-C [19], suggesting a role for ROS in the DNA damage response.

We employed *S. cerevisiae* as a model system to determine the role of ROS scavengers in the ROS-mediated DNA damage stress response. Increased levels of DNA damage could be due to a lack of DNA lesion removal by DNA repair systems or alterations of enzyme activities responsible for ROS scavenging. Perturbations of ROS scavenging enzymes could cause an increase in intracellular ROS levels. Such an increase in intracellular ROS levels could cause damage not only to DNA, but also proteins and other macromolecules that are involved in cell signaling. Previous studies have shown a correlation between increased levels of DNA damage and increased levels of ROS [19]. Defining the DNA damage response in ROS scavenging mutants (*sod1Δ*, *sod2Δ*, *cta1Δ*, and *ctt1Δ*) provides insight into ROS regulation in signaling and cytotoxicity.

MMS and UV-C cytotoxicity in ROS scavenging mutants.

To determine whether ROS scavenging is related to DNA damage-induced cell killing, we examined the cytotoxicity profiles of ROS scavenging mutants following exposure to MMS (0-55mM) in cells deficient in *SOD1*, *SOD2*, *CTA1*, and *CTT1*. MMS does not directly generate ROS and produces DNA alkylation products repaired primarily by BER. However, WT cells exposed to MMS produce increased ROS levels [19]. All strains (WT, *sod1Δ*, *sod2Δ*, *cta1Δ*, and

ctt1Δ) exhibited a dose-dependent cytotoxic response to MMS. There was no increase in cytotoxicity compared to WT cells in the ROS scavenging compromised (mutant) strains (Figure 2A). These results indicate that each ROS scavenging enzyme individually does not protect cells from the cytotoxic effects of MMS. This could be due to the presence of redundant, backup pathways that are still capable of protecting cells from indirect ROS cytotoxicity or that the cytotoxic effects of increased ROS levels are masked by the cytotoxic effects of MMS acting through other mechanisms.

We next examined the cytotoxicity profiles of the ROS scavenging mutants following exposure to UV-C (0-50 J/m^2). UV-C produces helix distorting DNA lesions repaired primarily by NER and does not directly generate ROS. However, following exposure to UV-C, WT cells displayed increased levels of ROS [19]. All strains (WT, *sod1Δ*, *sod2Δ*, *cta1Δ*, and *ctt1Δ*) exhibited increased cytotoxicity in response to increasing exposure doses of UV-C (Figure 2B). Exposure to UV-C caused increased cytotoxicity in *sod2Δ*, *cta1Δ*, and *ctt1Δ* strains compared to WT cells (Figure 2B). Notably, in response to low dose UV-C exposure (5 J/m^2), *ctt1Δ* strains exhibited a significant (p value = 0.0053) increase in cytotoxicity (65% survival) compared to WT cells (89% survival). In response to a relatively high dose of UV-C (25 J/m^2), *sod2Δ*, *cta1Δ*, and *ctt1Δ* exhibited a significant increase (p value >0.05% for all strains) in cytotoxicity (8%, 9%, and 1% survival) compared to WT cells (42% survival). These data indicate that the ROS eliminated by the ROS scavengers *SOD2*, *CTA1*, and CTT*1* play a role in UV-C induced ROS-mediated signaling or cytotoxicity.

Intracellular ROS levels in ROS scavenging mutants following exposure to MMS and UV-C.

To further investigate the role of ROS scavenging systems in the DNA damage-induced ROS response, we next employed a panel of fluorescent probes that detect different, specific subspecies of ROS. These probes include DHR, DHEt, HPF, and APR (Table 1). DHR is a

relatively non-specific probe as it detects a variety of ROS subspecies [25, 26]. In contrast, DHEt specifically detects $O_2^{\bullet-}$ [27, 28]. APR detects H_2O_2 [29, 30] and HPF detects $^{\bullet}OH$ [31]. Cells were exposed to a range of doses of MMS (0-55 mM) and then probed for specific ROS. Following exposure to MMS, an increase in the levels of $O_2^{\bullet-}$ was observed in all strains at the highest dose (55mM) examined, with a significantly elevated response following exposure to 25 and 55 mM MMS in the *sod1Δ* strain compared to WT cells at these same doses (indicated by "*" in Figure 3C). A dose-dependent increase in the levels of $^{\bullet}OH$ was observed for all strains following exposure to MMS (Figure 3D). However, in contrast with the DHEt-detected increase in $O_2^{\bullet-}$, the *sod1Δ* strain did not exhibit a significant increase in the levels of $^{\bullet}OH$ compared to WT cells. When examining the levels of multiple ROS subspecies with the fluorescent probe DHR, an increase in ROS levels were observed in *sod1Δ* and *cta1Δ* strains following exposure to the highest MMS dose (55mM) (Figure 3B). This increase was also observed at 25mM MMS in the *sod1Δ* strain. When cells were probed with the H_2O_2-specific probe, APR, there was no increase in the levels of H_2O_2 detected in any of the strains examined (Figure 2E). The DNA damage-induced ROS response following exposure to MMS in the ROS scavenging mutants indicates that Sod1 influences DNA damage-induced $O_2^{\bullet-}$ levels in cells suggesting that the production/location of ROS following exposure to MMS is cytoplasmic as Sod1 is located primarily in the cytoplasmic.

As previously reported, the increase in intracellular ROS levels does not correspond with to an increase in cell death [19]. Following exposure to MMS, there is no change in the cytotoxicity of any of the ROS scavenging-compromised strains compared to WT cells, yet there are different patterns in the levels of intracellular ROS observed. For example, following exposure to both 25 and 55 mM MMS there is no difference in the cytotoxicity profiles of WT and *sod1Δ* (Figure 3A), however there are significantly increased levels of ROS detected by DHR (indicated by an "# in Figure 3B), and specifically, in the levels of $O_2^{\bullet-}$ (indicated by "*" in

Figure 3C) in the *sod1Δ* strain. These data further substantiate the notion that the increases in intracellular ROS levels are in response to DNA damage and not a result of cell death.

To determine the role of the ROS scavenger in the response to a different class of DNA damaging agent we examined the intracellular ROS levels in the ROS scavenging mutants in response to a range of doses of UV-C (0-50 J/m^2). When cells were probed with the non-specific ROS probe, DHR, following exposure to UV-C, an increase was observed in WT and *cta1Δ* strains at all doses examined (Figure 4B). There was no significant increase in the intracellular levels of ROS in any of the other strains. This ROS response pattern differs from the pattern observed in these strains following exposure to MMS indicating that the DNA damage-induced ROS response may be dependent on the specific nature of DNA damage in the genome. An increase in the levels of $O_2^{\bullet-}$ was observed in all strains following exposure to UV-C (Figure 4C). Following exposure to UV-C, there is no significant increase in the intracellular levels of $O_2^{\bullet-}$ in *sod1Δ* mutants compared to WT cells in contrast to the pattern observed following exposure to MMS. Interestingly, there was a significant increase in $O_2^{\bullet-}$ levels following exposure to UV-C in the *sod2Δ*, *cta1Δ*, and *ctt1Δ* strains (indicated by "*" in Figure 4C). The increase in $O_2^{\bullet-}$ levels in the s *sod2Δ* strain could indicate that the production/location of the increased ROS in response to UV-C induced DNA damage is mitochondrial.

In a set of repair deficient strains (BER^-, NER^-, and BER^-/NER^-) following exposure to UV-C, we previously observed that the levels of $^{\bullet}OH$ and H_2O_2 increased at low doses followed by a decrease at higher doses [19]. A similar response was observed in the ROS scavenging mutants when probed for H_2O_2 (Figure 4D) and $^{\bullet}OH$ (Figure 4E) following exposure to UV-C. The increase in ROS following exposure to low doses of UV-C (2-5 J/m^2) is involved in signaling associated with cell survival and the stress response, while the increase in $O_2^{\bullet-}$, but not H_2O_2 and $^{\bullet}OH$, following exposure to high doses of UV-C (25-55 J/m^2) is involved in signaling for cell death.

The increase in $O_2^{\bullet-}$ levels following exposure to high doses of UV-C (25-50 J/m^2) correlate with increases in cell death. Increased cell death was observed following exposure to high doses of UV-C (25-50 J/m^2) in *sod2Δ*, *cta1Δ*, and *ctt1Δ* strains compared to WT cells. The *sod2Δ*, *cta1Δ*, and *ctt1Δ* strains also displayed increased $O_2^{\bullet-}$ levels following exposure to these same doses of UV-C (25-50 J/m^2) compared to WT cells. These results differ from those observed following exposure to MMS and indicate that the DNA damage-induced ROS response differs depending on the type of DNA damage induced.

Discussion

Oxidative stress has been historically defined as the imbalance of prooxidants and antioxidants which can lead to macromolecular damage (lipids, proteins, RNA, and DNA) and disruption of redox signaling [32, 33]. This definition is limiting in that it assumes that within the cell the balance is uniform and static. More recently, oxidative stress has been redefined as a condition that disrupts redox signaling and control such that the balance of pro- and antioxidants is defined regionally (subcellular localization or tissue-specific within the whole organism) and not system-wide [34]. Maintaining the redox balance in cells is essential for proper cellular functions and survival. Low concentrations of ROS generated under normal physiological conditions are utilized by the cell for signaling; however, when the redox balance shifts, and more ROS is produced, and deleterious consequences can occur including damage to proteins, lipids, and most importantly, to DNA [35]. Increased levels of $O_2^{\bullet-}$ have been associated with apoptosis and cell death in both yeast and mammalian cells [36-38].

Different types of intracellular ROS are both produced and scavenged by cells in a compartment-specific manner. ROS production and scavenging must be regulated in order to maintain a redox balance within the cell and different cellular compartments. ROS can be produced as byproducts of normal cellular metabolism, within mitochondria [11], and peroxisomes, as well as during inflammatory cell activation [39]. We propose that in response to increased levels of DNA damage there is an increase in intracellular ROS levels [19, 20] and address the mechanism of the ROS production, which is not yet understood. While some of the DNA damage-induced ROS is produced in mitochondria, other DNA damage-induced ROS could be generated via different mechanisms in the nucleus or cytoplasm. There is evidence that rho^0 cells with non-functional mitochondria and lacking mtDNA, display a dose-dependent increase in ROS levels in response to increasing doses of MMS. The levels of ROS increase in the rho^0 cells; however, not to the levels seen in WT cells at the same doses of MMS [40]. This increase in

ROS indicates that mitochondria only partly contribute to DNA damage-induced ROS production.

As ROS production tips the redox scale in one direction, ROS scavenging tips it in the other. At the same time increases or decreases in the levels of one sub-type of ROS could lead to an imbalance of the signaling pathways. There are several ROS scavengers present in cells including SODs, CATs, thioredoxins, and glutathiones. Each of these acts through a specific mechanism to maintain the balance of ROS in cells. In *S. cerevisiae* one SOD and one CAT is present in the mitochondria and one SOD and one CAT present in the mitochondria. By examining cells that are defective for different scavenging enzymes, we can identify the potential subcellular localization of the DNA damage-induced ROS.

In the present study, we examined the role of ROS scavengers in the DNA damage-induced ROS response. By examining the cytotoxicity profiles of the ROS scavenging mutants in response to DNA damaging agents that induce distinct types of damage we can determine whether any of the ROS scavengers is capable of protecting cells from the deleterious consequences of these agents and whether this protection is specific for the type of DNA damage. MMS, regardless of dose, is equally toxic to all strains and survival does not decrease in any of the SOD or CAT mutant strains compared to WT cells. These data indicate that, individually, the ROS scavenging enzymes examined plays a role in protecting cells from the deleterious effect of MMS.

UV-C appears to be more toxic to several of the mutant strains, *sod2Δ, cta1Δ,* and *ctt1Δ,* compared to WT cells (Figure 2B). This increase in cytotoxicity indicates that the mitochondrial scavenging enzymes, Sod2 and Cta1, as well as the cytosolic ROS scavenging enzyme, Ctt1, are important for protection against the deleterious effects of UV-C. The increase in ROS following exposure to UV-C may be due to an increase in ROS production in mitochondria rather that the cytoplasm. However, because of the redundant ROS scavenging systems present in cells, it is

difficult to fully appreciate the role of each individual enzyme in protecting cells from the deleterious consequences of DNA damage-induced ROS.

The ROS produced in response to MMS and UV-C could be different with respect to the sub-species produced as well as the sub-cellular localization. Alone, none of the scavenging enzymes are protective against MMS, suggesting that the ROS scavengers have overlapping specificity in protecting cells from the effects of MMS. However, following exposure to UV-C Sod1, Cta1, and Ctt1, all appear to play a more significant than Sod2 role in protecting cells from the deleterious consequences (i.e. cell killing) of UV-C. It is possible that the ROS produced in response to MMS are generated in the cytoplasm and cytosolic ROS are involved in cell survival stress associated signaling, while the ROS produced in response to UV-C is localized to mitochondria, and involved in cell death. Increased levels of mitochondrial ROS have been associated with apoptosis [36, 41].

To further explore the role of ROS scavenging in maintaining the redox state of cells following DNA damage and to address the possible role of ROS in MMS and UV-C induced cytotoxicity, we examined the intracellular ROS levels following exposure to MMS and UV-C. In response to DNA damage, intracellular ROS levels increase in a manner that is dependent on the type and dose of DNA damaging agent [19]. Different sub-species of ROS are induced to different extents following exposure to MMS or UV-C [19]. In the present study, we detected a dose-dependent increase in $O_2^{\bullet-}$ levels following exposure to MMS (Figure 3C). This increase is significantly elevated in *sod1Δ* mutants compared to WT cells. Sod1 is a cytosolic enzyme that scavenges $O_2^{\bullet-}$ [15]. Therefore, the increase in $O_2^{\bullet-}$ observed following exposure to MMS is primarily cytosolic and we hypothesize that it functions in cell signaling. A significant increase in the levels of ROS detected with the fluorescent probe DHR was also observed in the *sod1Δ* strains following exposure to MMS (Figure 3B). We propose that the general increase in ROS is likely due to the specific increase in $O_2^{\bullet-}$ as detected with DHEt.

Following exposure to UV-C, $O_2^{\bullet-}$ levels increase in a dose-dependent manner in all cell types. The increase in $O_2^{\bullet-}$ levels appears to be a general response to all types of DNA damage regardless of the strain background. However, the sub-cellular location of this increase may not be the same for each type of DNA damage. The levels of $O_2^{\bullet-}$ are similar in WT and *sod1Δ* strains; however, there is a significant increase in the levels of $O_2^{\bullet-}$ in *sod2Δ, cta1Δ,* and *ctt1Δ* strains at the highest exposure dose (50 J/m2) (Figure 4C). Interestingly, *sod1Δ* strains were the only ROS scavenging mutant for which the increase in cytotoxicty of UV-C was not observed. This increase in cytotoxicity may indicate that the cytosolic levels of $O_2^{\bullet-}$ do not increase in response to DNA damage produced by UV-C, but the levels of mitochondrial $O_2^{\bullet-}$ may increase. The levels of $^{\bullet}OH$ detected with HPF and H_2O_2 detected with APR following exposure to UV-C exhibited similar dynamics in ROS levels for all strains examined. Exposure to low to moderate doses of UV-C (5-25 J/m^2) increases cellular $^{\bullet}OH$ and H_2O_2, but at higher doses (55 J/m^2), the levels decrease (Figure 4D-E). It is possible that the increase in $^{\bullet}OH$ and H_2O_2 followed by a decrease is caused by an initial increase in ROS to allow for an ROS signaling events, followed by low levels of $^{\bullet}OH$ and H_2O_2 when the levels of DNA damage are elevated to the point that the cell switches from signaling for a survival associated response to signaling for cell death. Evidence exists supporting a role for ROS in signaling for DNA repair and cell death [36, 41].

Previously, we examined a set of repair deficient strains (BER^-, NER^-, and BER^-/NER^-). There is an increase in the intracellular levels of ROS regardless of the strain background following exposure to MMS and UV-C [19]. However, there were changes in the levels of the subspecies of ROS depending on the dose of DNA damaging agent utilized. Following exposure to low doses of DNA damaging agents an initial increase in the levels of ROS ($O_2^{\bullet-}$, $^{\bullet}OH$, and H_2O_2) was observed and is thought to be involved in cell stress response signaling. We propose that following exposure to high doses of DNA damaging agent the levels of DNA damage

increase to the extent that the cell is no longer capable of repairing all DNA damage present. At these levels of DNA damage, $O_2^{\bullet-}$ continues to increase and $^{\bullet}OH$ and H_2O_2 decrease. The increase in $O_2^{\bullet-}$ levels could be due to an increase in production of $O_2^{\bullet-}$ or to a decrease in the conversion of $O_2^{\bullet-}$ to H_2O_2 by SODs. The increase in $O_2^{\bullet-}$ levels is thought to signal cell death and potentially block cell survival signaling [19].

