Published in final edited form as:

Mech Ageing Dev. 2012 April ; 133(4): 147–156. doi:10.1016/j.mad.2012.03.009.

Yap1: A DNA damage responder in *Saccharomyces cerevisiae*

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Abstract

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 reactive oxygen species increase. One important function of such a response could be to initiate signal transduction processes. We have employed the model eukaryote *Saccharomyces cerevisiae* to delineate DNA damage sensing mechanisms. We report a novel, unanticipated role for the transcription factor Yap1 as a DNA damage responder, providing direct evidence that reactive oxygen species are an important component of the DNA damage signaling process. Our findings reveal an epistatic link between Yap1 and the DNA base excision repair pathway. Corruption of the Yap1-mediated DNA damage response influences cell survival and genomic stability in response to exposure to genotoxic agents.

Keywords

DNA damage signaling; Oxidative stress; Base excision repair; Reactive oxygen species; Genome instability

1. Introduction

Cells are continually exposed to both endogenous and exogenous sources of reactive oxygen species (ROS). High levels of ROS are detrimental to cells leading to oxidative stress and to impaired physiological function through damage to DNA, proteins, and lipids (Apel and Hirt, 2004; Cadenas and Sies, 1985; Scandalios, 2005). In addition, chronic exposure to ROS has been associated with several human pathologies including cancer (Dreher and Junod, 1996), neurodegenerative disorders (Droge, 2002), and cardiovascular disease (Alexander, 1995), as well as the process of aging (Harman, 1981).

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DNA damage signaling confers on eukaryotic cells an efficient and rapid response to genotoxic insults. Mechanistic studies of DNA damage signaling pathways involving the detailed dissection of the interactions between dozens of previously identified components has proven to be beneficial in both clinical applications as well as for our basic understanding of signal transduction mechanisms. However, the vast majority of past and current investigations have focused on DNA damage signaling processes caused by either DNA single or double strand breaks (SSBs and DSBs, respectively)(Ciccia and Elledge, 2010). Although a significant fraction of cellular DNA damage is, indeed, contributed by SSBs and DSBs, oxidative damage is a major source of the deleterious modifications of DNA and, in certain cases thought to be a precursor of SSBs and DSBs (Sage and Harrison, 2011). In humans, recent studies have shown that defects in base excision repair and /or aberrant repair of oxidative DNA damage could contribute to tumorigenesis (Kryston et al., 2011), (Nemec et al., 2010).

Elucidation of oxidative DNA damage signaling is not a straightforward task since oxidative modifications of DNA are efficiently repaired by base excision repair enzymes with overlapping specificities (Doetsch et al., 2001). However, previous studies from our group have demonstrated that DNA damage itself causes increases in cellular ROS (Evert et al., 2004), (Salmon et al., 2004),(Rowe et al., 2008). We hypothesized that DNA damage-induced moderate increases in ROS levels could be beneficial for cells if such levels function as a molecular switch of the redox status of transcriptional activators of DNA repair and/or ROS-scavenging genes. Here we report that signaling of non-oxidative, alkylation agent-induced DNA damage could be relayed by the transcription factor Yap1 providing insight into the mechanisms DNA base damage response networks.

In order to maintain genomic stability, cells have evolved a number of DNA damage management pathways that include direct reversal, base excision repair (BER), nucleotide excision repair (NER), mismatch repair, translesion synthesis, and recombination (Friedberg et al., 2006). As BER and NER are key systems for the removal of numerous deleterious lesions from the genome (Friedberg et al., 2006), 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 and abasic sites, such as those caused by oxidizing and alkylating agents (Lindahl et al., 1997). NER is thought to be the major pathway for the repair of DNA helix distorting lesions such as ultraviolet light (UV-C)-induced cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4 PP) as well as a variety of bulky adducts (Lindahl et al., 1997). In addition to its major role in repair of bulky DNA lesions, NER can also contribute to the repair of oxidative DNA damage (Evert et al., 2004; Friedberg et al., 2006).

Yeast is an informative model system for dissection of eukaryotic DNA repair pathways as there is a high degree of conservation with complex organisms. In yeast, BER and NER are the major repair pathways for many 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 DNA damaging agent cytotoxicity and ROS levels and determine whether modulation of ROS levels constitutes a general response to DNA damage or is limited to specific classes of DNA lesions.

We have recently documented that increased levels of DNA damage, regardless of type or degree of cytotoxicity, cause elevations in intracellular ROS (Rowe et al., 2008). These studies also revealed that cells deficient both in BER and NER harbor greatly elevated (~800-fold) levels of oxidative DNA damage, and substantially increased ROS levels (Salmon et al., 2004). Furthermore, intracellular ROS levels in both WT and repair-deficient

strains (BER⁻, NER⁻, and BER⁻/NER⁻ strains) are elevated in a dose-dependent manner following exposure to the DNA alkylating agent methyl methanesulfonate (MMS) or UV-C (Rowe et al., 2008). These studies indicate that DNA damage *per se* can cause an increase in intracellular ROS regardless of the type of DNA lesion or the primary pathway involved in its repair, suggesting that the ROS produced may function in DNA damage-induced signaling processes (Rowe et al., 2008).