Figure 5 depicts a model of how the cell responds to two distinct classes of DNA damage. Under normal physiological conditions (Figure 5A), the cell maintains the redox balance through the production and scavenging of ROS. Sod1 and Cta1 maintain the ROS balance in the mitochondria (blue), while Sod2 and Ctt1 maintain the ROS balance in the cytoplasm (green). When the cell is exposed to the alkylating agent MMS (Figure 5B), there is a dose-dependent increase in the intracellular levels of ROS which shifts the redox balance within the cell. The increase in $O_2^{\bullet-}$ levels was highest when the cells were deficient for Sod1, the cytosolic SOD. This finding suggests that increased production of $O_2^{\bullet-}$ in response to MMS is localized to the cytoplasm. Furthermore, we hypothesize that the increase in cytoplasmic $O_2^{\bullet-}$ is involved in pro-survival and stress responses signaling because there is no increase in cell death in any of the ROS scavenging mutant strains compared to WT (Figure 5B green). The response to DNA damage caused by UV-C (Figure 5C) differs from that of MMS. Although there is an increase in intracellular ROS levels following exposure to UV-C that shifts the redox balance of the cell, the increase in ROS is greater in *sod2Δ, cta1Δ,* and *ctt1Δ* strains compared to WT cells. Additionally, the cytotoxicity of the *sod2Δ, cta1Δ,* and *ctt1Δ* strains was increased compared to the WT cells. This increase in cytotoxicity would suggest that there is a correlation between the increase in $O_2^{\bullet-}$ and the increase in cytotoxicity. Previous reports have shown that unicellular eukaryotes undergo a caspase-independent apoptotic-like cell death process [37, 38]. One important feature of the cell death process (in both yeast and mammalian cells) is an increase in the intracellular levels of $O_2^{\bullet-}$ [36]. Therefore we hypothesize that the increase in $O_2^{\bullet-}$ observed

following exposure to UV-C is involved in cell death signaling. Our results support a model where $O_2^{\bullet-}$ localization in the cytoplasm is involved in pro-survival signaling and $O_2^{\bullet-}$ localized to the mitochondria contributes to cell death.

Although these studies provide insight into ROS mediated DNA damage responses and the subcellular localization of the ROS subspecies, much work remains to be done. For example, to fully understand the production of ROS in response to DNA damage, cells that are deficient in multiple ROS scavenging enzyme need to be examined. There are many redundant pathways that are capable of scavenging ROS within cells, and unless multiple pathways are compromised it may be difficult to delineate the mechanistic details of ROS signaling. It is possible that if the ROS scavenging pathways present in mitochondria (Sod1 and Cta1) were eliminated there would be increased sensitivity and increased intracellular levels of $O_2^{\bullet-}$ following exposure to UV-C . Similarly, if the cytosolic ROS scavenging enzymes (Sod2 and Ctt1) were eliminated then there may be increased intracellular levels of $O_2^{\bullet-}$ following exposure to MMS. Finally, another level of ROS processing in the cell is through non-enzymatic proteins such as thioredoxins and glutathiones [12, 42]. Cells deficient in these ROS scavengers should also be studied to better understand how the cell produces/handles ROS in response to DNA damage.

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Figure 1. ROS scavenging systems in *Saccharomyces cerevisiae*. There are several ROS scavenging systems present in yeast cells. These include the enzymes superoxide dismutase (SOD), catalase (CAT), and small molecules that function to neutralize ROS. SODs are responsible for the conversion of $O_2^{\bullet-}$ into H_2O_2. There are two SODs present, Sod1 (located in the cytoplasm) and Sod2 (located in the mitochondria). The conversion of $O_2^{\bullet-}$ into $^{\bullet}OH$ can occur both in the cytoplasm and the mitochondria via the Fenton Reaction. CATs are responsible for the conversion of H_2O_2 into H_2O and O_2. Two CATs are present in yeast cells, Cta1 (located in the mitochondria) and Ctt1 (located in the cytoplasm). Cta1 is also present in mitochondria. In addition, small molecules which are capable of converting H_2O_2 into H_2O and O_2 include the glutathiones and thiroedoxins [42].

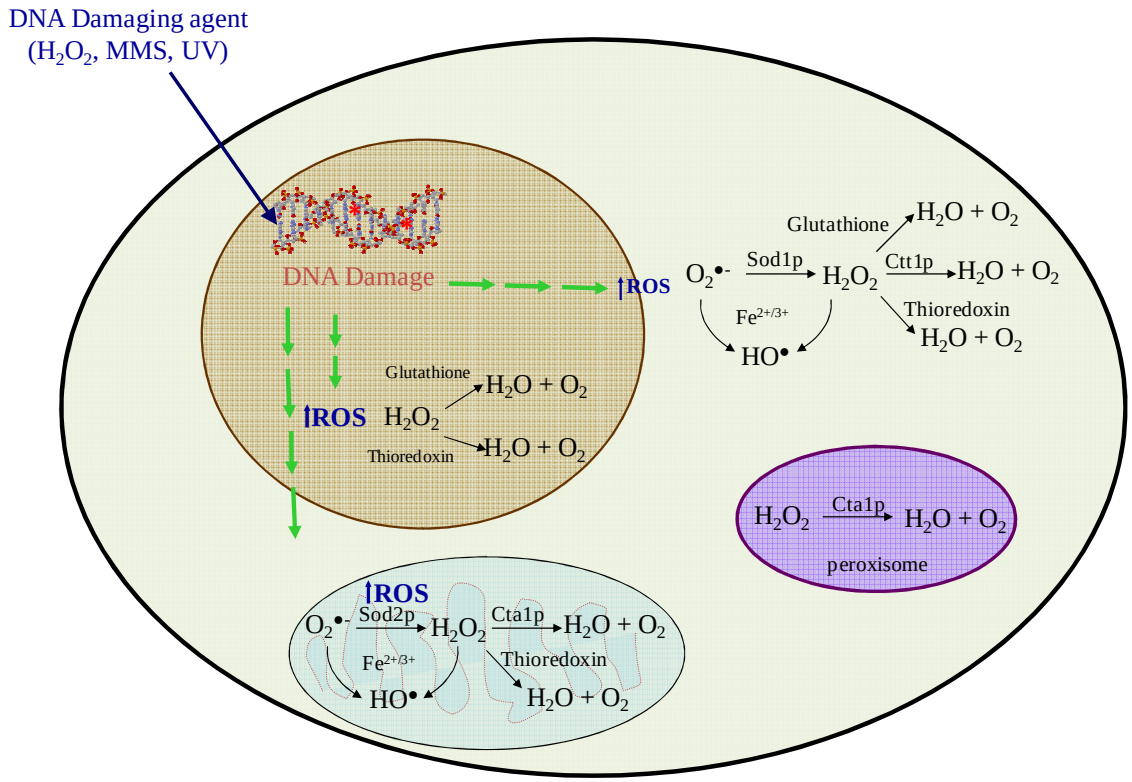

Figure 1. ROS scavenging systems in *Saccharomyces cerevisiae*.

Figure 2. MMS and UV-C cytotoxicity profiles of ROS scavenging mutants. WT (circles), *sod1Δ* (squares), *sod2Δ* (diamonds), *cta1Δ* (triangles), and *ctt1Δ* ("x"s). Cells were exposed to **(A)** 0, 0.5, 5, 25, and 55 mM MMS for 30 min at 30 °C or **(B)** 0, 2, 5, 25, 50 J/m^2 UV-C. The results represent an average of six different experiments. Error bars represent ± SD. Experimental details are provided in the text.

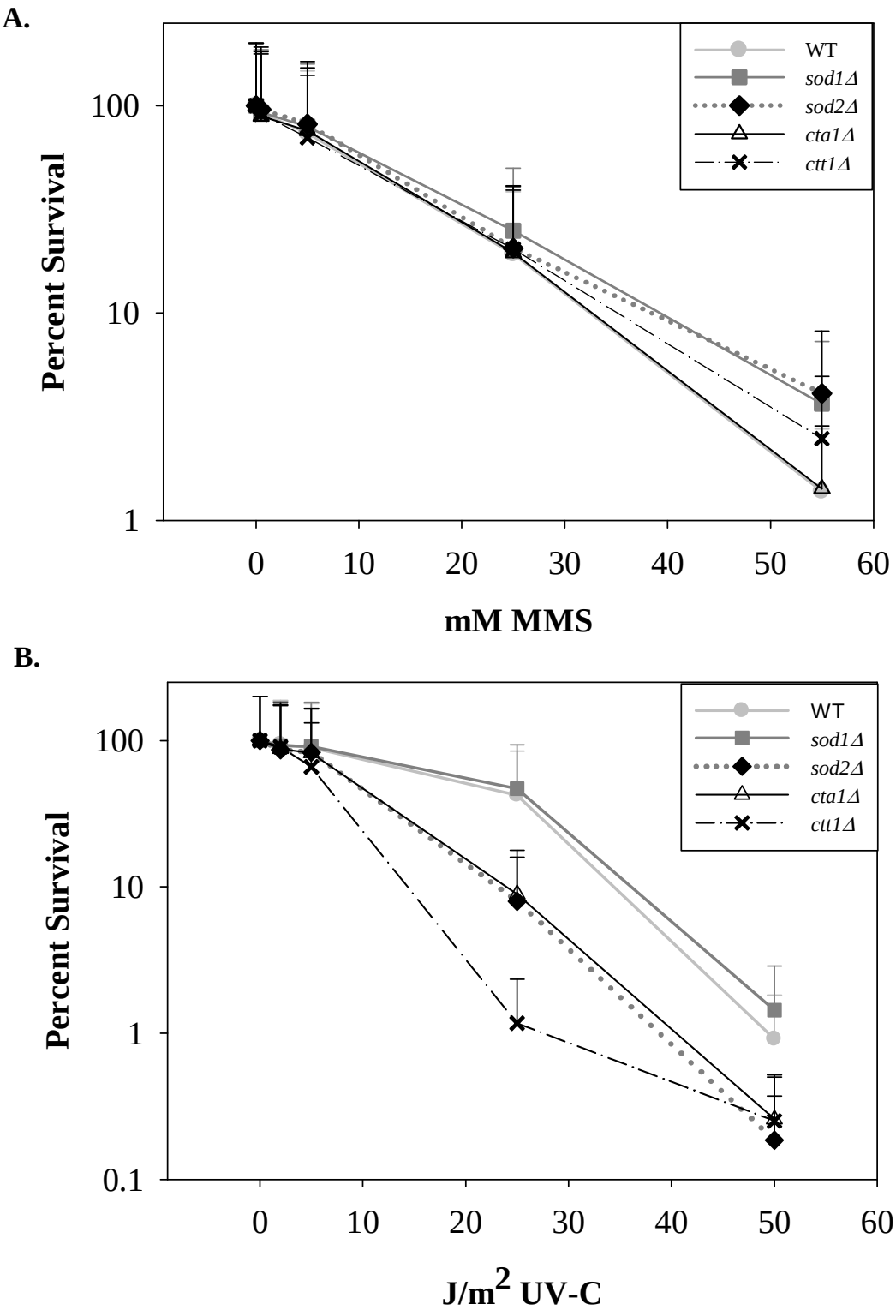

Figure 2. MMS and UV-C cytotoxicity of ROS scavenging mutants.

Table 1. ROS Fluorescent Probes

Fluorescent Probe	ROS detected	Reference
Dihydrorhodamine (DHR)	multiple species	(28,29)
Dihydroethidium (DHEt)	$O_2^{\bullet -}$	(34,35)
2-[6-(4'-Hydroxy)phenoxy-3H-xanthen-3-on-9yl] bezoic acid (HPF)	$^{\bullet}OH$	(36,37)
N-AcN-Acetyl-3,7-dihydroxyphenoxazine (Amplex Red (APR))	H_2O_2	(38)

Figure 3. ROS levels in scavenging mutants in response to DNA alkylation damage. A. MMS cytotoxicity of ROS scavenging mutants. WT (circles), *sod1Δ* (squares), *sod2Δ* (diamonds), *cta1Δ* (triangles), and *ctt1Δ* ("x"s). Cells were exposed to 0, 0.5, 5, 25, and 55 mM MMS for 30 min at 30 °C as described in the text. Results displayed are an average of six different experiments. **B – E.** Isogenic WT, *sod1Δ*, *sod2Δ*, *cta1Δ*, and *ctt1Δ* strains were first exposed to MMS (0-55 mM) for 30 min and then incubated with the indicated fluorescent probes (**B**) DHR, (**C**) DHEt, (**D**) HPF, or (**E**) APR for 2 hours. Following incubation, cells were analyzed for ROS levels by flow cytometry as described in Experimental Procedures. Fluorescence values were obtained from measurement of the mean peak values of the cytograms. Fluorescence values for each ROS probe are reported as fold changes relative to the WT strain (set to a value of 1.0). Error bars represent ± SD.

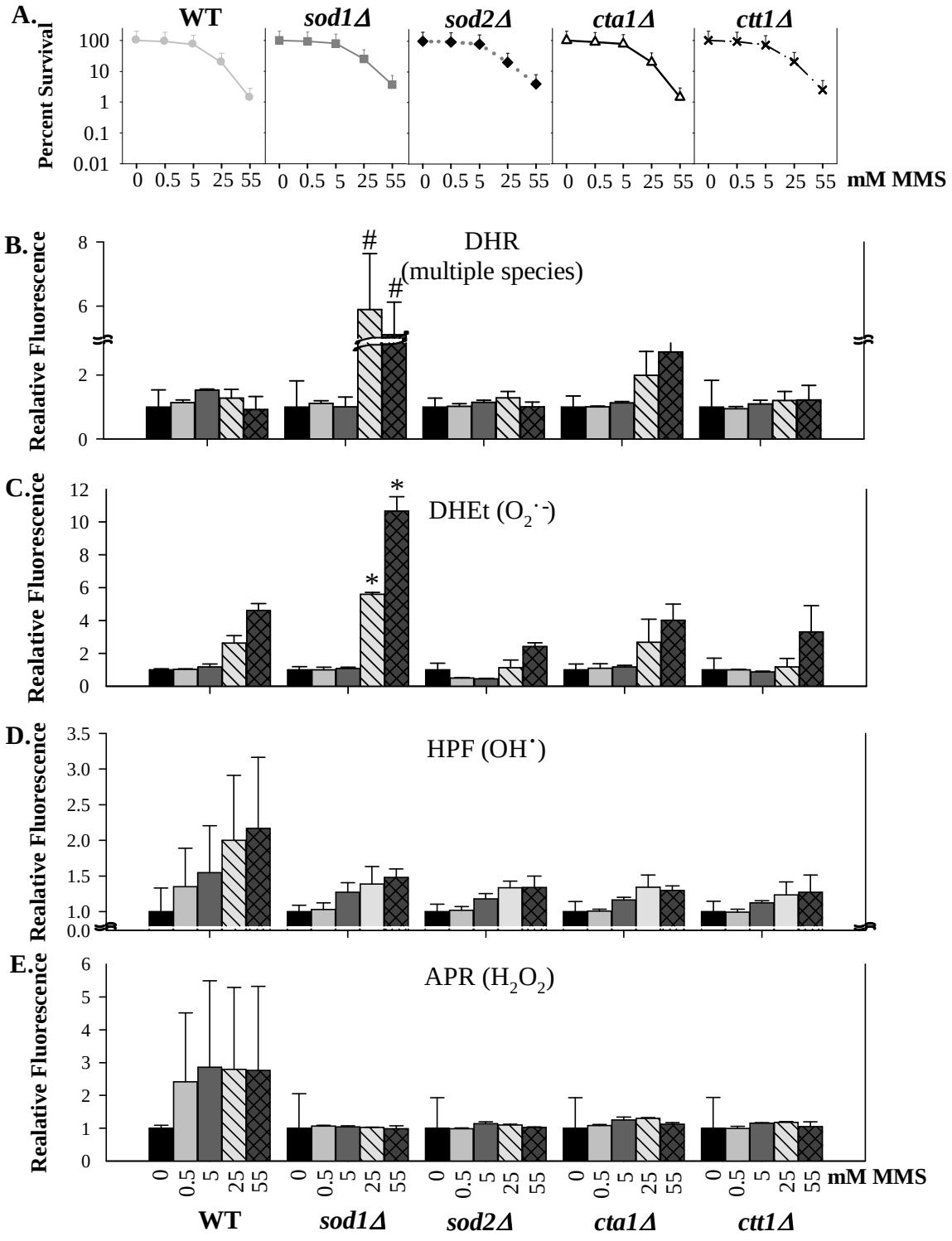

Figure 3. ROS levels in scavenging mutants in response to DNA alkylation damage (MMS).

Figure 4. ROS levels in scavenging mutants in response to UV-C induced DNA damage. A. UV-C cytotoxicity of ROS scavenging mutants. WT (circles), *sod1Δ* (squares), *sod2Δ* (diamonds), *cta1Δ* (triangles), and *ctt1Δ* ("x"s). Cells were exposed to 0, 2, 5, 25, and 50 J/m^2 UV-C as described in the text. Results displayed are an average of six different experiments. **B - E.** Isogenic WT, *sod1Δ*, *sod2Δ*, *cta1Δ*, and *ctt1Δ* strains were first exposed to UV-C (0-50 J/m^2) and then incubated with the indicated fluorescent probes (**B**) DHR, (**C**) DHEt, (**D**) HPF, or (**E**) APR for 2 hours. Following incubation, cells were analyzed for ROS levels by flow cytometry as described in Experimental Procedures. Fluorescence values were obtained from measurement of the mean peak values of the cytograms. Fluorescence values for each ROS probe are reported as fold changes relative to the WT strain (set to a value of 1.0).

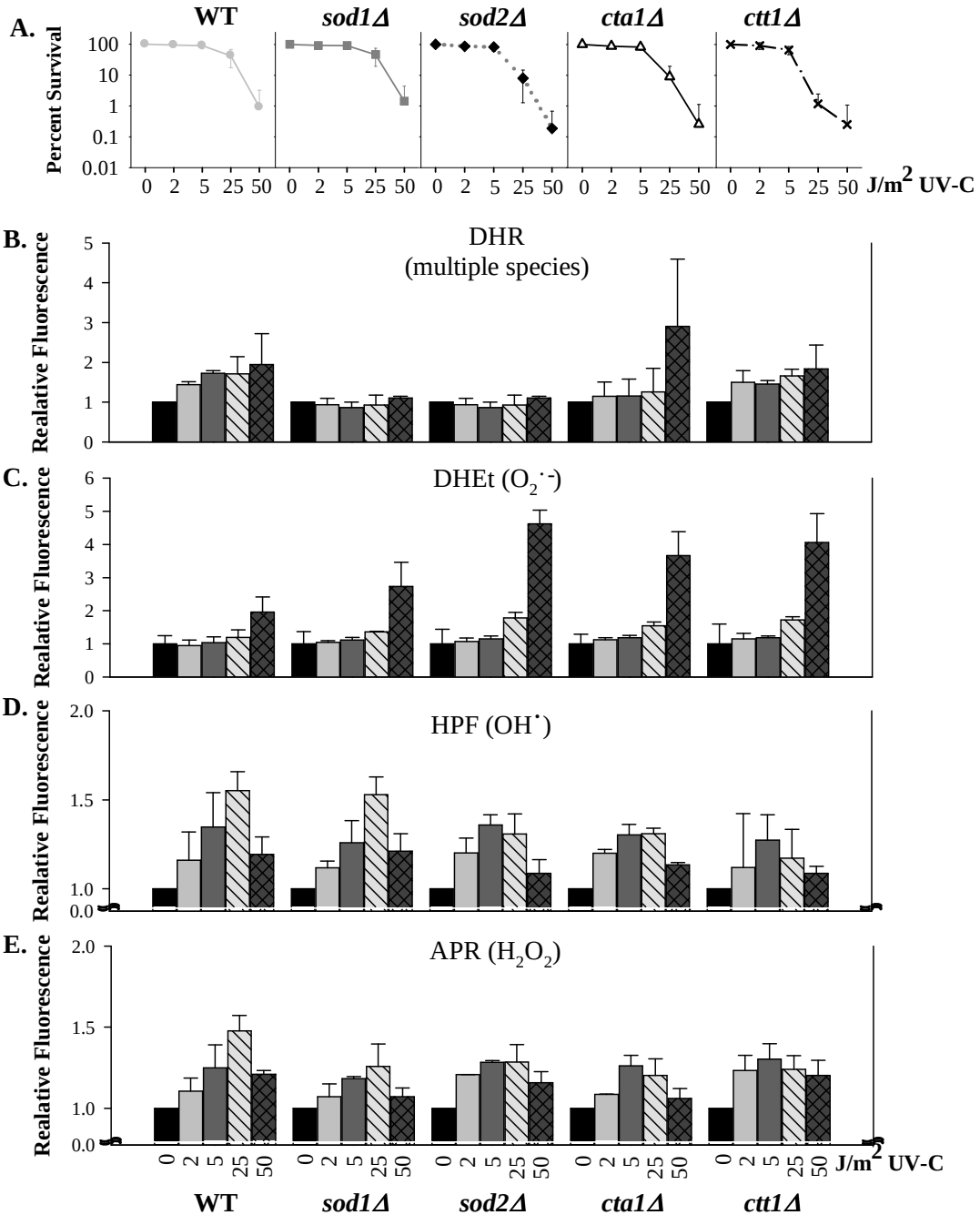

Figure 4. ROS levels in scavenging mutants in response to UV-C induced DNA damage.

Figure 5. Model for ROS scavenging systems in the DNA damage response. A. Under normal cellular growth conditions, in the absence of genomic DNA damage, the redox state of the cell is maintained through the production and scavenging if ROS. ROS can be produced in the mitochondria, cytoplasm, and the nucleus. In order to maintain the proper balance of ROS the ROS scavengers, SODs and CATs convert ROS into less toxic molecules. There are SODs and CATs present in both the mitochondria (blue) as well as the cytoplasm (green). The $O_2^{\bullet-}$ and H_2O_2 present in the cell can be employed in signaling processes to maintain normal cell growth and metabolism. **B.** Following exposure to high doses of MMS (25-55 mM) there is an increase in intracellular $O_2^{\bullet-}$ levels. The increase in $O_2^{\bullet-}$ levels could be involved in cellular signaling of DNA damage. In *sod1Δ* strains following exposure to high doses of MMS (25-55 mM) there is an additional increase in the levels of $O_2^{\bullet-}$ compared to WT cells, indicating that the location of the $O_2^{\bullet-}$ increase is in the cytoplasm. There is no increase in the levels of cytotoxicity in *sod1Δ* strains following exposure to MMS compared to WT cells. Therefore, the increase in $O_2^{\bullet-}$ levels is likely to be associated with cell survival and other stress responses. **C.** Following exposure to high doses of UV-C (25-50 J/m^2) there is an increase in the intracellular levels of $O_2^{\bullet-}$. An additional increase in $O_2^{\bullet-}$ compared to WT cells following exposure to UV-C was observed in the *sod2Δ*, *cat1Δ*, and *ctt1Δ* strains. The *sod2Δ*, *cat1Δ*, and *ctt1Δ* strains also displayed an increase in cytotoxicity at the high doses of UV-C (25-50 J/m^2) compared to WT cells. Therefore, the increase in $O_2^{\bullet-}$ levels following exposure to UV-C is thought to be localized to the mitochondria where it functions in cell death response.

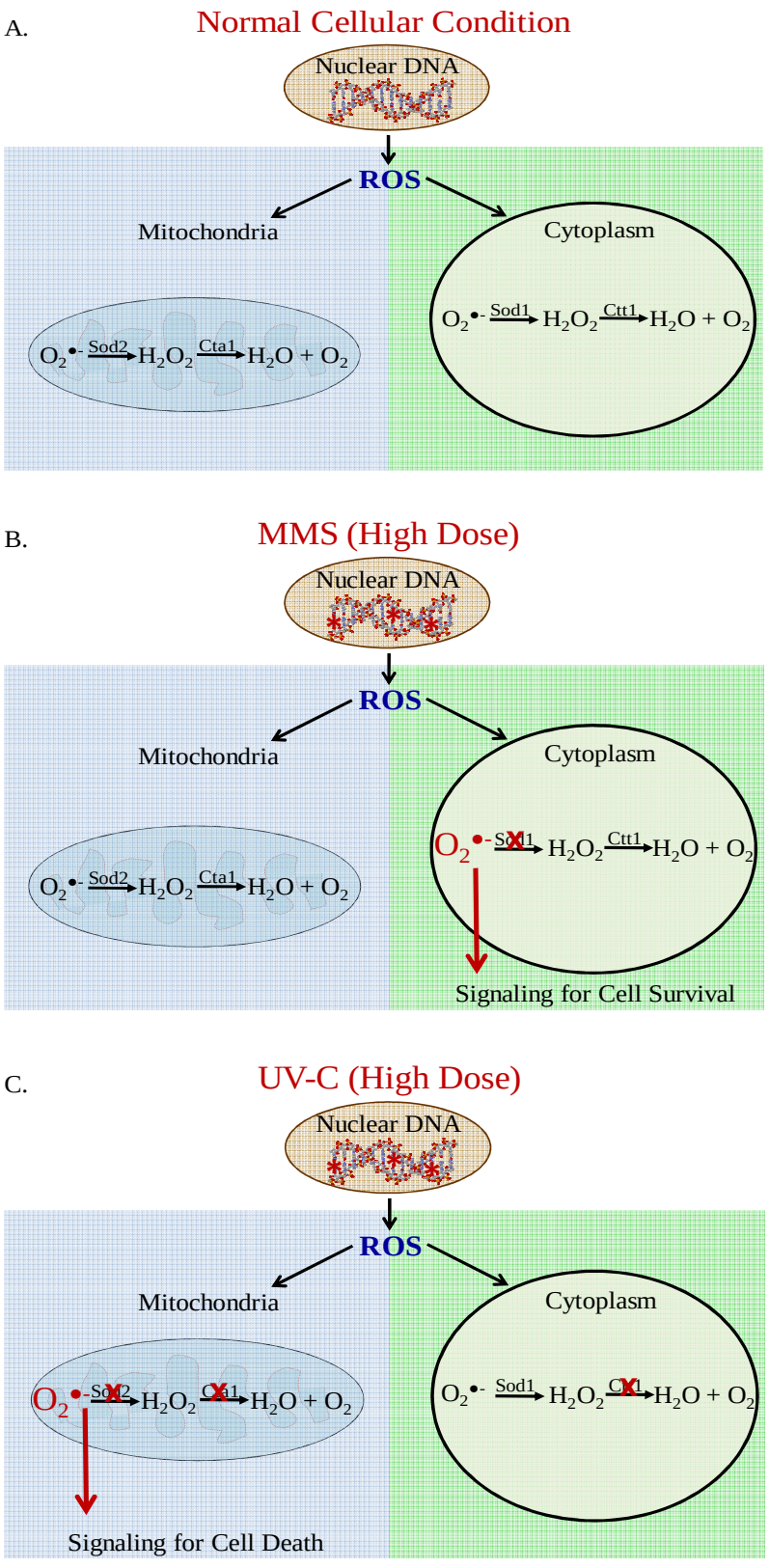

Figure 5. Model for ROS scavenging systems in the DNA damage response.

CHAPTER IV

Yap1: A DNA damage responder in *Saccharomyces cerevisiae*

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(Manuscript to be submitted)

Abstract

Cellular DNA is continually damaged by both endogenous and exogenous sources of reactive oxygen species (ROS) and the DNA must be repaired with high efficiency and fidelity for normal DNA transactions to occur. Exceeding cellular repair capacity by introducing high levels of damage or compromising the functions of the repair systems is thought to be a causative factor for human diseases including cancer, neurodegenerative disorders, cardiovascular disease, as well as the process of aging. Activation of signaling pathways in response to genotoxic stress is crucial for cells to properly repair DNA damage. In response to DNA damage, intracellular levels of ROS increase. It has been proposed that an important function of ROS generated following DNA damage is to initiate specific signal transduction processes that are responsible for appropriate management of oxidative stress. In this study we have employed *Saccharomyces cerevisiae* as a model eukaryotic system to examine the ability of DNA damage-induced ROS to activate the transcription factor, Yap1. By examining the subcellular localization of Yap1 and several biological endpoints (cytotoxicity, intracellular ROS levels, and genomic stability) we are able to assess the involvement of Yap1 in the DNA damage-induced ROS response. Determination of the subcellular localization of Yap1in response to three distinct classes of DNA damaging agents (hydrogen peroxide (H_2O_2), methyl methanesufonate (MMS), and ultraviolet light (UV-C)) revealed that Yap1 relocalizes to the nucleus in response to H_2O_2 and MMS, but not UV-C. These results indicate that an important component of the DNA damage-induced stress response involves the production of ROS and activation of Yap1 that is specific for DNA lesions that are repaired by the base excision repair (BER) but not the nucleotide excision repair (NER) pathway. In addition to its role in oxidative stress responses, we have shown thatYap1 is involved in the DNA damage response and is important for cell survival and maintaining genomic stability.

Introduction

Cells are continually exposed to both endogenous and exogenous sources of reactive oxygen species (ROS). High levels of ROS have been considered detrimental to cells leading to oxidative stress and to impaired physiological function through damage to DNA, proteins, and lipids [1-3]. In addition, chronic exposure to ROS has been associated with several human pathologies including cancer [4], neurodegenerative disorders [5], and cardiovascular disease [6], as well as the process of aging [7]. In contrast, recent studies have demonstrated the benefit of ROS as signaling molecules [3, 8-10]. At low levels, ROS is involved in cell signaling processes for normal cellular function [11]. ROS-mediated signaling is likely to be an important component in the response to genotoxic insults, including those caused by oxidative stress [12].

In order to maintain genomic stability, cells have evolved a number of DNA damage handling pathways that include direct reversal, base excision repair (BER), nucleotide excision repair (NER), mismatch repair, translesion synthesis, and recombination [13]. As BER and NER are key systems for the removal of numerous deleterious lesions from the genome [13], the influence of ROS signaling on the function of these pathways may be crucial for an appropriate cellular response to genotoxic stress. BER is primarily responsible for the repair of small, non-bulky lesions (primarily base modifications) and abasic sites, such as those caused by oxidizing and alkylating agents [14]. NER is thought to be the major pathway for the repair of DNA helix distorting lesions such as cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4 PP) induced by ultraviolet light (UV) as well as bulky lesion [14], NER can also contribute to the repair of oxidative DNA damage as well [13, 15].

In yeast, BER and NER are the major repair pathways for most types of DNA lesions, and the specific range of DNA damages that are primarily handled by each are generally distinct. By examining cells deficient in one or both of these repair pathways it is possible to delineate the relationship between cytotoxicity and ROS levels and determine whether such responses constitute a general response to DNA damage or are limited to specific classes of DNA lesions.

We have recently documented that increased levels of DNA damage, regardless of type, cause elevations in intracellular ROS [12]. These studies also revealed that cells deficient in both BER and NER harbor significantly elevated (~800-fold) [15] levels of oxidative DNA damage, and increased ROS levels (80 %) compared to DNA repair-proficient strains [16]. Furthermore, intracellular ROS levels in both WT and repair-deficient strains (BER^-, NER^-, and BER^-/NER^- deficient strains) are elevated in a dose-dependent manner following exposure to the DNA alkylating agent methyl methanesulfonate (MMS) or UV-C [12]. These studies indicate that DNA damage per se can cause an increase in intracellular ROS independent of the class of DNA lesion or the pathway involved in its repair, suggesting that the ROS produced may function in signaling processes [12].

Yap1, a transcription factor, is activated by oxidative stress [17-21]. Under normal cellular conditions Yap1 is localized in the cytoplasm due to constitutive nuclear export by the nuclear export receptor, Crm1 [20, 22]. However, under conditions of increased oxidative stress, up to three intermolecular disulfide bonds form in Yap1 blocking the binding of Crm1, inhibiting Yap1 nuclear export, leading to Yap1 accumulation in the nucleus [23, 24]. Over 70 genes have been shown to be directly activated by Yap1, including superoxide dismutase (*SOD1*), catalase (*CTT1*), and thioredoxin (*TRX2*) [25]. Several studies suggest that genes involved in DNA repair, replication, and check point control can be activated by Yap1, including *NTG1*, *POL1*, *MAG1*, *MEC1*, and *POL3* [26, 27]. Yap1 was reported to be induced in response to hydrogen peroxide (H_2O_2) exposure in a screen to identify DNA damage-inducible transcripts in cells with different DNA repair backgrounds [16]. In addition,Yap1 accumulates within the nucleus in response to MMS exposure [12]. These findings suggest that Yap1 may be directly involved in the DNA damage response.

The objectives of this study were to determine the involvement of Yap1 in the DNA damage response and to define the types of DNA damage capable of Yap1 activation. We examined biological endpoints in a set of isogenic repair-proficient (WT, *yap1Δ*) and repair-

deficient (BER$^-$, BER$^-$ *yap1Δ*, NER$^-$, and NER$^-$ *yap1Δ*) strains with and without Yap1. Determination of the subcellular localization of Yap1 in repair-proficient and -deficient cells exposed to different DNA damaging agents revealed that Yap1 accumulates to the nucleus in response to H_2O_2 and MMS, but not UV-C. In addition, cytotoxicity, intracellular ROS levels, and genomic stability endpoints were determined. Our results suggest that ROS generated in response to DNA damage likely functions in signaling processes that activate Yap1. This response appears to function for types of DNA damage primarily repaired by the BER pathway.

Experimental Procedures

Strains, Media, and Growth Conditions – Two sets of isogenic repair deficient strains were utilized in these studies. Each isogenic set contains a repair proficient strains (wild type – WT), DSC025, LAR009, LAR017, and LAR025. Strains deficient in BER were deleted for *NTG1*, *NTG2*, and *APN1*and include DSC035, LAR013, LAR021, and LAR029. Strains deficient for NER were deleted for *RAD1* and include DSC036 and LAR011. *yap1Δ* strains include LAR010, LAR018, and LAR026. BER$^-$ *yap1Δ* strains include LAR014, LAR022, and LAR030. NER$^-$ *yap1Δ* strains include LAR014. All LAR strains were derived from hDNP42 (all genotype listed in Table 1). All of the DSC derived strains were constructed as previously reported [28]. All hDNP42 diploid strain was constructed by transformation of the hDNP19 with PCR fragment containing *natNT2* gene, conferring resistance to nourseothricin. Plasmid pYM17 (Euroscarf) was used as a template for amplification of *natNT*2 gene [29]. The sequence of the primers is available upon request. The DSC derived strains were utilized in Yap1 localization studies and measurement of $O_2^{\bullet-}$ levels. The hDNP42 derived strains were utilized in the cytotoxicity, mutation rate, and CHEF gel electrophoresis studies. All yeast strains were grown in YPD media (1% yeast extract, 2% peptone, 2% dextrose and 2% agar for plates). All YPD media were supplemented with 0.005% adenine sulfate. For selection of strains containing the Yap1-GFP plasmid, the strains were grown on SD-minimal –URA media (0.5% ammonium sulfate, 0.17% yeast nitrogen base without amino acids, 2% dextrose, 0.14% minimal Ura drop out mix, and 2.5% agar for plates) [30]. All strains are listed in Table 1.