The transcription factor Yap1 is activated by oxidative stress (Coleman et al., 1999; Delaunay et al., 2000; Delaunay et al., 2002; Moye-Rowley et al., 1989; Rodrigues-Pousada et al., 2004). Under normal cellular growth conditions Yap1 is localized in the cytoplasm due to constitutive nuclear export by the classical nuclear export receptor, Crm1 (Delaunay et al., 2002; Yan et al., 1998). However, in response to oxidative stress, intermolecular disulfide bonds form in Yap1 blocking the binding of Crm1. In absence of Crm1 Yap1 cannot be exported to the cytoplasm and accumulates in nucleus where it activates transcription of over 70 genes (Gulshan et al., 2005; Okazaki et al., 2007). The best characterized group of transcriptional targets of Yap1 are the genes involved in ROS scavenging, such as superoxide dismutase (*SOD1*), catalase (*CTT1*), and thioredoxin (*TRX2*) (Temple et al., 2005). Several studies indicate that genes involved in DNA repair, replication, and cell cycle check point control can also be activated by Yap1, including *NTG1*, *POL1*, *MAG1*, *MEC1*, and *POL3* (Monteiro et al., 2008). We have recently reported that as the endogenous levels of ROS increase following exposure to MMS, Yap1 accumulates within the nucleus (Rowe et al., 2008). Collectively, these findings suggest that Yap1 may be directly involved in the DNA damage response via ROS signaling.

The goal of the present study was to determine the involvement of Yap1 in the DNA damage response. We examined several biological endpoints in a set of isogenic repair-proficient (WT, *yap1Δ*) and repair-deficient (BER⁻, BER⁻ *yap1Δ*, NER⁻, and NER⁻ *yap1Δ*) strains with or without functional Yap1. We determined the sub-cellular localization of Yap1 in cells exposed to different DNA damaging agents and found that Yap1 accumulates within the nucleus in response to MMS and H₂O₂, but not UV-C exposure. Analysis of the role of Yap1 in modulating cytotoxicity, intracellular ROS levels, and levels of chromosomal aberrations caused by these agents allowed us to conclude that ROS generated in response to DNA damage mediates signaling processes resulting in Yap1 activation. Such activation appears to be invoked in response to certain types of DNA damage, specifically, DNA damage repaired by BER.

2. Material and Methods

2.1 Strains, media, and growth conditions

All the strains utilized in this study (with exception of DSC0025, DSC0035 and DSC0036) are haploid isogenic derivatives of heterozygous diploid hDNP42 (Supplemental Table 1). Two sets of isogenic haploid strains were obtained by sporulation and dissection of independent isolates of the parental diploid hDNP42. Each isogenic set contains a repair proficient strains (wild type – WT and WT *yap1Δ*) and repair deficient strains (BER⁻, NER⁻, BER⁻ *yap1Δ*, and/or NER⁻ *yap1Δ*). hDNP42 diploid strain was constructed by the replacement of one copy of *YAP1* gene in heterozygous hDNP19 diploid with a PCR fragment containing the *natNT2* gene flanked by *YAP1* upstream and downstream sequences, conferring resistance to nourseothricin and deletion of the entire *YAP1* open reading frame. Plasmid pYM17 (Euroscarf) was used as a template for amplification of *natNT2* gene (Degtyareva et al., 2008). Primer sequences are available upon request. Isogenic strains DSC0025 (WT), DSC0035 (BER⁻) and DSC0036 (NER⁻) had been described previously (Swanson et al., 1999) and were utilized in Yap1 localization studies and measurement of O₂^{•-} levels. The hDNP42-derived strains were utilized in the

cytotoxicity, mutation rate, and chromosomal aberration (karyotype analysis) studies. All yeast strains were grown in YPD media (1% yeast extract, 2% peptone, 2% dextrose). All YPD media were supplemented with 0.5% adenine sulfate. For selection of strains containing the Yap1-GFP plasmid, the strains were grown on synthetic complete media lacking uracil (0.5% ammonium sulfate, 0.17% yeast nitrogen base without amino acids, 2% dextrose). Synthetic complete media lacking arginine and supplemented with 60 mg/ml L-canavanine was used for mutation rates measurement. Solid media contained 2% agar (Guthrie and Fink, 1991).

Strains referred to in the following text as BER⁻ strains contain disruptions of three genes *NTG1*, *NTG2*, and *APN1* (due to the redundancy in functional activities of BER enzymes lack of one or two BER genes is very efficiently compensated by other BER proteins (Swanson et al., 1999). NER⁻ strains contain a disruption of the *RAD1* gene. Strains that also have a disruption in *YAP1* gene are referred to as *yap1Δ*, BER⁻ *yap1Δ*, and NER⁻ *yap1Δ* strains.

2.2 Cell growth and viability

Liquid YPD media was inoculated with yeast cells and grown at 30 °C for ~24 hrs to saturation ($> 7 \times 10^7$ cells/mL). 50 mL of liquid YPD was inoculated with an appropriate amount of cells, such that the culture would reach a density of 2×10^7 cells/mL 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.

2.3 Yap1 Cellular Localization Studies

For studies examining the sub-cellular localization of Yap1, strains DSC0025, DSC0035 and DSC0036 (WT, BER⁻, and NER⁻ strains respectively) were transformed with a centromeric plasmid pLR1 (Rowe et al., 2008) encoding Yap1-GFP fusion protein. Cells transformed with the plasmid were grown to mid-log phase ($\sim 2 \times 10^7$ cells/mL) as described above in YPD at 30 °C overnight, counted, and washed twice with H₂O. The density of the cells was adjusted to 2×10^7 cells/mL in H₂O. Cells were stained with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen) to visualize DNA in nuclei and mitochondria. Cells were incubated with 1 μL DAPI (100 nM) per 1 mL of cells for 5 min, washed once with H₂O, and then re-suspended in the original volume in H₂O. Cells were then exposed to MMS (0.5 or 25 mM), H₂O₂ (0.5 or 25 mM), or UV-C (2 or 25 J/m²). Cells were incubated in MMS or H₂O₂ 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 experiment. Cells were subjected to fluorescence confocal microscopy (Zeiss LSM510 META) and images were analyzed using Carl Zeiss LSM Image Browser software.

2.4 Measurement of O₂^{•-} levels

O₂^{•-} levels were detected using the fluorescent probe dihydroethidium (DHEt) (Benov et al., 1998; Carter et al., 1994). Cells were grown to mid-log phase ($\sim 2 \times 10^7$ cells/mL) in YPD at 30 °C overnight. Cells were counted in haemocytometer, washed twice in H₂O and then adjusted to 2×10^7 cells/mL in H₂O. 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 O₂^{•-} measurements. Immediately following fluorescent probe addition, cells were incubated in the dark at 30 °C.

for 2 hrs. Cells were subsequently washed twice with H₂O and then re-suspended in 2 mL phosphate buffered saline (PBS) and assessed for fluorescence intensity employing a BD™ LSR II flow cytometer (BD Biosciences).

2.5 Measurement of Mutation Rates

Several independent cultures for all the strains of different genotypes were grown to saturation (2×10^8 cells/mL) in 5 mL YPD at 30 °C, washed twice in H₂O and resuspended in 1 mL of water. Cells were then plated onto synthetic complete media, supplemented with L-canavanine and lacking arginine for selection of canavanine-resistant mutants. In order to calculate the mutation rates an appropriate dilution of cells was plated onto YPD to obtain more than 20 colonies per plate. The plates were then incubated at 30 °C until colonies appeared. Two independently obtained strains of the same genotype were used for analysis. Median mutation rates and confidence limits were calculated for total of 18-20 cultures of the same genotype as previously described (Chen and Kolodner, 1999).

2.6 Detection of Chromosomal Aberrations by CHEF

The analysis was performed as previously described (Degtyareva et al., 2008). Briefly, haploid strains of all genotypes were streaked for single colonies on the plates with rich medium (YPD). Eight independent colonies of each genotype were designated as a “founders” for the corresponding lineages (lanes) and re-streaked onto YPD plates. One colony from these founders’ progeny was re-streaked onto YPD plates. At the conclusion of the experiment, the cells were passaged (re-streaked) 20 times. DNA isolation and CHEF gel electrophoresis was performed as previously described to determine large scale chromosomal rearrangements (Narayanan et al., 2006). Patterns of chromosome migration after the 10th and 20th passages were compared to the founders’ karyotypes. Visible changes in chromosome size were scored as a chromosome aberration.

3. Results

Yap1 activation in response to oxidative stress is well documented and can be caused by direct oxidation and subsequent retention of Yap1 within the nucleus (Coleman et al., 1999; Delaunay et al., 2000; Delaunay et al., 2002; Moye-Rowley et al., 1989; Rodrigues-Pousada et al., 2004). A major objective of these studies was to address an unknown function of Yap1 in DNA damage signal transduction. Previously, we demonstrated that genotoxic stress caused by DNA damaging agents triggers an increase in intracellular ROS levels (Rowe et al., 2008). Increases in both endogenous oxidative DNA damage and intracellular levels of ROS were also observed in DNA repair compromised cells (BER⁻/NER⁻) not exposed to exogenous DNA damaging agents, indicating that compromising DNA repair causes accumulation of endogenous DNA damage and leads to increase in intracellular ROS (Evert et al., 2004; Rowe et al., 2008; Salmon et al., 2004). These findings allowed us to hypothesize that Yap1 is involved in DNA damage signal transduction via the following steps: i) DNA damage leads to an increase in ROS levels; ii) ROS cause conformational changes in Yap1, followed by its accumulation in the nucleus; iii) Nuclear Yap1 activates the transcription of ROS scavenging and DNA repair genes.

3.1 Dynamics of Yap1 sub-cellular compartmentalization in response to different types of DNA damage

Fluorescence microscopy was utilized to determine the localization of Yap1-GFP in response to various DNA damaging agents. MMS produces non-bulky base lesions while UV-C induces larger, helix distorting DNA lesions that are primarily repaired by BER and NER, respectively (Friedberg et al., 2006). Although MMS and UV-C produce specific types of DNA lesions, neither is itself a reactive oxygen species, thus they cannot directly

activate Yap1-mediated oxidative stress responses. However, both MMS and UV-C cause an increase in intracellular ROS levels (Rowe et al., 2008).