Strains referred to in the following text as BER$^-$ deficient contain disruptions in the *NTG1*, *APN1*, and *APN2* genes and NER$^-$deficient contain a disruption in the *RAD1* gene. Strains that also have a disruption in *YAP1* gene are referred to as *yap1Δ*, BER$^-$ *yap1Δ*, and NER$^-$ *yap1Δ* deficient strains.

Cell Growth and Viability - Liquid YPD media was inoculated with yeast cells and grown at 30 °C for ~24 hrs to saturation (> 7 x 10^7 cells/mL). A hemacytometer was used to determine the density of the cells and 50 mL YPD was inoculated with an appropriate amount of cells so that the culture would reach a density of 2 x 10^7 after 12 hrs of growth at 30 °C. To determine cell viability cultures, were plated on YPD after exposure to MMS or UV-C and incubated for 48 hrs at 30 °C. Cultures were diluted to a density that would yield approximately 100-200 colonies per plate.

YAP1-GFP Plasmid Construction - For studies examining the subcellular localization of Yap1, cells derived from SJR751 strain (WT, BER^-, and NER^-) were transformed with pLR1 plasmid as previously described [12].

Yap1 Cellular Localization Studies - Cells transformed with the YAP1-GFP plasmid, pLR1, were grown to mid-log phase (~2 x 10^7 cells/mL) as described above (*Cell Growth and Viability)* in YPD at 30 °C overnight, counted, and washed twice with H_2O. The density of the cells was adjusted to 2 x 10^7 cells/mL in H_2O. Cells were stained with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen) to visualize DNA in nuclei and mitochondria by incubating cells with 1 μL DAPI (100 nM) per mL of cells for 5 min, washed once with H_2O, and then resuspended to the original volume in H_2O. Cells were then exposed to H_2O_2, MMS, or UV-C. Cells were incubated in H_2O_2 or MMS throughout the time course. Cells were exposed to UV-C at the start of the time course and then placed in the dark for the duration of the time course. Cells were subjected to fluorescence confocal microscopy (Zeiss LSM510 META) and images were analyzed using Carl Zeiss LSM Image Browser software.

Measurement of $O_2^{\bullet-}$ *Levels* - $O_2^{\bullet-}$ levels were detected using the fluorescent probe DHEt [31, 32]. Cells were grown to mid-log phase (~2 x 10^7 cells/mL) in YPD at 30 °C overnight. Cells were counted (hemacytometer), washed twice in H_2O and then adjusted to 2 x 10^7 cells/mL in H_2O. Cells were then exposed to various doses of either MMS or UV-C as described in the

text. For experiments involving UV irradiation, cells (15 mL) were placed in a 15 mm petri dish and exposed to UV-C. Immediately after UV exposure, cells were placed in the dark to prevent photoreactivation. For experiments involving MMS, cells (3 mL) were placed in the dark and exposed to MMS for 30 min at 30 °C. Following exposure to MMS or UV-C, 1 mL aliquots were used for survival measurements and 2 mL were incubated with 25 μg/mL DHEt for $O_2^{\bullet-}$ measurements. Immediately following fluorescent probe addition, cells were incubated in the dark at 30 °C for 2 hrs. Cells were subsequently washed twice with H_2O and then resuspended in 2 mL phosphate buffered saline (PBS) and assessed for fluorescence intensity employing a BD™ LSR II flow cytometer (BD Biosciences).

Measurement of Mutation Rates- Cells were grown to saturation ($2x10^8$ cells/mL) in 5 mL YPD at 30 °C. Cells were then washed twice in H_2O and concentrated to 1mL. Cells were then plated on SD-Arg+CAN (0.5% ammonium sulfate, 0.17% yeast nitrogen base without amino acids, 2% dextrose, 0.14% minimal Arganine drop out mix, 0.006% Canavanine, and 2.5% agar) and YPD at an appropriate dilution to result in growth of greater than 20 colonies per plate from at least two independent segregants. The plates were then incubated at 30 °C until colonies appeared. Colonies were counted and mutation rates were calculated as previously described [33].

Replicative Aging Experiments and CHEF Gel Electrphoresis – Replicative aging experiments were carried out by serially passaging haploid strains on rich medium (YPD) for multiple generations (20 generations) as previously described in [29]. Cells from colonies (originating from a single cell) obtained from each passage was streaked on YPD and incubated at 30 °C for 48 hours. CHEF gel electrophoresis was performed as previously described to determine large scale chromosomal rearrangements [34]. The karyotype was determined for the first or "founder" generation and then for subsequent (10^{th} and 20^{th}) generations.

Results

A major objective of these studies was to address the involvement of the transcription factor Yap1 in the cellular DNA damage response. Previously, we determined that an increase in intracellular ROS levels is a genotoxic stress response caused by different classes of DNA damaging agents such as MMS and UV-C [12]. Increases in both endogenous oxidative DNA damage and intracellular levels of ROS were also observed in DNA repair compromised cells (BER^-/NER^- deficient) [12, 15, 16]. Increased levels of ROS are capable of triggering nuclear accumulation and activation of Yap1 through a conformational change that blocks the binding of the nuclear export factor Crm1, therefore allowing Yap1 to accumulate in the nucleus [18, 21]. Yap1 is believed to upregulate the expression of several genes involved in DNA repair, such as *NTG1* and *MAG1* [26, 27]. In the present investigation, we addressed the possible involvement of Yap1 in signaling by DNA damage-induced ROS through the activation of Yap1.

Yap1 nuclear relocalization in response to DNA alkylation damage.

Fluorescence microscopy of isogenic strains deficient in different DNA repair was utilized to determine the localization of Yap1-GFP in response to different classes of DNA damaging agents that are processed by different repair pathways such as BER and NER (Figure 1). The alkylating agent, MMS, produces non-bulky base damages that are primarily repaired by BER, while UV-C induces larger, helix distorting DNA lesions that are primarily repaired by NER [13]. Although each of these DNA damaging agents produces specific types of DNA lesions, neither of these agents itself is an ROS, and thus cannot directly activate the oxidative stress response or Yap1.

Both MMS and UV-C can cause an increase in intracellular ROS levels [12]. To examine the involvement of Yap1 in the DNA damage response, we exposed repair-proficient (WT) and repair-deficient (BER^- and NER^-) strains to low and high doses of MMS. Yap1 relocalizes to the nucleus in repair-proficient (WT) cells exposed to high doses (25 mM), but not

low doses (0.5 mM) of MMS (Figure 2A and [12]). Nuclear localization of Yap1 was observed in 12% of WT cells exposed to 0.5 mM MMS. When WT cells were exposed to 25 mM MMS the number of cells displaying Yap1 nuclear localization increased to 32% (Figure 2A). Yap1 localized to the nucleus in WT cells following exposure to H_2O_2 within the first few minutes of exposure (Figure 1 and 2A) confirming previous reports [19, 20, 35]. H_2O_2-induced Yap1 relocalization occurred faster compared to MMS-induced relocalization (Figure 2A). In contrast, no change in localization of Yap1 was observed in WT cells following UV-C exposure (Figure 2A). We next examined the localization of Yap1 in BER$^-$ and NER$^-$ deficient strains. In BER$^-$ deficient cells exposed to a low dose (0.5 mM) of MMS, a higher percentage of cells displayed nuclear accumulation of Yap1 to the nucleus as compared to WT cells (Figure 2B). A delay in the localization of Yap1 to the nucleus was observed in BER$^-$ deficient cells following MMS exposure, requiring 60 minutes for 47% of cells to display nuclear localization, a response similar to that observed for MMS-exposed WT cells (Figure 2B). The relocalization of Yap1 to the nucleus in NER$^-$ deficient cells was influenced by H_2O_2 exposure and high, but not low doses of MMS, and was similar to the responses observed for both WT and BER$^-$ deficient cells (Figure 2C). Localization of Yap1 was unchanged in BER$^-$ and NER$^-$deficient cells following exposure to UV-C (Figures 2B and C respectively). Collectively, these data suggest that the response of Yap1 to DNA damage is specific for the type of DNA damage present and also indicate that the activation of Yap1 is occurs in cells containing high levels of unrepaired DNA damage.

Yap1-mediated protection from oxidative and alkylating DNA damage cytotoxicity.

The involvement of Yap1 in the protection from deleterious consequences of exposure to MMS, UV-C, and H_2O_2 was investigated by examining the cytotoxicity of repair-proficient and -deficient cells with and without functional Yap1 following exposure to these agents. MMS and UV-C cannot directly produce intracellular ROS, so it is unlikely that these agents can directly activate Yap1. However, it is possible that Yap1 activation could be indirectly induced by DNA

damage. In WT and NER$^-$ deficient cells, Yap1 does not protect cells from the cytotoxic effects of MMS (Figure 3A and 3C). However, BER$^-$ deficient cells were protected from the cytotoxic effects of MMS by Yap1. BER$^-$ deficient cells exposed to 5 mM MMS exhibited 74% survival while BER$^-$ y*ap1Δ* deficient cells exhibited 23% survival (Figure 3B). These results indicate that Yap1 functions in protecting cells from increased levels of MMS-induced alkylating base damage.

To address the involvement of Yap1 in the in the protection of cells from other classes of DNA lesions, we examined the cytotoxicity of the repair-proficient and -deficient strains with and without functional Yap1 following exposure to UV-C. In response to a range of doses of UV-C (0-25 J/m^2), all strains exhibited increased cytotoxicity to increasing doses of UV-C (Figure 3D-3F). In contrast to the responses observed following MMS exposure, Yap1 did not protect the cells from the cytotoxic effects of UV-C in either the DNA repair-proficient or -deficient strains. These results indicate that Yap1 (in BER$^-$ deficient strains) is likely to function in the response caused by DNA lesions that are primarily repaired by BER, but not NER.

Previous studies have established that H_2O_2 oxidizes and directly activates Yap1 [19]. Yap1 mutants displayed increased sensitivity to H_2O_2 compared to the WT and repair-deficient strains at low doses (0.5-5 mM H_2O_2) suggesting Yap1 influences the cytotoxic effects of oxidative stress (Figure 3G-3I). WT cells following exposure to 0.5 mM H_2O_2 exhibited greater (85%) survival compared to *yap1Δ* cells, which exhibited lower (35%) survival at the same exposure dose (Figure 3G). These results indicate that Yap1 is able to protect cells from the H_2O_2-induced cytotoxicity.

The cytotoxic response of Yap1 mutants as well as Yap1 relocalization following exposure to three different classes of DNA damaging agents suggests a role for Yap1 as a DNA damage responder. Yap1 activation in response to oxidative stress is well documented and can be caused by direct activation of Yap1 and subsequent retention of Yap1 within the nucleus. In this

study the activation of Yap1 occurs indirectly through DNA damage followed by an increase in intracellular ROS levels. MMS and UV-C, unlike H_2O_2, are not direct sources of ROS which makes it possible to address the role of Yap1 in DNA damage signaling. The relocalization of Yap1 to the nucleus following exposure to MMS is observed in BER$^-$ deficient cells at lower doses than in WT and NER$^-$ deficient cells. Increased cytotoxicity to MMS in BER$^-$ deficient cells resulted in a similar pattern; BER$^-$ deficient cells were more sensitive to MMS compared to NER$^-$ deficient cells. These results suggest that Yap1 responds to DNA lesions that are primarily repaired by the BER pathway. Yap1 is activated in response to DNA damage-induced ROS produced in response to alkylating DNA damage and therefore is involved in the DNA damage signaling response.

Yap1 modulation of DNA repair

The activation of Yap1 could be due to the increase in intracellular ROS that occurs following exposure to MMS or due to the involvement of Yap1 in the genotoxic stress response. To probe the involvement of Yap1 in the maintenance of genomic stability, we determined the level of genetic instability in repair-proficient and -deficient strains with and without functional Yap1 via a forward mutation assay utilizing the *CAN1* locus.

Following analysis of forward mutation rates (*CAN1* resistance), a significant 2.5-fold increase in mutation rate was observed in *yap1Δ* strains as compared to WT strains (Table 2). A 15-fold increase in mutation rate was also observed in the BER$^-$ deficient strain compared to WT cells. In contrast, there was no increase in mutation rates in the NER$^-$ deficient strain (Table 2). However, the deletion of *YAP1* causes a 2-fold increase in mutation rates in NER$^-$ *yap1Δ* deficient strains, but no increase in the BER$^-$ *yap1Δ* deficient strain when compared to NER$^-$ and BER$^-$, respectively. These results suggest that Yap1 has a moderate anti-mutator effect. This anti-mutator effect is less detectable in BER$^-$ deficient cells because it is possible that Yap1 and BER

function in the same pathway. BER utilizes the protein Ntg1 in its repair process, and there is some evidence that *NTG1* is upregulated by Yap1, therefore in the BER$^-$ *yap1Δ* deficient strains where *NTG1* and *YAP1* are removed, no further increase in mutation rates is observed.

Role of Yap1 in prevention of large-scale chromosomal aberrations

Large-scale chromosomal aberrations are known to be consequences of increased levels of intracellular ROS [36] and chronic unrepaired DNA damage [29]. We followed the occurrence of chromosomal aberrations in replicative aging experiments described in materials and methods and [29]. Briefly, in replicative aging studies we can evaluate the stability of the genome via CHEF gel electrophoresis to separate the chromosomes of DNA repair-proficient and -deficient strains with and without functional Yap1 that have been serially passaged (subcultured) for multiple generations on rich medium (YPD). The genomic DNA was analyzed for each founder strain (p0) and again at the 10th (p10) and 20th (p20) generations for chromosomal aberrations. A representative image of ethidium bromide-stained gels is shown in Figure 4.

Previous studies revealed that cells compromised in BER alone or in conjunction with NER (BER$^-$/NER$^-$ deficient), which have elevated levels of intracellular ROS [12, 16], also have increased chromosomal aberrations [29]. In the present studies, a significant increase in chromosomal aberrations was observed in *yap1Δ*, BER$^-$deficient, and BER$^-$ *yap1Δ* deficient strain compared to WT cells (Figure 4). However, we did not observe an increase in chromosomal rearrangements in BER$^-$ *yap1Δ* deficient strain compared to BER$^-$ deficient strain. It is possible that Yap1 and Ntg1 (BER) function in the same signaling pathway in response to DNA damage, either through the known roles (stated above) or through an unknown function of

Ntg1 and Yap1. Therefore, when BER$^-$ *yap1Δ* strains are examined there is no increase in the levels of mutation rates or chromosomal aberrations.

Another possible reason that an increase was not observed in BER$^-$ *yap1Δ* deficient strain compared to BER$^-$ deficient strain is the number of chromosomal rearrangements is likely to be underestimated due to the fact that a single chromosomal is scored per isolate, regardless of the total number of chromosomal size changes observed per isolate. For example in BER$^-$ *yap1Δ* deficient strain (Figure 4 BER$^-$ *yapΔ* lane 4) three rearrangements are observed: an extra chromosomal band above chromosome XII, XVI, a decrease in chromosome II size, and an increase in chromosome V, VIII size. The rearrangements could be the result of independent events or the result of one translocation event. These data further support the concept of Yap1 as a DNA damage responder that influences genomic stability.

Yap1 modulation of intracellular ROS levels induced by DNA damage.

To further define the involvement of Yap1 in the DNA damage response, we examined the levels of intracellular $O_2^{\bullet-}$ in repair-proficient and -deficient strains with and without functional Yap1. Endogenous levels of $O_2^{\bullet-}$ were determined for all strains, and a significant increase in $O_2^{\bullet-}$ levels in *yap1Δ* (23%), BER$^-$ deficient (30%) and BER$^-$ *yap1Δ* deficient (65%) cells was observed compared to WT cells (Figure 6). The increase in intracellular ROS has been shown to correspond with an increase in DNA damage [12], suggesting that the increase in ROS observed in the *yap1Δ* cells is likely due to an increase in the levels of oxidative DNA damage.

The intracellular $O_2^{\bullet-}$ levels were determined following exposure to a range of doses of MMS (0-25 mM) and UV-C (0-25 J/m^2). The $O_2^{\bullet-}$ levels increased with increasing exposure to doses of both MMS and UV-C regardless of the strain background (Figure 6A-F). There was a significant increase in $O_2^{\bullet-}$ levels in BER$^-$ *yap1Δ* cells as compared to BER$^-$ deficient cells following exposure to both MMS and UV-C (Figure 6B and 6E). There was no increase in *yap1Δ*

or NER$^-$ *yap1Δ* cells following exposure to MMS or UV-C (Figure 6A, 6D, 6C, and 6F) as compared to WT and NER$^-$ deficient cells, respectively. These results support the concept that the increase in $O_2^{\bullet-}$ level is due to the increase in DNA damage regardless of the strain background. The increase in $O_2^{\bullet-}$ levels in the BER$^-$ *yap1Δ* deficient cells above the BER$^-$ deficient cells is due to the endogenous increase in $O_2^{\bullet-}$ levels, and is not further increased following exposure to DNA damaging agents.

Discussion

Repair of DNA damage is essential for proper cellular function and survival. In unicellular organisms, DNA mutations can lead to changes in protein function that can contribute to a growth survival advantage, however, in multicellular organism, such as humans, mutations that lead to growth survival advantages can often lead to deleterious outcomes such as cancer [37]. Cancer have elevated levels of ROS and increased levels of DNA damage that are believed to contribute to tumorgenesis and resistance to therapeutic agents [38, 39].

Increases in DNA damage cause an increase in intracellular ROS levels [12, 16]. This observation raises the obvious issue of the biological purpose for generating molecules that are capable of damaging macromolecules. High levels of ROS can be deleterious to cells; however they are also involved in a variety of cell signaling pathways [3, 8-10]. Signaling molecules are defined as being able to be generate a specific response, being short lived, and able to act specifically [40]. ROS can be generated at the time of receptor activation as seen in as a specific response to DNA damage [12], they are short lived within cells, and they can act with specificity. ROS target the amino acids of proteins [10]. In *Escherichia coli* the redox sensitive transcription factors SoxR and OxyR are able to distinguish between $O_2^{\bullet-}$ and H_2O_2. SoxR possesses an iron-sulfur center that readily reacts with $O_2^{\bullet-}$ [41], while OxyR contains cystine residues that react with H_2O_2 [42]. In yeast, ROS alter protein structure of Yap1 through direct interaction with cysteine sulfhydryl groups allowing for intramolecular disulfide bonds to form, blocking the binding of Crm,1 and allowing Yap1 to accumulate in the nucleus [10]. In this study, we explored the possible role of Yap1 as a responder to DNA damage-induced ROS signal transduction.

The increase in intracellular ROS observed following DNA damage is dependent on the type of DNA damage and the repair proficiency of the cell [12]. Depending on the DNA damage induced there are changes in the levels of ROS subspecies. Several lines of evidence suggest that

the DNA damage-induced increases in ROS are used by the cell in signaling processes to maintain genomic integrity [12, 15, 16]. Yap1 is a transcription factor known to be involved in the oxidative stress response in yeast [17-21]. To our knowledge, the involvement of Yap1 in the DNA damage response has not been previously investigated.

To assess the possible role of Yap1 in DNA damage-induced ROS signaling, we assessed the changes in localization of Yap1 following exposure to MMS and UV-C, which produce two distinct classes of DNA lesions. Previously, we determined that there are significantly higher levels of ROS in cells following exposure to MMS compared to UV-C [12]. In this study, we found that Yap1 relocalizes to the nucleus following exposure to MMS, but not UV-C. This finding supports the idea that the increase in intracellular ROS following DNA damage is biologically relevant because it leads to the relocalization of Yap1 to the nucleus and only occurs in response to base damages repaired by BER (i.e. alkylating DNA damage).

An important role of Yap1 as a "sensor" of alkylating DNA damage was further revealed in cell survival measurements following exposure MMS, UV-C, and H_2O_2. Exposing cells to H_2O_2 not only induces DNA damage, but also immediately oxidizes Yap1 resulting in activation of the oxidative stress response. Cells lacking functional Yap1 are sensitized to the cytotoxicity of H_2O_2 regardless of the repair proficiency. To address the role of Yap1 specifically in the response to DNA damage we utilized MMS and UV-C as neither agent directly produces ROS. Cells deficient in BER were more resistant to MMS cytotoxicity compared to BER$^-$ *yap1Δ* cells. However, there was no difference in the cytotoxicity profiles of WT and NER$^-$ deficient cells whether Yap1 was present or not. Survival of cells following exposure to DNA damaging agents appears to be modulated by Yap1 in response to MMS, but not UV-C.

The relevance of DNA damaged-induced ROS signaling is further verified by our finding that Yap1 protects cells against mutations and large-scale chromosomal rearrangements. Examination of the genetic stability by assessment of mutation rates and large scale chromosomal rearrangements revealed that Yap1 functions in reducing mutation rates, and large chromosomal

rearrangements (Table 3 and Figure 4). Mutation rates were increased approximately 2-fold in both *yap1Δ* and NER$^-$ *yap1Δ* deficient strains compared to WT and NER$^-$deficient strains, respectively. However, there was no increase in the mutation rates for BER$^-$ *yap1Δ* deficient strains as compared to BER$^-$ deficient strains. A similar pattern was observed for the effects of Yap1 on large scale chromosomal rearrangements. Three strains (*yap1Δ*, BER$^-$ deficient, and BER$^-$ *yap1Δ* deficient) all possess significantly higher levels of chromosomal rearrangements compared to WT cells with no further increased observed in BER$^-$ *yap1Δ* deficient strains. These results indicate that Yap1 also functions in the maintenance of the genome; which appears to be related to BER. Ntg1 seems to be involved in both the BER pathway and the response of Yap1 to DNA damage. The participation of Ntg1 is known for BER; however, the connection between Ntg1 and Yap1 is unknown. There is indirect evidence that Yap1 upregulates *NTG1* [26, 27] and that Yap1 is required for the upregulation of Ntg1. If either Yap1 or Ntg1 is deleted, then there is an increase in genomic instability, but if both are deleted, then there is no further increase in genomic stability because Yap1 and Ntg1 function in the same pathway.

No increase in chromosomal rearrangements was observed in NER$^-$ *yap1Δ* deficient strains compared to WT cells. Previous reports indicate that deletion of *RAD1* causes an increase in genomic stability [43]. In NER- deficient cells, which are *rad1Δ*, there is no increase in chromosomal aberrations compared to WT cells. The increase in genomic stability conferred by the deletion of *RAD1* overshadowed the expected increase in genomic instability observed with the removal of Yap1.

The results of this study lead to the following proposed following model for the function of Yap1 as a DNA damage responder. H_2O_2 rapidly activates Yap1 through the oxidation of cysteine residues resulting in the sequestration of Yap1 in the nucleus. Upon accumulation within the nucleus, Yap1 functions as a transcription factor to activate genes that are involved in the ROS scavenging (*SOD1*, *CTT1*, and *TRX2*) [25]. The activation of these genes contributes to

returning the cell to normal redox homeostasis. In *yap1Δ* mutants, regardless of the DNA repair proficiency of the cell, increased susceptibility to the toxic effects of H_2O_2 was observed due to the fact that Yap1 is directly activated by H_2O_2 and induces a protective oxidative stress response. In the absence of Yap1, cells can no longer upregulate genes that scavenge ROS causing an increase in cytotoxicity regardless of the DNA repair capacity. Because of the dual effects of H_2O_2 in directly inducing oxidative stress and production of oxidative DNA damage, it is difficult to understand the involvement of Yap1 in the DNA damage response versus the oxidative stress response.

To further delineate the role of Yap1 as a DNA damage responder, we employed DNA damaging agents (MMS and UV-C) that are not known to directly activate Yap1. DNA helix distorting lesions caused by UV-C are not capable of activating Yap1. However, helix non-distorting lesions produced by MMS elicit an increase in intracellular ROS [12] via a mechanism that has yet to be determined. This increase in ROS activates Yap1 causing accumulation within the nucleus (Figure 2) where it functions as a transcription factor and upregulates genes involved in ROS scavenging, check point control, and/or DNA repair. The upregulation of these genes can then be involved in the maintenance of the genome.

In cells that are compromised either in BER or Yap1, the response to DNA damage changes. In cells that do not contain functional Yap1 there is an increase in large chromosomal rearrangements, as well as a small increase in mutation rates, suggesting that even under normal growth conditions Yap1 plays a role in maintaining genomic integrity. In cells that are deficient in BER there is activation of Yap1 following exposure to low doses of MMS (0.5 mM) in contrast to no activation in exposed WT and NER$^-$ deficient cells. In addition, increased cytotoxicity is observed in cells that are BER$^-$ *yap1Δ* deficient compared to BER$^-$ deficient cells, suggesting that Yap1 does not occur in response to all types of DNA damage, and operates when the DNA damage causes an increase in $O_2^{\bullet -}$ levels.

These studies provide new insight into the DNA damage response and how the DNA-damage signal is transduced into a stress response pathway that involves Yap1. Here we were able to define a new regulator of the DNA damage response, Yap1. However, there are still many questions that need to be answered. Including how and where the DNA damaged-induced ROS are produced? Does Yap1 directly upregulate genes involved in DNA damage repair? By further exploring the involvement of Yap1 in the DNA damage response we can gain a new understanding of how cells respond to DNA damage.

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Table 1. Genotypes of strains

Strains	Relevant genotypes and/or description
hDNP42 [1]	*MAT a/ α rad1::kanMX4/RAD1 ntg1::hphMX4/NTG1 ntg2::BSD/NTG2 apn1::TRP1/APN1 yap1::natNT/ YAP1 DSF1:: URA3/ DSF1 his7-1/his7-1 lys2D5'::LEU-lys2D3'/lys2D5':: LEU-lys2D3' ade5-1/ade5-1 trp1-289/trp1-289 ura3-52/ura3-52*
LAR009 [2]	*MATa his7-1 lys2Δ5'::LEU-lys2Δ3' ade5-1 trp1-289 ura3-52*
LAR010 [2]	*MATa his7-1 lys2Δ5'::LEU-lys2Δ3' ade5-1 trp1-289 ura3-52 yap1:: natNT*
LAR011 [2]	*MATa his7-1 lys2Δ5'::LEU-lys2Δ3' ade5-1 trp1-289 ura3-52 rad1::kanMX4*
LAR012 [2]	*MATa his7-1 lys2Δ5'::LEU-lys2Δ3' ade5-1 trp1-289 ura3-52 rad1::kanMX4 yap1:: natNT*
LAR013 [2]	*MATa his7-1 lys2Δ5'::LEU-lys2Δ3' ade5-1 trp1-289 ura3-52 ntg1::hphMX4 ntg2::BSD apn1::TRP1*
LAR014 [2]	*MAT α his7-1 lys2Δ5'::LEU-lys2Δ3' ade5-1 trp1-289 ura3-52 ntg1::hphMX4 ntg2::BSD apn1::TRP1 yap1:: natNT*
LAR017 [2]	*MAT α his7-1 lys2Δ5'::LEU-lys2Δ3' ade5-1 trp1-289 ura3-52*
LAR018 [2]	*MATa his7-1 lys2Δ5'::LEU-lys2Δ3' ade5-1 trp1-289 ura3-52 yap1:: natNT*
LAR021 [2]	*MATa his7-1 lys2Δ5'::LEU-lys2Δ3' ade5-1 trp1-289 ura3-52 ntg1::hphMX4 ntg2::BSD apn1::TRP1*
LAR022Δ [2]	*MATa his7-1 lys2D5'::LEU-lys2D3' ade5-1 trp1-289 ura3-52 ntg1::hphMX4 ntg2::BSD apn1::TRP1 yap1:: natNT*
LAR025 [2]	*MAT α his7-1 lys2Δ5'::LEU-lys2Δ3' ade5-1 trp1-289 ura3-52*
LAR026 [2]	*MAT α his7-1 lys2Δ5'::LEU-lys2Δ3' ade5-1 trp1-289 ura3-52 yap1:: natNT*
LAR029 [2]	*MAT α his7-1 lys2Δ5'::LEU-lys2Δ3' ade5-1 trp1-289 ura3-52 ntg1::hphMX4 ntg2::BSD apn1::TRP1*
LAR030 [2]	*MATa his7-1 lys2D5'::LEU-lys2D3' ade5-1 trp1-289 ura3-52 ntg1::hphMX4 ntg2::BSD apn1::TRP1 yap1:: natNT*
DSC025 [3]	*MAT ade2-101oc his3200 ura3Nco lys2Bgl leu2-R*
DSC035 [4]	*MAT α ade2-101oc his3Δ200 ura3ΔNco lys2ΔBgl leu2-R ntg1Δ::LEU2 ntg2Δ::hisG apn1D1::HIS3*
DSC036 [5]	*MAT α ade2-101oc his3Δ200 ura3ΔNco lys2ΔBgl leu2-R rad1Δ::hisG*

[1] hDNP42 is isogenic with hDNP19 [29].

[2] These strains are haploid derivatives of hDNP42

[3] This strains is the same as SJR751 [28]. Full genotype is shown.

[4] This strains is the same as SJR867 [28]. Full genotype is shown.

[5] This strains is the same as SJR868 [28]. Full genotype is shown.

Figure 1. Fluorescence microscopy of Yap1 localization. Yap1-GFP was visualized by direct fluorescence microscopy in WT cells following no treatment (Control) or exposure to 25 mM MMS for 25 min. Cells were stained with DAPI to visualize the position of the chromatin within nuclei. The Merge image indicates the overlap (yellow) between the Yap1-GFP signal (green) and DAPI (red) staining of nuclei. Corresponding DIC images are also shown

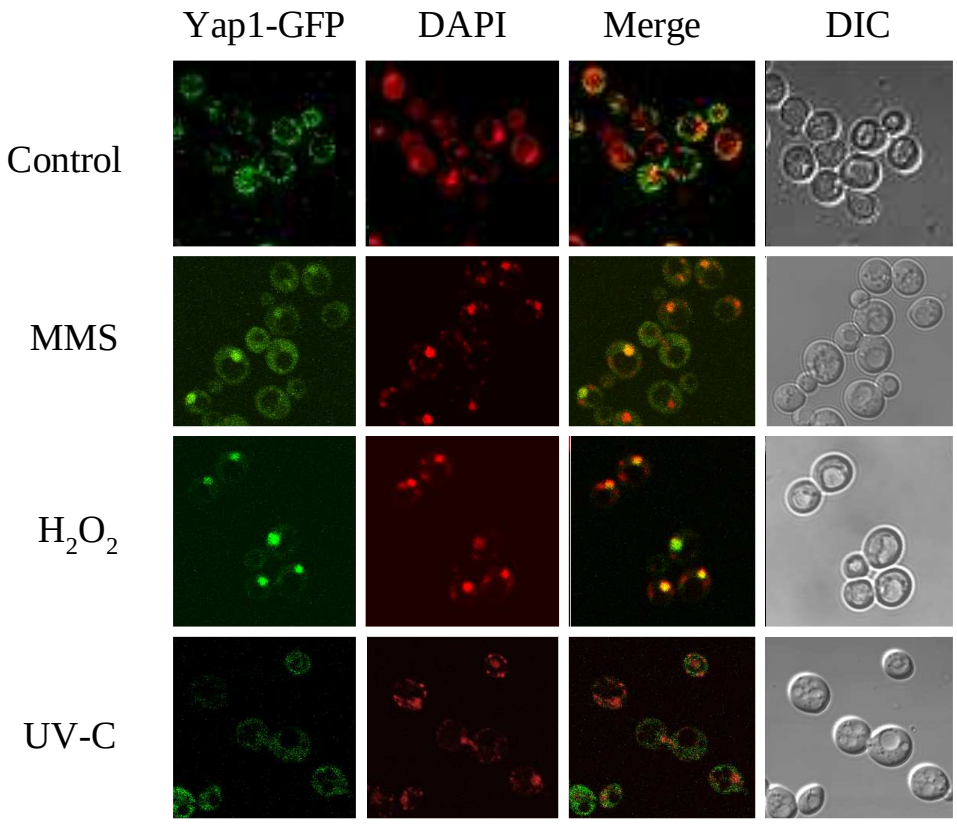
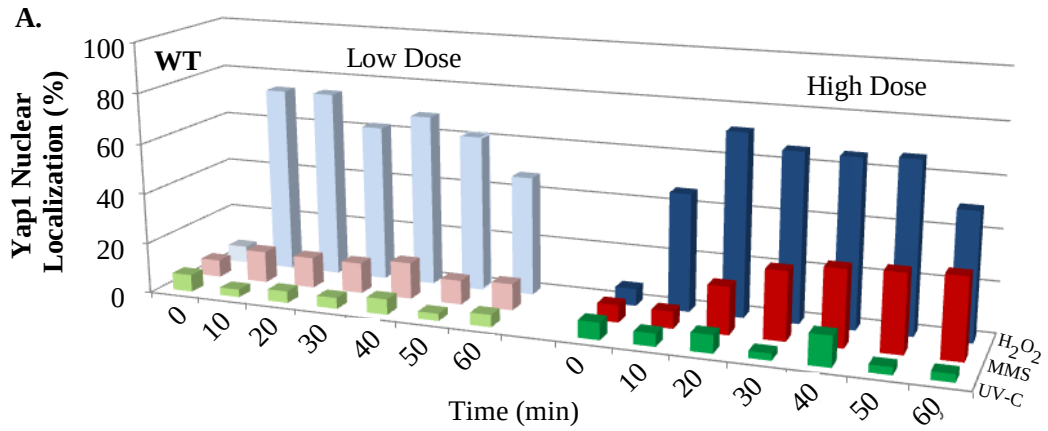
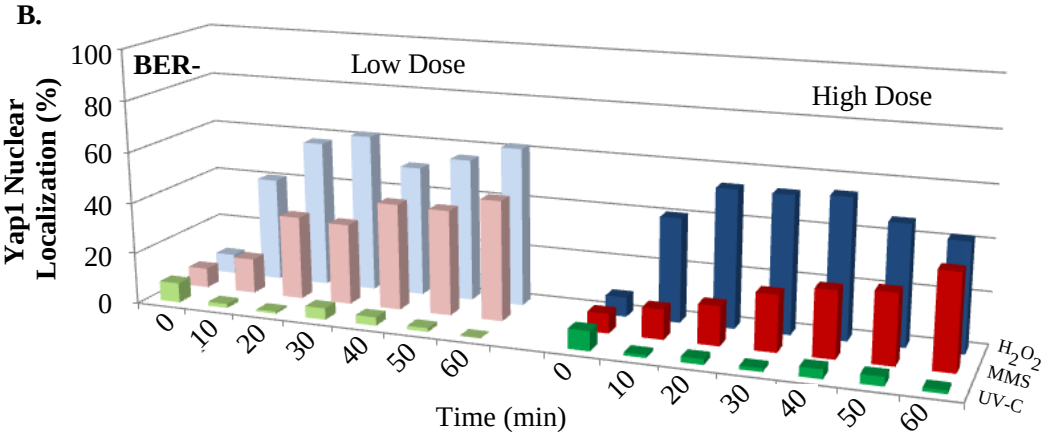
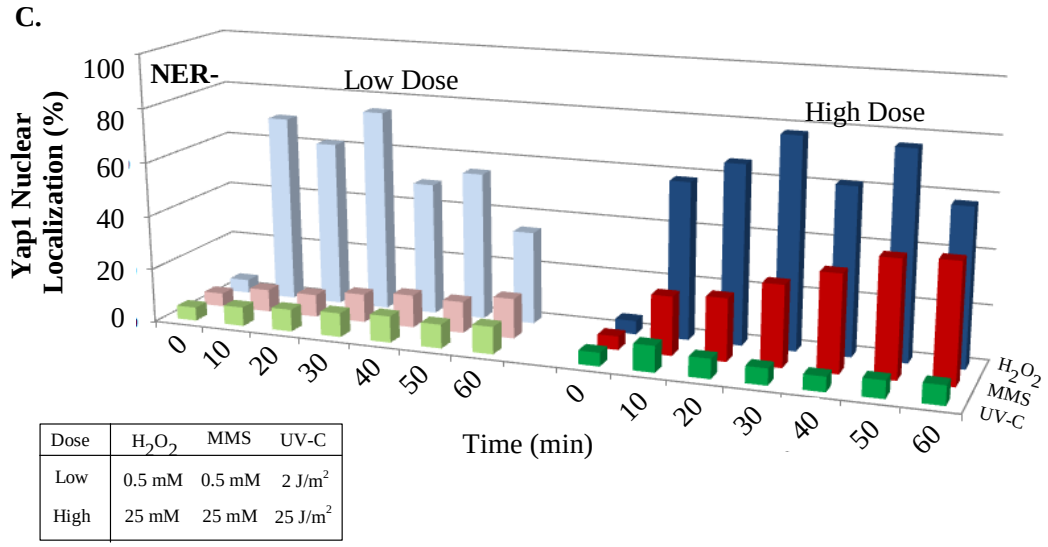