To investigate the involvement of Yap1 in the DNA damage response, we exposed repair-proficient (WT) and repair-deficient BER⁻ (*ntg1Δ ntg2Δ apn1Δ* triple mutant) and NER⁻ (*rad1Δ* mutant) strains to both low and high doses of MMS. At a low exposure dose of 0.5mM MMS, only 12% of cells exhibited nuclear localization of Yap1. When WT cells were exposed to 25 mM MMS, the number of cells displaying Yap1 nuclear localization increased to 32% (Figure 1A). In agreement with previous reports (Azevedo et al., 2003; Delaunay et al., 2000; Delaunay et al., 2002), following treatment of cells with H₂O₂, Yap1 re-localized to the nucleus within the first few minutes after exposure (Figure 1A-C). MMS-induced re-localization occurred slower compared to H₂O₂-induced re-localization (Figure 1A, right panel). The dynamics of MMS-induced Yap1 re-localization suggests that the mechanism of Yap1 activation by MMS is different from that of Yap1 induction by H₂O₂. Furthermore, the delay in nuclear accumulation of Yap1 following MMS exposure indicates that the signaling of DNA damage to Yap1 involves additional steps, that require time to be transduced. Despite the fact that exposure to UV-C increases intracellular levels of ROS (Rowe et al., 2008), no change in localization of Yap1 was observed in WT cells following UV-C exposure (Figure 1A), indicating that Yap1 is selectively sensitive to different types of DNA damage.

We next examined the localization of Yap1 in BER⁻ and NER⁻ strains. In BER⁻ cells exposed to a low dose (0.5 mM) of MMS (Figure 1B, left panel), a higher percentage of cells displayed nuclear accumulation of Yap1 compared to WT cells (Figure 1A left panel) exposed to the same dose of MMS at the same time points following exposure (Figure 1B). The dynamics of Yap1 re-localization in BER⁻ deficient cells in response to a low dose of MMS (Figure 1B left panel) was similar to that observed in wild type cells exposed to a high dose of MMS (Figure 1A, right panel). For example, 60 min following exposure of BER⁻ cells to a low dose of MMS, 47% of cells displayed Yap1 accumulation in the nucleus, compared to 37% in WT cells exposed to a high dose of MMS. At the same time only 12% of WT cells exposed to low dose of MMS showed accumulation of Yap1 in the nucleus after 60 min. As expected, the re-localization of Yap1 to the nucleus in NER⁻ cells was stimulated by H₂O₂ exposure (Figure 1C). However, low doses of MMS did not change the sub-cellular distribution of Yap1 in NER⁻ cells (Figure 1C left panel) and in contrast to BER⁻ cells, the dynamics of re-localization was similar to that of WT cells for both low and high doses of MMS (compare Figure 1A and 2C). Localization of Yap1 was unchanged in BER⁻ and NER⁻ cells as well as in WT cells following exposure to UV-C (Figure 1B and 2C). Collectively these results suggest that Yap1 is activated in response to specific types of DNA damage, namely, DNA damage repaired by BER.

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

We investigated the involvement of Yap1 in the protection from cytotoxicity following exposure to MMS, UV-C, and H₂O₂ in DNA repair-proficient and -deficient cells. In WT and NER⁻ cells, Yap1 does not protect cells from the cytotoxic effects of MMS (Figure 2A and C). However, BER⁻ cells were protected from the cell killing effect of MMS by Yap1. BER⁻ and BER⁻ *yap1Δ* cells exposed to 5 mM MMS exhibited 75% and 25% survival, respectively (Figure 2B). These findings support the conclusion that Yap1 is a component of the DNA damage response for base damage induced by alkylating agents.

To address the involvement of Yap1 in the protection of cells from other classes of DNA lesions, we examined the cytotoxicity of UV-C in the same DNA repair-proficient and -deficient strains. As expected, all strains exhibited increased cytotoxicity in response to increasing doses (0-25 J/m²) of UV-C (Figure 2D-F). In contrast to its role in protection

from MMS-induced damage, Yap1 did not influence UV-C –induced cytotoxicity in either the DNA repair-proficient or -deficient strains. These results further support the idea that Yap1 functions as a DNA damage responder for lesions that are primarily processed by BER (e.g. alkylating damage) but not NER (e.g. UV damage) and points to a novel BER-specific role for this transcription factor. In addition, our studies show that in the absence of Yap1, DNA repair deficient strains are sensitized to H₂O₂ (Figure 2G-I) as well as wild type strains.

3.3 Influence of Yap1 on genomic stability

The finding that Yap1 functions in DNA damage signaling suggests that it might play a role in the maintenance of genomic stability. We investigated contribution of Yap1 in prevention of mutations by measuring the mutation rates in DNA repair-proficient and -deficient strains via a forward mutation assay utilizing the *CAN1* locus (Degtyareva et al., 2008). Deletion of Yap1 caused a significant, 2-fold, increase in mutation rate in WT and NER⁻ cells, but did not further increase mutability of BER⁻ cells (Table 1). These results suggest that Yap1 has a moderate anti-mutator effect. The absence of an observed anti-mutator effect of Yap1 in BER⁻ cells in which the *NTG1* gene is deleted could be explained by the fact that the *NTG1* gene promoter contains a putative Yap1 binding sequence (Monteiro et al., 2008; Teixeira et al., 2006). Ntg1 is a major DNA glycosylase responsible for repair of oxidative base damage in yeast (Friedberg et al., 2006) and if epistatic interaction between *YAP1* and *NTG1* occurs, the anti-mutator role of Yap1 would be undetectable in *ntg1Δ* cells.

3.4 Role of Yap1 in prevention of large-scale chromosomal aberrations

Increased levels of intracellular ROS and accumulation of chronic, unrepaired DNA damage result in large-scale chromosomal aberrations characterized by amplification, deletions, and translocations (Degtyareva et al., 2008). To further address the role of Yap1 in prevention of the genome destabilization, we followed the occurrence of chromosomal aberrations by analysis of the changes in karyotypes of yeast cells serially passaged on solid media under non-selective conditions (Material and Methods). To evaluate the number of chromosomal aberrations in a population of haploid yeast cells, CHEF gel electrophoresis was utilized as previously described (Degtyareva et al., 2008).

Subcultures originating from a single colony in the progeny of the “founder” were established. The cultures were maintained until full-size colonies formed resulting from multiple divisions of the original colony cells. For every passage, a single colony from each line was streaked onto a fresh plate containing rich media. Colonies from the 10th (p10) and 20th (p20) passages were analyzed for chromosomal aberrations (Figure 3A). The chromosomal electrophoretic mobility patterns were analyzed following the 10th and 20th passage and compared to previous passages (0 and 10th passages, respectively) to assess changes in chromosome sizes (Figure 3B and Supplemental Table 2).

Previous studies revealed that cells compromised in BER alone or in conjunction with NER (BER⁻/NER⁻ cells), that harbor elevated levels of intracellular ROS (Rowe et al., 2008; Salmon et al., 2004) (Figure 4), also have increased frequencies of chromosomal aberrations (Degtyareva et al., 2008). In the present study, an increase in intracellular ROS levels was observed in *yap1Δ*, BER⁻, and BER⁻ *yap1Δ* strains, but not NER⁻ or NER⁻ *yap1Δ* strains (Figure 4). The strains with elevated ROS levels also displayed a significant increase in chromosomal aberrations compared to WT cells (indicated by asterisks in Figure 3B). In a DNA repair-proficient background Yap1 protects cells from chromosome aberrations. However, no increase in chromosomal aberrations was observed in the BER⁻ deficient *yap1Δ* strain compared to BER⁻ strain alone. A possible reason that an increase was not

detected in the $\text{BER}^- \text{ yap1}\Delta$ strain compared to the BER^- strain is epistatic interaction between Yap1 and Ntg1 in BER pathway and is consistent with observed interactions between Yap1 and BER pathway in prevention of small mutational changes in the *CAN1* locus. Furthermore, the number of chromosomal rearrangements is likely to be underestimated due to the fact that the number of chromosomes with size changes observed per isolate (one lane of the gel) was scored as one change, despite the occurrence of multiple size changes per lane. This was done because, for example, a change in the size of two chromosomes could be a result of one destabilizing event (e.g. reciprocal translocation). These data provide further evidence that Yap1 is a DNA damage responder that influences genomic stability.

3.5 Yap1 modulation of intracellular $\text{O}_2^{\bullet-}$ levels induced by DNA damage

To further define the involvement of Yap1 in the DNA damage response, we examined the levels of intracellular $\text{O}_2^{\bullet-}$ in DNA repair-proficient and -deficient strains with and without functional Yap1. Endogenous levels of $\text{O}_2^{\bullet-}$ were determined for all strains, and a significant increase in $\text{O}_2^{\bullet-}$ levels in *yap1* Δ , BER^- , and $\text{BER}^- \text{ yap1}\Delta$ cells was observed compared to WT cells (Figure 4). Such an increase in intracellular ROS has been shown to be accompanied by an increase in DNA damage (Rowe et al., 2008), suggesting that the increase in ROS observed in *yap1* Δ cells is likely due to an increase in the levels of oxidative DNA damage produced endogenously. Intracellular $\text{O}_2^{\bullet-}$ levels were also measured following exposure to a range of doses of MMS (0-25 mM) and UV-C (0-25 J/m²). Consistent with our previous observations (Rowe et al., 2008), the $\text{O}_2^{\bullet-}$ levels increased with increasing exposure doses of both MMS and UV-C regardless of the strain background (Figure 5 A-F). Notably, there was a significant increase in $\text{O}_2^{\bullet-}$ levels in $\text{BER}^- \text{ yap1}\Delta$ cells compared to BER^- cells following exposure to both MMS and UV-C (Figure 5B and 6E), indicating that Yap1 plays an important role in sensing $\text{O}_2^{\bullet-}$ levels in BER^- cells. These findings suggest that in addition to its well-documented role in transcriptional activation of ROS-scavenging genes, Yap1 regulates the genes of the BER pathway. Lack of such activation in BER^- deficient *yap1* Δ cells leads to further DNA damage, which in turn causes a significant increase in $\text{O}_2^{\bullet-}$ levels. Likewise, the increase in $\text{O}_2^{\bullet-}$ levels in $\text{BER}^- \text{ yap1}\Delta$ cells following exposure to increasing doses of UV-C likely reflects the contribution of BER in the repair of non-pyrimidine dimer (non-bulky) base lesions (e.g. monobasic pyrimidine photohydrates) (You et al., 1999). These data are consistent with our observation that Yap1 is activated by ROS only in response to specific types of DNA damage, (e.g. MMS-induced), that are processed by the BER pathway. The lack of a significant increase in $\text{O}_2^{\bullet-}$ levels in $\text{NER}^- \text{ yap1}\Delta$ cells compared to NER^- cells (Figure 5F) confirms the specific role of Yap1 in up-regulating the BER pathway. If Yap1 is involved only in activation of the BER component, and does not target NER genes, the increase in ROS levels caused by UV-C-induced DNA damage cannot be decreased by up-regulation of the NER pathway. Thus, no difference in the relative levels of $\text{O}_2^{\bullet-}$ in NER^- and $\text{NER}^- \text{ yap1}\Delta$ cells is observed.