Figure 1. Fluorescence microscopy of Yap1 localization.

Figure 2. Yap1 localization in repair-proficient and -deficient cells after exposure to DNA damaging agents. Graphical representation of fluorescence microscopy analysis assessing Yap1-GFP localization in **A.** WT cells. Cells were exposed to low doses (5 mM H_2O_2 (blue), 5 mM MMS (red), and 2 mM UV-C (green)) and high doses (25 mM H_2O_2 (light blue), 25 mM MMS (pink), and 25 mM UV-C (light green)) of DNA damaging agent and fluorescence images were obtained. Cells were counted and evaluated in 10 min interval groups. Cells with nuclear localization alone or nuclear plus cytoplasmic localization for Yap1 are represented in the bars graphs for every 10 minute interval for the duration of the experiment (60 min) are indicated. **B.** BER^- deficient cells. Cells were exposed to low doses (5 mM H_2O_2 (blue), 5 mM MMS (red), and 2 mM UV-C (green)) and high doses (25 mM H_2O_2 (light blue), 25 mM MMS (pink), and 25 mM UV-C (light green)) of DNA damaging agent and fluorescence images were obtained. Cells were counted and evaluated in 10 min interval groups. Cells with nuclear localization alone or nuclear plus cytoplasmic localization for Yap1 are represented in the bars graphs for every 10 minute interval for the duration of the experiment (60 min) are indicated. **C.** NER^- deficient cells. Cells were exposed to low doses (5 mM H_2O_2 (blue), 5 mM MMS (red), and 2 mM UV-C (green)) and high doses (25 mM H_2O_2 (light blue), 25 mM MMS (pink), and 25 mM UV-C (light green)) of DNA damaging agent and fluorescence images were obtained. Cells were counted and evaluated in 10 min interval groups. Cells with nuclear localization alone or nuclear plus cytoplasmic localization for Yap1 are represented in the bars graphs for every 10 minute interval for the duration of the experiment (60 min) are indicated.







Dose	H_2O_2	MMS	UV-C
Low	0.5 mM	0.5 mM	2 J/m^2
High	25 mM	25 mM	25 J/m^2

Figure 2. Yap1 localization in repair-proficient and -deficient cells after exposure to DNA damaging agents.

Figure 3. Cytotoxicity profiles of cells with different DNA repair capacities with and without functional Yap1 following exposure to DNA damaging agents. A.-C. Sensitivity of DNA repair deficient strains with and without functional Yap1 to H_2O_2. **(A)** WT (circles) and *yap1Δ* (triangles) **(B)** BER$^-$ (circles) and BER$^-$ *yap1Δ* (triangles) **(C)** NER$^-$ (circles) and NER$^-$ *yap1Δ* (triangles) cells were exposed to 0, 0.5, 5, and 25 mM H_2O_2 for 30 min at 30 °C. **D.-F.** Sensitivity of DNA repair deficient strains with and without functional Yap1 to MMS. **(D)** WT (circles) and *yap1Δ* (triangles) **(E)** BER$^-$ (circles) and BER$^-$ *yap1Δ* (triangles) **(F)** NER$^-$ (circles) and NER$^-$ *yap1Δ* (triangles) cells were exposed to 0, 0.5, 5, and 25 mM MMS for 30 min at 30 °C. **G.-I.** Sensitivity of DNA repair deficient strains with and without functional Yap1 to UV-C. **(A)** WT (circles) and *yap1Δ* (triangles) **(B)** BER$^-$ (circles) and BER$^-$ *yap1Δ* (triangles) **(C)** NER$^-$ (circles) and NER$^-$ *yap1Δ* (triangles) cells were exposed to 0, 2, 5, and 25 J/m^2 UV-C. The results are an average of three independent experiments. Error bars represent ± SD. Experimental details are provided in the text. Asterisks (*) above bars indicate statistical significance of a p value <0.05 as compared to Yap1 proficient strain.

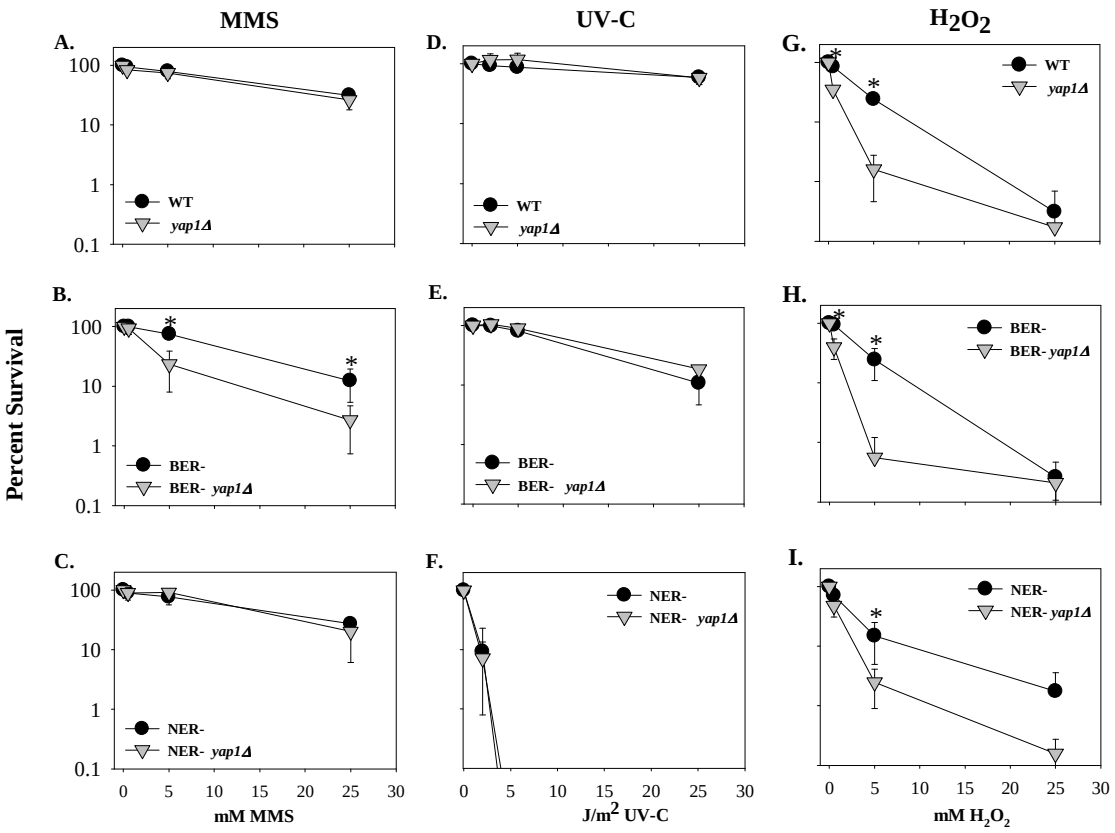


Figure 3. Cytotoxicity profiles of cells with different DNA repair capacities with and without functional Yap1 following exposure to DNA damaging agents.

Table 2. Mutation Rates of *yap1 Δ* strains [a]

Strain	Mutation rates (10-7) (95% confidence limits) for:			
	YAP1 strains		*yap1 Δ* strains	
Wild Type	3.1 (2.4-4.7)	(1.0)[b]	7.1 (5.0-8.2)	(1)[c]
BER- *(ntg1 ntg2 apn1)*	47.9 (40.7-72.8)	(15.4)[b]	51.8 (35.4-79.1)	(7.3)[c]
NER- (*rad1*)	5.2 (4.2-7.6)	(1.7)[b]	9.2 (7.9-10.3)	(1.3)[c]

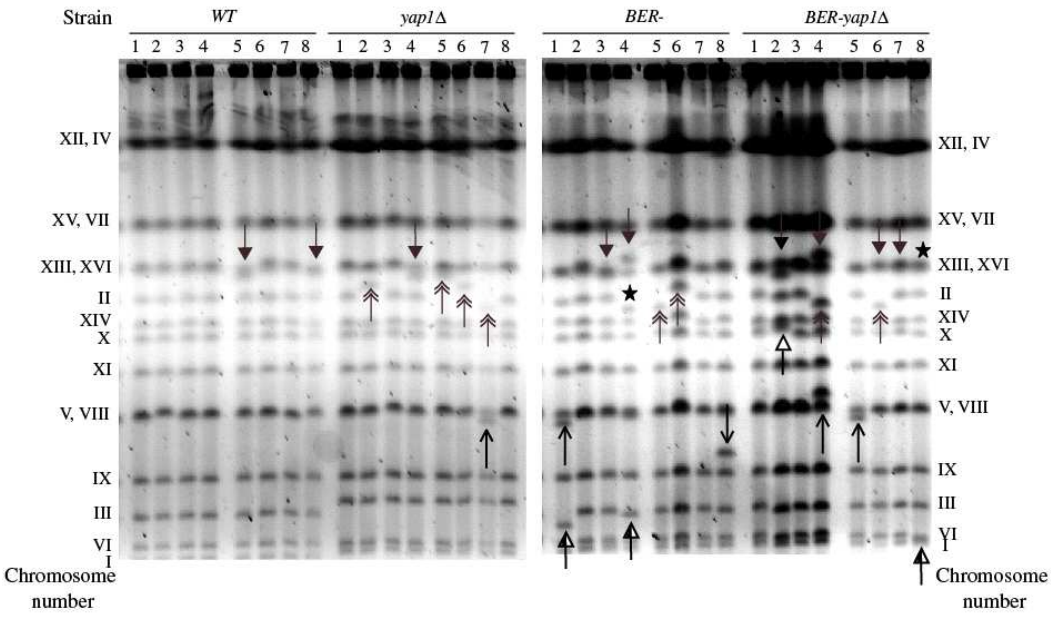
[a] Median mutation rates were determined for 18-24 cultures from two independent segragants of the same genotype as described in Methods and Materials

[b] Fold change over WT in the *YAP1* strains

[c] Fold change over WT in the *yap1 Δ* strains

Figure 4. CHEF Gel analysis of replicative aging population. A. Representative **e**thidium bromide stained CHEF gel separating the yeast chromosome of the 20th generation of WT, *yap1Δ*, BER$^-$, and BER$^-$ *yap1Δ* strains. Arrows represent changes in chromosomes: "↑" chromosome XIII, XVI; "↑" chromosome II; "↑"chromosome X; "↑"chromosome V, VIII; "↑"chromosome III or I. Stars (★) depict chromosomes that were altered in the founder generation (p0). **B.** Number of lineages with chromosomal aberrations. CHEF gel analysis was performed on the founder generation (p0) to determine the karyotype of each strain, and again at the 10th and 20th generations. The graph represents the number of aberrations that were first detected in the 10th and 20th generation. Isolates with chromosomal aberrations are listed at the end of each bar and the total number of isolates examined is listed in parentheses. Asterisks (*) beside bars indicate statistical significance of a p value <0.05 compared to WT cells. Pound sign (#) beside bars indicate significance of a p value <0.05 compared to *yap1Δ* strain.

A.



B.

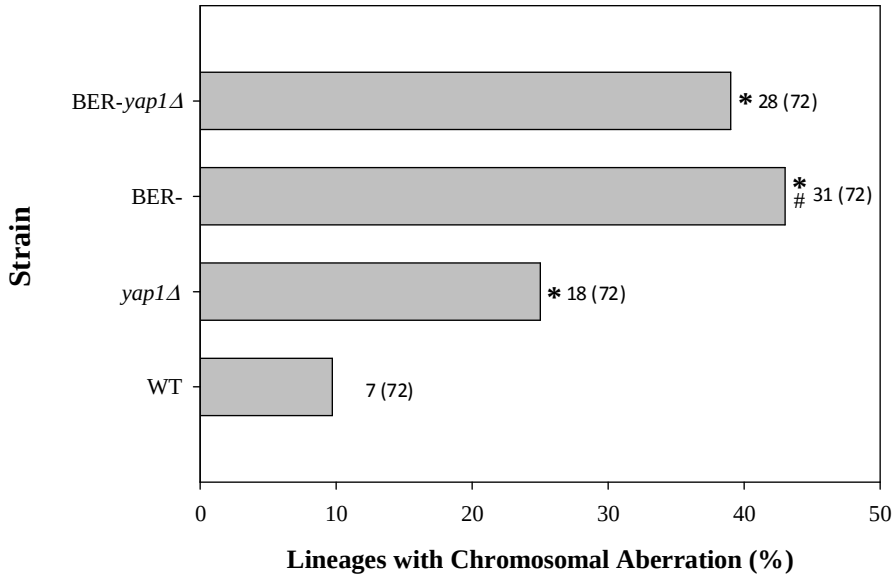


Figure 4. CHEF Gel analysis of replicative aging population.

Table 3. Frequencies of large-scale chromosomal rearrangments in haploid strains

	No. of rearrangements (no. of linages analyzed) for passage:			Total no. or rearrangments in p10 and p20 (Total no. of lineages analyzed)
Strain:	0	10	20	
WT	0 (36)	2 (36)	5 (36)	7 (72)
yap1 Δ	21 (36)	13 (36)	5 (36)	18 (72)
NER$^-$	4 (16)	0 (16)	1 (16)	1 (32)
NER$^-$ yap1D	0 (16)	1 (16)	2 (16)	3 (32)
BER$^-$	17 (36)	18 (36)	13 (36)	31 (72)
BER$^-$ yap1D	17 (36)	16 (36)	12 (36)	28 (72)

Figure 5. Endogenous levels of superoxide in isogenic DNA repair-proficient and -deficient strains with and without functional Yap1. Isogenic WT, *yap1Δ*, BER$^-$, BER$^-$ *yap1Δ*, NER$^-$, and NER$^-$ *yap1Δ* strains were incubated with the fluorescent probe, DHEt, for 2 hours as described in Experimental Procedures. Following incubation, cells were analyzed for ROS levels by flow cytometry as described in the text. Fluorescence values were obtained by evaluating of the mean peak values of the cytograms and are reported as the fold change relative to the WT (repair proficient) strain (set to a value of 1.0). Error bars represent ± SD. Asterisks above bars indicate statistical significance of a p value <0.05 as compared to WT strain. "#" above bars indicate statistical significance of a p value <0.05 as compared to BER$^-$ deficient strain.

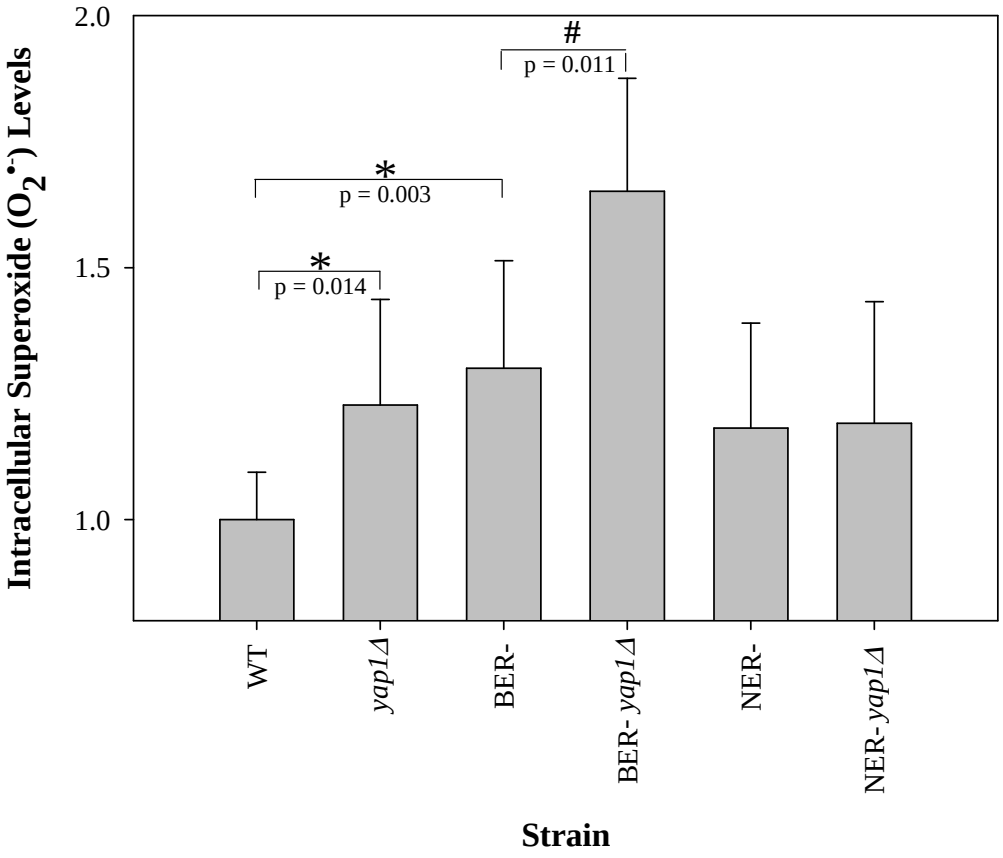


Figure 5. Endogenous levels of superoxide in isogenic DNA repair-proficient and – deficient strains with and without functional Yap1

Figure 6. Superoxide levels in DNA repair-proficient and -deficient strains with and without functional Yap1 in response to DNA damage. A.-C. Intracellular ROS levels in DNA repair deficient strains with and without functional Yap1 following exposure to MMS. **(A)** WT (black bars) and *yap1Δ* (gray bars) **(B)** BER$^-$ (black bars) and BER$^-$ *yap1Δ* (gray bars) **(C)** NER$^-$ (black bars) and NER$^-$ *yap1Δ* (gray bars) strains were exposed to MMS (0-25 mM) for 30 min. **D.-E.** Intracellular ROS levels in DNA repair deficient strains with and without functional Yap1 following exposure to UV-C. **(D)** WT (black bars) and *yap1Δ* (gray bars) **(E)** BER$^-$ (black bars) and BER$^-$ *yap1Δ* (gray bars) **(F)** NER$^-$ (black bars) and NER$^-$ *yap1Δ* (gray bars) strains were exposed to MMS (0-25 J/m^2). Following exposure to the DNA damaging agent cells were then incubated with the fluorescent probe, DHEt, for 2 hours. Cells were analyzed for ROS levels by flow cytometry as described in Experimental Procedures. Fluorescence values were obtained by evaluating the mean peak values of the cytograms and are reported as fold changes relative to the parental (functional Yap1) strain (set to a value of 1.0). Error bars represent ± SD. "$_*$" symbols above bars indicate statistically significant (p value <0.05) differences between the Yap1-functional strains versus the Yap1-deficient strains within a particular dose of MMS.

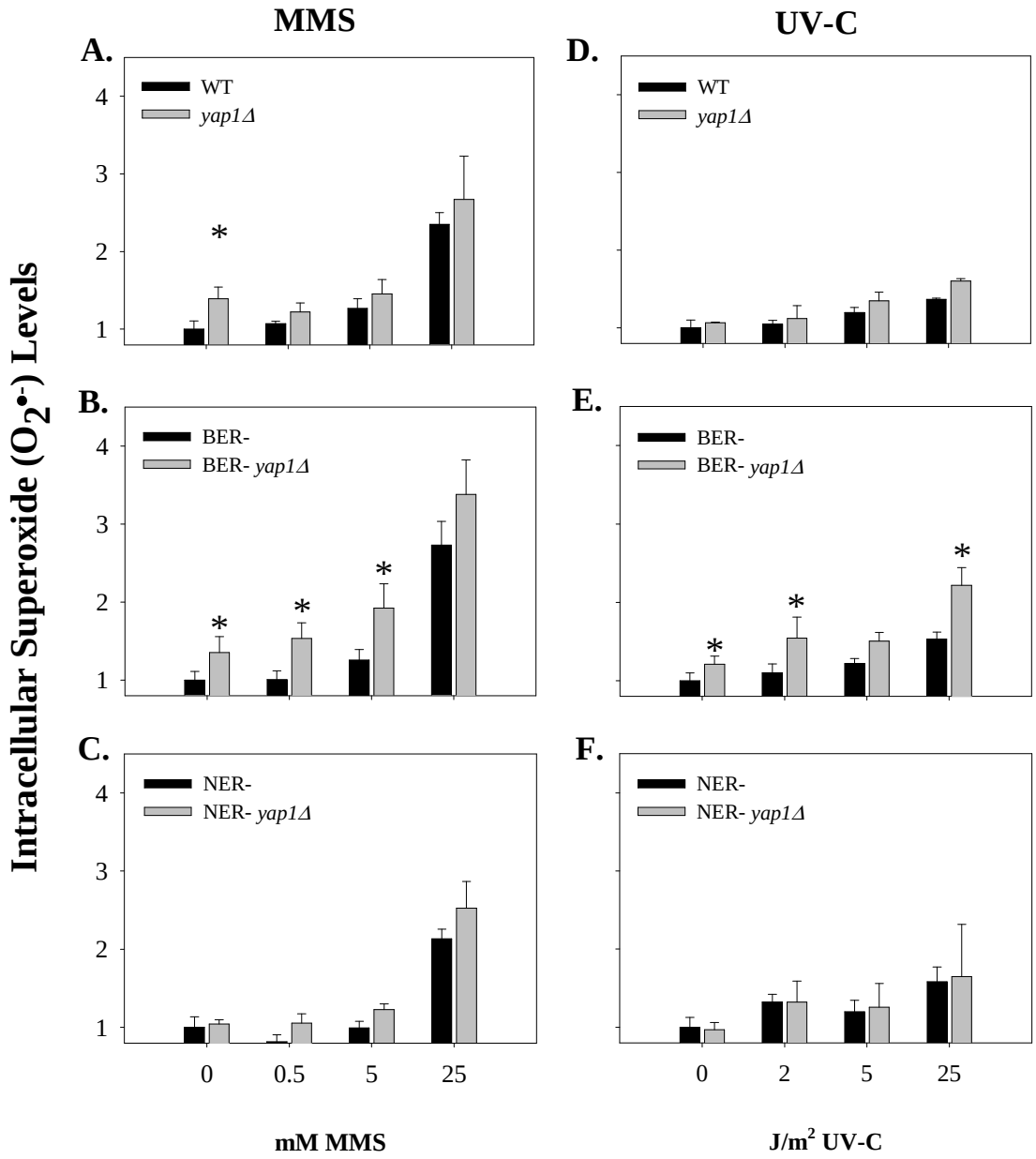


Figure 6. Superoxide levels in DNA repair-proficient and -deficient strains with and without functional Yap1 in response to DNA damage.

Figure 7. Model of the role of Yap1 in the DNA damage response. Yap1 is known to be activated in response to oxidative stress very rapidly (left side – **bold** arrows). In response to oxidative stress Yap1 is relocalized to the nucleus where it activates a number of genes involved in ROS scavenging (*SOD1*, *CTT1*, and *TRX2*). The activation of these genes contributes to returning the cell to redox homeostasis. Following exposure to DNA damaging agents (right side – small arrows) that produce helix distorting lesions (UV-C) Yap1 is not activates and does not relocalization to the nucleus. Following exposure to DNA damaging agents that produce helix non-distorting lesions (MMS) there is an increase in intracellular ROS levels through unknown mechanisms. The DNA damage-induced ROS are responsible for the activation of Yap1. The activation of Yap1 is slow compared to the activation of Yap1 in response to H_2O_2, taking up to 30min for relocalization to the nucleus. Once activated Yap1 can upregulate genes involved in ROS scavenging, check point control (*MEC1*, *POL1*, and *POL3*), and DNA repair (*NTG1* and *MAG1*). The upregulation of these genes can then be involved in the maintenance of the genome.

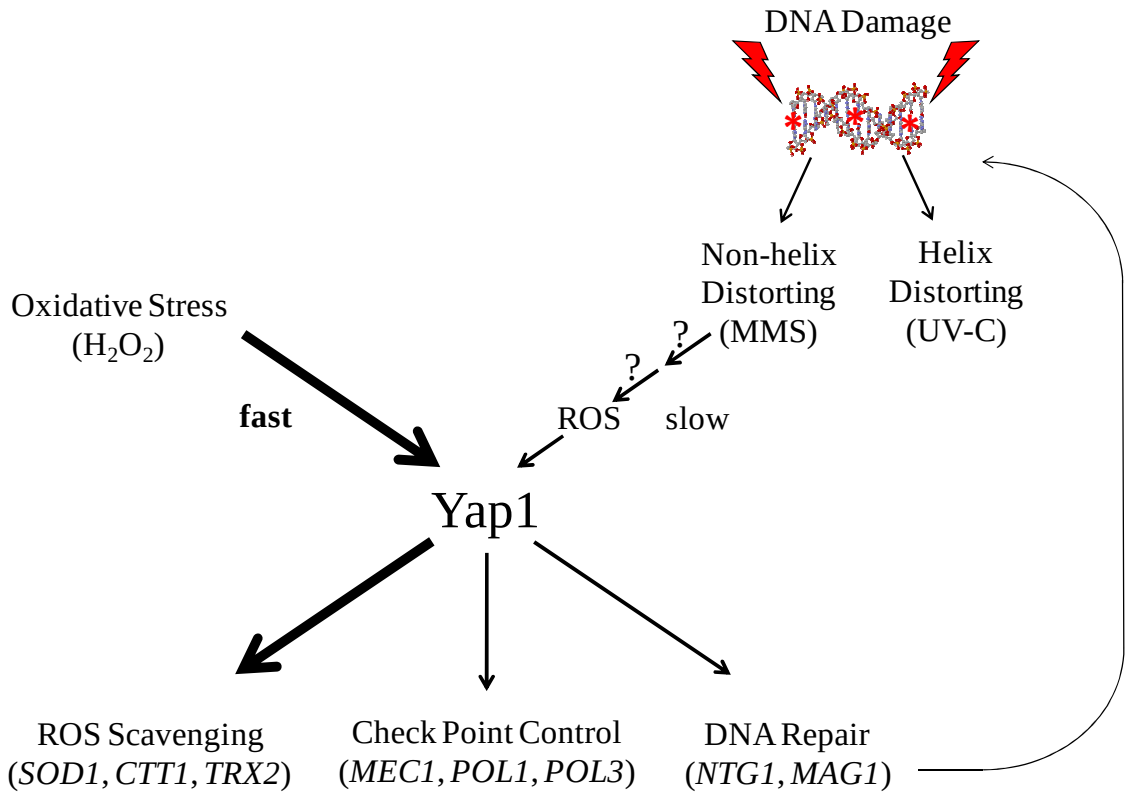


Figure 7. Model of the role of Yap1 in the DNA damage response.

CHAPTER V

Conclusions and Future Directions

Conclusions

Living in an oxygen rich environment poses the unique challenge of balancing a need for oxygen to sustain life and dealing with its reactive nature. Reactive oxygen species (ROS), as the name implies, are reactive oxygen molecules that are produced from molecular oxygen [1]. ROS can cause damage to cellular macromolecules including proteins, lipids, RNA, and DNA [2]. While cells have developed ways to directly eliminate destructive ROS through scavenging pathways [3], and repair damage to macromolecules via pathways such as DNA repair [4], they have also evolved to utilize ROS in signaling mechanisms [5].

An increase in intracellular ROS levels leads to a state of oxidative stress. Normal cellular conditions are characterized by a balance of pro- and antioxidants in the cell [2, 6]. Maintaining this redox balance in cells is important to preserve normal functions. Under non-stress conditions low levels of ROS are involved in signaling pathways; however, as levels increase, the redox balance shifts, leading to deleterious consequences [7]. Human degenerative conditions and diseases are associated with increased levels of ROS and compromised repair pathways. A few examples include neurodegenerative conditions [8], xeroderma pigmentosum [4], and cancer [9].

In the studies presented, we examined the role of ROS in the genotoxic stress response. In the absence of exogenous DNA damaging agents severely repair-compromised cells (BER-/NER-deficient) exhibit an increase in the oxidative DNA damage that is associated with an increase in the intracellular levels of ROS [10-12]. Additionally, there is a dose-dependent increase in the intracellular levels of ROS in response to MMS in repair-proficient and -deficient cells [10]. This observation raised an important question. Is the increase in intracellular ROS a biologically relevant general genotoxic stress response to DNA damage, and if so, is the increase in ROS specific for damages primarily repaired by BER?

By examining the intracellular levels of ROS in cells deficient in DNA repair (BER-, NER-, and BER-/NER-deficient strains) or in ROS scavenging (*sod1Δ*, *sod2Δ*, *cta1Δ*, *ctt1Δ*), we can begin to define the relationship between ROS and the genotoxic stress response. The levels of ROS increased in all strains examined (WT, BER-, NER-, BER-/NER-, *sod1Δ*, *sod2Δ*, *cta1Δ*, and *ctt1Δ*) in response to both MMS and UV-C, indicating that the increase in ROS levels is a general genotoxic stress response. However, the increase in the subspecies of ROS was not uniform and differed depending on the nature of the DNA damage. In the repair-deficient strains (WT, BER-, NER-, and BER-/NER-), following exposure to either MMS or UV-C, there was a dose-dependent increase in the levels of $O_2^{\bullet-}$. The increase in $O_2^{\bullet-}$ levels following exposure to MMS were elevated compared to the levels of $O_2^{\bullet-}$ observed following exposure to UV-C, indicating that while this is a general genotoxic stress response, there are differences in how the cell responds to different types of DNA damage. The intracellular levels of H_2O_2 and $^{\bullet}OH$ follow a similar pattern whether the cells were exposed to MMS or UV-C. At low doses (0.5-5 mM MMS and 2-5 J/m2) there was an increase in both H_2O_2 and $^{\bullet}OH$; however, following exposure to high doses (25-55 mM MMS or 25-50 J/m2) there was little to no increase in the levels of H_2O_2 and $^{\bullet}OH$. These results support our proposed model where increased intracellular ROS is dependent on the levels of DNA damage present in the cell (Chapter 2, Figure 7). At low to moderate levels of DNA damage (within the cellular capacity to repair the DNA damage) intracellular levels of $O_2^{\bullet-}$, H_2O_2, and $^{\bullet}OH$ are increased and are involved in signaling processes for maintaining cell survival and stress response activities. As the levels of DNA damage increase (exceeding the repair capacity of the cell), a continued increase in $O_2^{\bullet-}$ levels occurs, but an increase in the levels of H_2O_2 or $^{\bullet}OH$ is absent. Increased levels of $O_2^{\bullet-}$ are associated with cytotoxicity, and the continued increases in $O_2^{\bullet-}$ levels are involved in the cell death response, potentially blocking signaling for cell survival and stress response activities.

One possibility for the difference in the cellular response to MMS and UV-C is that the intracellular location of the ROS differs depending on the type of DNA damage induced. By examining ROS scavenging mutants (*sod1Δ*, *sod2Δ*, *cta1Δ*, and *ctt1Δ*), the location of the intracellular ROS can be explored. ROS scavenging enzymes are located within discrete cellular components. Sod1 is localized mainly to the cytoplasm [13], Cta1 is localized to the cytoplasm and the peroxisome [14], and Sod2 and Ctt1 are localized to the mitochondria [13, 15]. The ROS scavenging mutant cells displayed a unique spectrum of ROS levels dependent on the type of DNA damage induced. Exposure to MMS resulted in a significant increase in the levels of $O_2^{\bullet-}$ in the *sod1Δ* strain that was not observed in WT cells or the other ROS scavenging mutants. Exposure to UV-C caused a significant increase in the levels of $O_2^{\bullet-}$ in the *sod2Δ*, *cta1Δ*, and *ctt1Δ* strains compared to WT that was not observed in the *sod1Δ* strain. These data indicate that while there is a general increase in the levels of ROS following exposure to MMS and UV-C, this increase in ROS is distinct for each type of DNA damage and that the subcellular localization of ROS may be different. We hypothesize that in response to MMS (where we observe an increase in $O_2^{\bullet-}$ levels when Sod1 is eliminated) the increase in intracellular ROS is localized to the cytoplasm where it functions in cell survival and stress response signaling, In addition, in response to UV-C (where we observe an increase in $O_2^{\bullet-}$ levels when *SOD2* is deleted) the increase in intracellular ROS is localized to mitochondria where it may function to signal cell death. This notion is supported by previous reports that showed that an increase in mitochondrial $O_2^{\bullet-}$ levels is associated with cell death [16].