4. Discussion

Increases in DNA damage cause an increase in intracellular ROS levels (Rowe et al., 2008; Salmon et al., 2004). This observation raises the obvious issue of the biological relevance for generating reactive species 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 (Apel and Hirt, 2004; D'Autreaux and Toledano, 2007; Genestra, 2007; Kamata and Hirata, 1999). Signaling molecules are defined as being able to generate a specific short-lived response (Forman et al., 2004). ROS meet the requirement of signaling molecules in that they are short-lived and can be generated at the time of receptor activation.

ROS can also target specific amino acids of proteins to alter their function (D'Autreaux and Toledano, 2007). Several known examples for ROS subspecies specificity have been observed in both *Escherichia coli* and yeast. In *E. coli*, the redox sensitive transcription factors SoxR and OxyR are capable of distinguishing between $O_2^{\bullet-}$ and H_2O_2 , respectively. SoxR possesses an iron-sulfur center that readily reacts with $O_2^{\bullet-}$ (Hidalgo and Dimple, 1994), while OxyR contains cystine residues that react with H_2O_2 (Zheng et al., 1998). In yeast, ROS alter the protein structure of Yap1 through direct interaction with cysteine sulfhydryl groups allowing for intramolecular disulfide bonds to form, blocking the binding of Crm1 resulting in Yap1 accumulation in the nucleus (D'Autreaux and Toledano, 2007). Interestingly, interactions between specific and distinct pairs of cysteines residues allow Yap1 to discriminate between oxidative stress caused by peroxide and diamide (Wemmie et al., 1997), suggesting possibility of a differentiated response to distinct stress cues. Thus, we explored the possible role of Yap1 as a responder to DNA damage-induced ROS-mediated signaling.

The increase in intracellular ROS observed following DNA damage is dependent on the type of DNA damage and the repair proficiency of the cell (Rowe et al., 2008). Depending on the DNA damage induced, there are changes in the spectrum and the levels of ROS subspecies (Rowe et al., 2008). Several lines of evidence suggest that the DNA damage-induced increases in ROS levels are used by the cell in signaling processes to maintain genomic integrity (Evert et al., 2004; Rowe et al., 2008; Salmon et al., 2004). As a transcription factor known to be the primary mediator of differential oxidative stress responses in yeast (Coleman et al., 1999; Delaunay et al., 2000; Delaunay et al., 2002; Moye-Rowley et al., 1989; Rodrigues-Pousada et al., 2004), Yap1 appears to be a strong candidate for transducing ROS-induced DNA damage signaling in yeast.

To assess the role of Yap1 as a DNA damage responder, we determined the changes in localization of Yap1 following exposure to MMS and UV-C, that produce two distinct classes of DNA lesions while neither agent directly produces ROS. Previously, we determined that there are substantially higher levels of ROS in cells following exposure to MMS compared to UV-C (Rowe et al., 2008). In this study, we found that Yap1 re-localizes 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 intended to cause of Yap1 nuclear accumulation and only occurs in response to DNA damage of the repaired by BER (e.g. alkylating DNA damage). These experiments also revealed that Yap1 protects cells specifically from MMS, but not UV-C-induced DNA damage in a BER⁻ background. However, there was no difference in the DNA damage cytotoxicity in WT and BER-deficient cells whether functional Yap1 was present or not. A mechanistic explanation of Yap1-dependent MMS sensitivity in BER⁻ strains was revealed by the studies of Yap1 re-localization in response to exposure to different types of DNA damage. Assuming that Yap1 accumulation in the nucleus results in activation of the relevant downstream targets (Coleman et al., 1999; Delaunay et al., 2002; Moye-Rowley et al., 1989; Rodrigues-Pousada et al., 2004), we analyzed the sub-cellular compartmentalization of Yap1 in response to exposure to DNA damaging agents. In agreement with the results of the cytotoxicity experiments, we found that even though Yap1 re-localizes to the nucleus following exposure to high doses of MMS regardless of the DNA repair status of the cells, in BER⁻ cells re-localization is induced by a low dose exposure to MMS. In addition, exposure to UV-C, (induction of DNA damage repaired by NER), did not result in Yap1 accumulation in the nucleus. Taken together, these results indicate that Yap1 responds to DNA lesions that are primarily repaired by the BER pathway. The relevance of DNA damaged-induced ROS signaling is further verified by our findings that Yap1 protects cells against mutations and large-scale chromosomal rearrangements. Assessment of mutation rates and large scale chromosomal rearrangements revealed that Yap1 functions in reducing both types of

genomic instability events (Table 1 and Figure 3). Our data also suggest that Yap1 does not function directly in DNA repair, but likely regulates DNA repair. Therefore, as expected, we detected a modest, but significant increase in genomic instability in *yap1Δ* strains. The observed absence of an effect of Yap1 in the prevention of genomic instability in BER[−] strains indicates that Yap1 functions in the maintenance of genomic stability in a regulatory pathway that is likely to be epistatic with BER.

The results of this study suggest the following model of Yap1 involvement in DNA damage-induced stress response and prevention of genomic instability (Figure 6). Accumulation of DNA lesions leads to an increase in the levels of intracellular ROS. This could occur as a result of exposure to exogenous agents (i.e. MMS or UV-C) or as a consequence of diminished capacity to repair DNA damage produced endogenously. Depending on the specific type of DNA lesions, which are processed by either BER (Figure 6, left panel) or NER (Figure 6, right panel), increases in the relative levels of sub-types of intracellular ROS (e.g. O₂^{•−}, H₂O₂, or OH[•] could vary. We have arbitrarily designated these as sub-types A and B. We propose that levels of ROS sub-type A increase more dramatically than levels of sub-type B in response to accumulation of MMS-induced alkylating base damage, whereas UV-induced damage causes a relatively modest increase of both sub-types. Non-bulky base lesions of the type produced by MMS or endogenous oxidative damage in BER[−] cells provoke a shift in the relative levels of ROS, such that the levels of sub-type A (left panel, red vertical bar) exceeds the threshold level causing Yap1 oxidation (horizontal dashed line). Oxidation of Yap1 leads to its accumulation in the nucleus where it functions as a transcription factor. Yap1 activation causes an up-regulation of transcription of BER and ROS-scavenging genes. Transcriptional activation of BER and ROS scavenging pathways facilitates DNA repair, prevents further DNA damage, protects cells from genomic instability, and restores the ambient levels of different ROS sub-types within the cell. In contrast, although accumulation of DNA lesions processed by NER increases the endogenous levels of ROS (35), relative concentrations of sub-type A that can cause Yap1 activation does not reach threshold levels required for oxidation of specific cysteine residues. Thus, Yap1 continues to cycle between the nucleus and cytoplasm and does not activate transcription of its target genes.

To our knowledge this is the first report of Yap1 functioning as a DNA damage responder and a novel transcriptional regulator of BER pathway. This study provides the framework for further elucidation of the DNA damage-induced ROS response in cells, including the elaboration of the sensor/effector pathways leading from DNA damage to ROS production.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Dr. Katherine Hales and Dr. Adam Marcus and the Cell Imaging and Microscopy Core of the Emory Winship Cancer Institute for assistance with the microscopy experiments and Dr. Robert Karaffa and The Emory University School of Medicine Core Facility for Flow Cytometry for their assistance with the flow cytometry experiments. We also thank Dr. Anita Corbett for plasmids, helpful discussions and critical reading of the manuscript. This work was supported by NIH Grant ES 011163 (PWD).

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Highlights

- Yap1 is involved in DNA damage signal transduction.
- DNA damage signaling is relayed to Yap1 via reactive oxygen species.
- Evidence that Yap1 senses specific types of DNA damage handled by base excision repair

Figure 1. Cellular DNA repair capacity modulates the dynamics of Yap1 re-localization
Graphical representation of fluorescence microscopy analysis assessing Yap1-GFP localization in **A.** WT cells, **B.** BER⁻ cells, and **C.** NER⁻ cells. Cells were exposed to low doses (0.5 mM H₂O₂ (light blue), 0.5 mM MMS (pink), and 2 J/m² UV-C (light green)) and high doses (25 mM H₂O₂ (blue), 25 mM MMS (red), and 25 J/m² UV-C (green)) of specified DNA damaging agent. Fluorescence images of cells were analyzed throughout 10 min intervals following exposure. Cells were counted and evaluated in 10 min interval groups. Percent of cells with nuclear localization alone or nuclear plus cytoplasmic localization for Yap1 among all cells analyzed are displayed as bars graphs.