To explore ways in which signaling can occur via increases in intracellular ROS in response to DNA damage, the activation of Yap1 through nuclear localization was examined following exposure to MMS and UV-C in repair-proficient (WT) and -deficient strains (BER^- and NER^-). Yap1 is a transcription factor that is activated in response to oxidative stress, regulating

the transcription of numerous (>70) genes including some that are involved in the oxidative stress response, such as *SOD1*, *CTT1*, and *TRX1* [17]. There is some evidence that Yap1 can also activate genes involved in DNA repair, replication, and check point control, including *NTG1*, *POL1*, *MAG1*, *MEC1*, and *POL3* [18, 19]. In the event that the increase in ROS that was observed following exposure to MMS and UV-C functions in signaling, one would expect to see the activation of Yap1 in response to DNA damage. Interestingly, Yap1 nuclear localization was influenced by exposure to MMS, but not UV-C. The magnitude of the increase in ROS produced in response to MMS and UV-C differ (10-fold), suggesting that a threshold level is required before Yap1 is activated. These findings also indicate that the ROS signaling that occurs following a DNA damaging event is likely to reflect the class of DNA damage introduced into the genome.

The activation of Yap1 in response to MMS could be due to the fact that the increase in intracellular ROS (compared to that observed following exposure to UV-C) activates the oxidative stress response or that Yap1 is more directly involved in genomic stability. To further explore the potential role of Yap1 in the DNA damage response, we determined the cytotoxicity profiles, mutation rates, and chromosomal aberration frequencies of repair-proficient and -deficient strains with and without functional Yap1 (WT, *yap1Δ*, BER-, BER- *yap1Δ*, NER-, and NER- *yap1Δ*) following exposure to MMS and UV-C. There was an increase in cytotoxicity following exposure to MMS in BER- *yap1Δ* strains compared to BER-deficient strains that was not observed in any of the other strains, or in any strain following exposure to UV-C. These results indicate that Yap1 is involved in the DNA damage response to damage primarily repaired by BER, but not by NER. Examining the activation of Yap1 with other DNA damaging agents that produce lesions repaired by BER or NER would further delineate whether the activation of Yap1 is specific to MMS or if any DNA damage primarily repaired by BER can activate Yap1.

Cells deficient in *YAP1*, BER or both accumulate more nuclear mutations compared to WT cells; however, there was no difference between the mutation rates of the BER- *yap1Δ* cells and the BER-deficient cells. A similar pattern was observed for the occurrence of chromosomal aberrations. An increase in the number of chromosomal aberrations was observed in the *yap1Δ*, BER-deficient, and BER- *yap1Δ* deficient strains compared to WT; however, there was no increase in chromosomal aberrations between BER- *yap1Δ* deficient cells and BER-deficient cells. These findings indicate that Yap1 and BER likely function in the same DNA damage response pathway. Thus, the elimination of one component has the same effect (epistasis) as elimination of two components that function in the same pathway. There is evidence that Yap1 regulates transcription of *NTG1*, one of the genes deleted to generate the BER-deficient strain [18, 19]. It is possible that the regulation of *NTG1* is one point of overlap between Yap1 and the BER pathway in response to DNA damage. It is possible that Yap1 is interacting with other proteins involved in the BER pathway as well, since the removal of *NTG1* alone has no discernible phenotype.

Proposed model of the DNA damage-induced ROS signaling

Based on the information emerging from this project, a model for the DNA damage-induced ROS signaling response was developed (Figure 1). We hypothesize that in response to different types of DNA damage the intracellular levels of ROS are increased. The increase in ROS could be caused by induction of unidentified cellular oxidases. There are several candidates both in yeast (flavoprotein-like molecules such as Pst1, Yhb1, Yor356W, Rfs1, and Ycp4) and mammalian cells (xanthine oxidase or NADPH oxidases) that could be responsible for such ROS production. The present study did not directly address any possible candidates for the production of ROS, but examined several relationships between the type and levels of DNA damage and ROS.

While there is a general increase in the levels of ROS following exposure to DNA damaging agents, the level of increase in ROS and sub-type of ROS increased are dependent on the levels of DNA damage produced (model presented in Chapter II, Figure 7). In response to repairable levels of DNA damage, an increase in the levels of $O_2^{\bullet-}$, H_2O_2, and $^{\bullet}OH$ was observed. We hypothesize that it is associated with stress response signaling. In response to high levels of DNA damage (exceeding the repair capacity of the cell) there is a further increase in the levels of $O_2^{\bullet-}$, but not in H_2O_2 and $^{\bullet}OH$. Such changes in the levels or different sub-species of ROS could lead to cell death.

The increase in ROS is also dependent on the type of DNA damage produced. As discussed above, in response to UV-C the relative increase in ROS is lower compared to the response to MMS (Chapter II, Figure 3-4). Either because the ROS levels are lower or the production of ROS is confined to a specific sub-cellular compartment (i.e. mitochondria) the ROS produced in response to UV-C does not result in activation of Yap1. The ROS may activate other unknown proteins in the cell, similar to Yap1, which transduces the DNA damage-induced ROS response to orchestrate DNA repair or check point functions.

Following exposure to MMS, there is an increase in ROS levels above that observed following exposure to UV-C that can activate Yap1. The increased levels of ROS and/or the location of the newly-generated ROS (i.e. cytoplasm) are responsible for the activation of Yap1. Once activated, Yap1 can then modulate transcription of ROS scavenging, DNA repair, and/or check point control genes. There is a possibility that Yap1 may play a more direct role in the DNA repair process and interact directly with BER proteins. While the direct interaction of Yap1 with BER is speculative, the deletion of *YAP1* from WT cells causes an increase in genomic instability. When BER is compromised in conjunction with the deletion of *YAP1* there is no further increase in genomic instability. Therefore Yap1 is functioning epistatically with the BER pathway.

If the extent of the DNA damage is exceeding the repair capacity of the cell, due to either disruption of the DNA damage response pathways (DNA repair, check point control, or DNA damage responder such as Yap1) or the increase in the dose of DNA damaging agent, the increase in ROS levels (specifically $O_2^{\bullet -}$) could lead to increased oxidative stress and chromosomal instability or to cell death. While the present studies have given us new insight into the DNA damage response events there are still many issues to be addressed.

Future Directions

Future studies should include further investigations into Yap1 as a mediator in the DNA damage response. There are six active cysiteine residues in Yap1 and the formation of one disulfide bond between two of these residues is required to block the binding of Crm1 and allows for nuclear accumulation of Yap1. The formation of one or two additional disulfide bonds is required for the transcription factor activity of Yap1 [20]. By mutating the cysteine residues required for nuclear accumulation of Yap1 and/or transcription factor activation, the role of Yap1 in the DNA damage-induced ROS response could be further delineated. The examination of the mutant Yap1 proteins would distinguish whether the nuclear localization of Yap1 or the transcription factor activity of Yap1 is necessary for its role as a DNA damage responder. If genomic instability is observed in mutant Yap1 cells (those that are able to accumulate in the nucleus, but cannot function as a transcription factor) then an additional unknown function of Yap1 would be elucidated.

There is indirect evidence that Yap1 is able to activate DNA repair and check point control genes, such as *NTG1*, but this has not been shown directly. Chromatin-IP analysis would demonstrate if Yap1 binds to the genes directly. To further support the idea that Yap1 regulates genes involved in DNA repair and check point control, RT-PCR and western blot analysis could be utilized following exposure to DNA damaging agents. If Yap1 is upregulating these genes

then an increase in mRNA production and protein would be expected. These studies would demonstrate that Yap1 is involved in the DNA damage response through the regulation of specific DNA repair genes. Another level of control of Yap1 is through its oxidation. The protein Gpx3 facilitates the oxidation of Yap1 in response to stress. By examining the dynamics of Yap1 in strains lacking Gpx3 (or other proteins that are involved in the oxidative regulation of Yap1) further information on how Yap1 is regulated in response to DNA damage-induced ROS could be obtained. The formation of the disulfide bonds is important for the function of Yap1 and by investigating the multiple levels of how this is regulate an understanding of the response of Yap1 to different types of stress (oxidative versus genomic) could be gained.

The results presented here support the idea that ROS generated in response to MMS is produced/localized in the cytoplasm whereas ROS generated in response to UV-C is produced/localized in mitochondria. To further elucidate the sub-cellular localization of ROS following DNA damage, repair deficient and ROS scavenging mutants that lack mtDNA (rho^0) should be examined following exposure to a range of doses of MMS and UV-C. These rho^0 strains should be unable to contribute mitochondrial ROS, allowing the distinction between ROS produced in the mitochondria and ROS produced elsewhere in the cell. Also, due to the fact that there is overlap in the function of ROS scavengers within the cells, determining the levels of the ROS subspecies in ROS scavenging mutants deleted for multiple pathways should be examined to help further elucidate the role of regulation of ROS in the DNA damage response. Preliminary experiments examining the intracellular $O_2^{\bullet-}$ levels in multiple ROS scavenging mutants are currently in progress in our laboratory.

Discovery and characterization of DNA-damage induced ROS generators is the most intriguing and important challenge of the future studies. While at this time there are no obvious candidates for ROS generators in yeast cells beyond those involved in the mitochondrial respiratory chain, there are several possible flavoprotein candidates. Flavoproteins typically

catalyze oxidation-reduction reactions [21]. Several possible candidates are Pst2, Yhb1, Yor356W, Rfs1, and Ycp4, which are all flavoprotein-related proteins [22]. *PST2* is regulated by Yap1 in response to oxidative stress [23]. Pst2 localizes to the mitochondria, cytoplasm and colocalizes with membrane rafts [23]. Yhb1 plays a role in the oxidative and nitrosative stress responses and localizes to the cytoplasm and mitochondria [24]. Yor356W has similarity to flavoprotein-type oxidoreductases and localizes to the mitochondria [25]. Rfs1 is a protein of unknown function that has similarity to the flavodoxin-like fold protein family and localizes to the cytoplasm [26]. Ycp4 is also a protein of unknown function that has similarity to flavodoxins [27]. Ycp4 localizes to the mitochondria, cytoplasm and also colocalizes with membrane rafts [27]. The primary candidates would be those that localize to the cytoplasm, as previous work by Salmon *et. al.* has shown that rho^0 cells still exhibit an increase in intracellular ROS levels following exposure to MMS [28] and therefore not all ROS is produced in the mitochondria in response to DNA damage.

The long term goal of this project would be to move these studies to a mammalian system as these are more closely related to humans and the ultimate goal is to fully understand the cells response to DNA damage in humans and how this may be related to disease. Preliminary experiments investigating the intracellular ROS levels in mammalian cells that were repair-deficient have been carried out, and increases in intracellular ROS levels occur following exposure to MMS (unpublished results). Mammalian cells, like yeast cells, contain activator protein (AP-1) that is similar to Yap1 in yeast. In mammalian cells, the AP-1 protein is formed as a heterodimer of proteins such as Fos and Jun [29]. The regulation of AP-1 is similar to Yap1 in that it is also regulated based on its oxidative state [30]. Examination of the activation (localization) of AP-1 following exposure to DNA damaging agents would provide support for the need to further investigate proteins that are not known to be directly involved in the DNA damage response.

In conclusion, this project has provided new insight into the DNA damage-induced ROS stress response. Additionally, it was found that Yap1 is a DNA damage responder and functions in maintaining genomic stability. These finding are important because new insight into the DNA damage response has been gained. Historically, studies have focused on DNA repair pathways individually and dissected the mechanism required for repair of specific lesions. More recently, the interconnection between DNA repair pathways has been examined. These previous studies have given us insight into the overlap of repair between multiple pathways. The studies presented here probe further into the regulation of the DNA repair pathways and reveal that there is a difference in the cellular response to certain classes of DNA lesions. While there may be overlap in the function of DNA repair pathways, the present studies have demonstrated that the cellular response to that DNA damage differs depending on the nature of the genotoxic stress. In humans, the ability to repair DNA damage is important to maintain our health and disruption of DNA repair systems can contribute to diseases such as cancer. By understanding the basic elements of the regulation of DNA repair we can begin to explore new ways to prevent and/or treat these diseases.

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Figure 1. Proposed model for DNA damage-induced ROS signaling. DNA damage caused by MMS or UV-C produces an increase in intracellular ROS levels. The increase in intracellular ROS, produced by cellular oxidases (currently unidentified), functions in distinct pathways whether it is produced following exposure to MMS or UV-C. Following exposure to UV-C there is an increase in intracellular ROS that potentially activates responder proteins that relay the message to initiate DNA repair and check point control pathways. Following exposure to MMS, the increased ROS activates Yap1. Yap1 functioins as a DNA damage responder and can regulate genes that are involved in the ROS scavenging (*SOD1*, *CTT1*, and *TRX1*), DNA damage repair (*NTG1* and *MAG1*), and check point control pathways (*MEC1*, *POL1*, and *POL3*). Disruption of DNA damage response pathways (DNA repair pathways or Yap1) can lead to further increases in intracellular ROS and lead to genomic instability or cell death depending on the magnitude of response provided in the cell and the type of DNA damage induced.

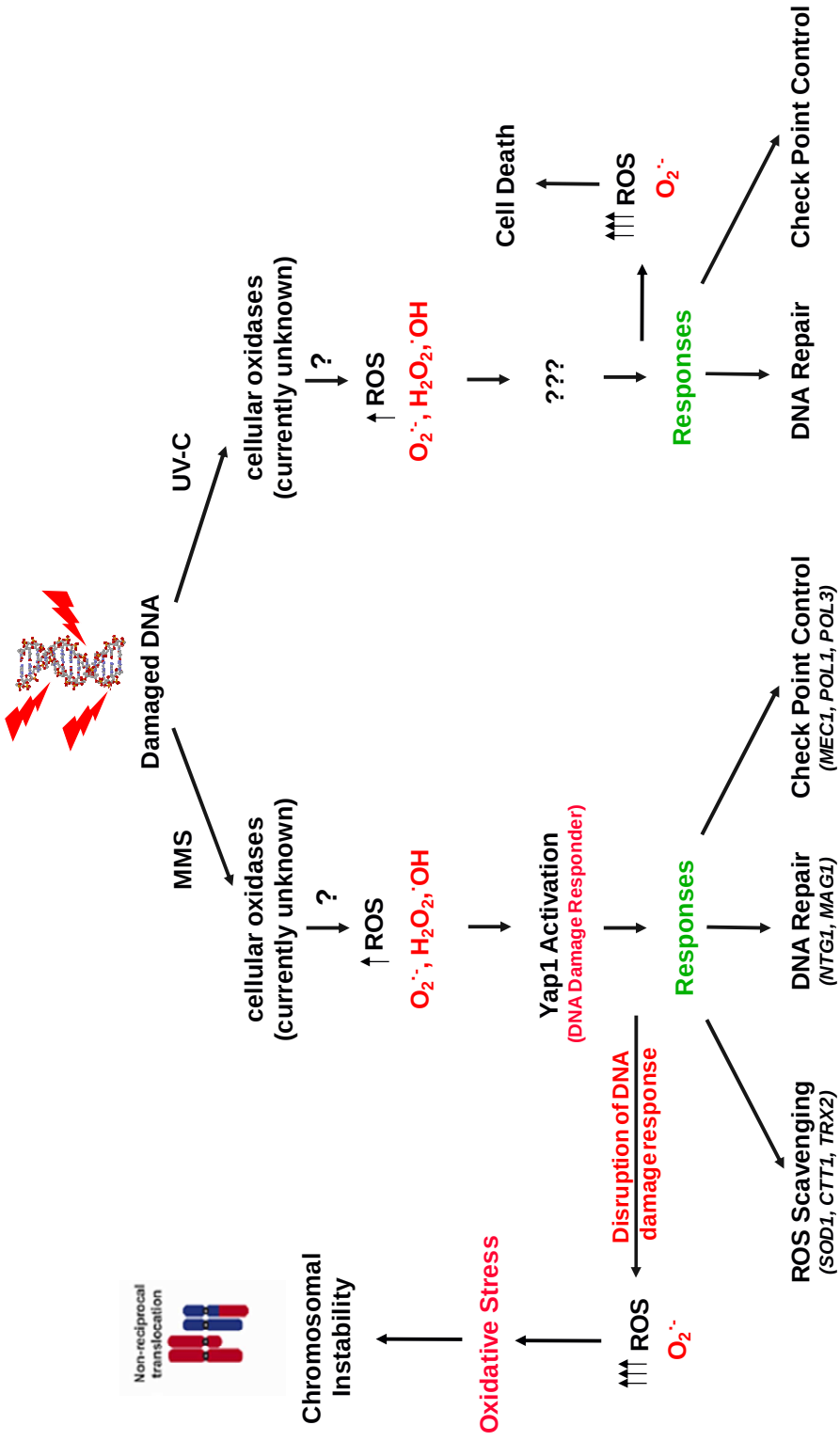

Figure 1. DNA damage-induced ROS signaling.