Figure 2. Effect of Yap1 in prevention of DNA damaging agent cytotoxicity. A.-C Dose-dependent MMS sensitivity of DNA repair deficient strains with and without functional Yap1. (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 MMS for 30 min at 30°C. **D.-F.** Dose-dependent UV-C sensitivity of DNA repair deficient strains with and without functional Yap1. (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, 2, 5, and 25 J/m² UV-C. **G.-I.** Dose-dependent H₂O₂ sensitivity of DNA repair deficient strains with and without functional Yap1 to. (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 H₂O₂ for 30 min at 30°C. The results are an average of three independent experiments. Error bars represent ± SD. Asterisks (*) above bars indicate statistical significance of a p value <0.05 compared to Yap1 proficient strain.

Figure 3. Effect of Yap1 in prevention of large-scale chromosomal rearrangements

A. Representative negative image of ethidium bromide stained CHEF gels separating individual yeast chromosomes after 20 passages under non-selective growth conditions of WT, *yap1Δ*, BER⁻, and BER⁻ *yap1Δ* strains. Arrows indicate size changes affecting specific chromosomes: “▲” chromosome XIII, XVI; “♣” chromosome II; “♠” chromosome X; “↑” chromosome V, VIII; “♣” chromosome III, I. Stars (★) depict chromosomes that were altered in the founder passage (p0). **B.** Frequencies of chromosomal aberrations. CHEF gel analysis was performed on the founder passage (p0) to assess the karyotype of each strain, and at the 10th and 20th passage corresponding to approximately 250 and 500 generations, respectively (see Materials and Methods for details). The bar graph displays the number of aberrations that were first detected in the 10th and 20th passage. 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.

Figure 4. Effect of Yap1 on endogenous superoxide levels in different DNA repair backgrounds Isogenic WT, *yap1Δ*, *BER-*, *BER- yap1Δ*, *NER-*, and *NER- yap1Δ* strains were incubated with the fluorescent probe for $O_2^{\bullet-}$, DHEt, for 2 hours. Following incubation, cells were analyzed for $O_2^{\bullet-}$ levels by flow cytometry as described in Material and Methods. The mean peak of fluorescence intensity values of the cytograms reflecting the accumulated levels of superoxide are reported as the fold change relative to the WT (repair proficient) strain (arbitrarily set to a value of 1.0). Data presented is the average of at least three independent measurements. Error bars represent $\pm$ SD. Asterisks above bars indicate statistical significance of a p value <0.05 compared to WT strain. “#” above bars indicate statistical significance of a p value <0.05 compared to the *BER-* strain.

Figure 5. Effect of Yap1 on DNA damage-induced superoxide levels in different DNA repair backgrounds

A.-C. Intracellular $O_2^{\bullet-}$ levels in strains with and without functional Yap1 following exposure to MMS. Intracellular superoxide levels in: **(A)** DNA repair proficient (WT) strains; **(B)** BER⁻; and **(C)** NER⁻ strains exposed to MMS (0-25 mM) for 30 min. **D.-F.** Intracellular ROS levels strains with and without functional Yap1 following exposure to UV-C (0-25 J/m²) in **(D)** DNA repair proficient (WT); **(E)** BER⁻; **(F)** NER⁻ strains. Black bars represent *YAP1* strains, gray bars represent *yap1Δ* strains. Superoxide levels were measured as described in Material and Methods. Data is presented as in Figure 4. Error bars represent $\pm$ SD. “*” symbols above bars indicate statistically significant (p value <0.05) differences between the Yap1-functional strains versus the Yap1-deficient strains for a particular exposure dose of MMS or UV-C.

Figure 6. Proposed role of Yap1 in ROS-mediated DNA damage signaling and prevention of genomic instability

Exposure to exogenous DNA damaging agents or accumulation of endogenously produced DNA lesions, which are the substrates for BER (such as MMS-induced base damage) causes dramatic increase in relative levels of ROS sub-type A (red bar), but only a modest increase in ROS sub-type B (dark blue bar). When the level of Yap1-activating ROS sub-type A reaches a threshold level (dashed horizontal line) sufficient for Yap1 oxidation, Yap1 accumulates in the nucleus, where it up-regulates genes involved in BER and ROS-scavenging pathways. Such activation induces DNA repair, prevents further DNA damage and subsequently protects cells from genome destabilization. In the absence of DNA damage the normal balance between different sub-types of ROS is restored. Upon induction of DNA damage processed by NER (such as UV-C-induced damage) intracellular ROS levels increase (dark blue and red vertical bars), but do not reach the levels necessary for Yap1 activation.

Table 1
Spontaneous mutation rates in *yap1A* strains^a

Strain	Mutation rates (10^{-7}) (95% confidence limits)			
	<i>YAP1</i> strains		<i>yap1Δ</i> strains	
Wild Type	3.1 (2.4-4.7)	(1.0) ^b	7.1 (5.0-8.2)	(2.2) ^b
BER ⁻ (<i>ntg1 ntg2 apn1</i>)	47.9 (40.7-72.8)	(15.4) ^b	51.8 (35.4-79.1)	(16.7) ^b
NER ⁻ (<i>rad1</i>)	5.2 (4.2-7.6)	(1.7) ^b	9.2 (7.9-10.3)	(3.0) ^b

^aMedian mutation rates were determined for 18-24 cultures from two independent segregants of the same genotype as described in Materials and Methods.

^bFold change over wild type