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Interaction of the Intra-S Phase Checkpoint and the Cellular Oxidative Stress Response in *Saccharomyces cerevisiae*

by

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Abstract

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The response to DNA damage in *Saccharomyces cerevisiae* and other eukaryotic cells is regulated by the DNA damage checkpoints, a group of proteins that act to maintain genomic stability. While mechanisms of checkpoint activation are poorly understood, it is possible that an increase in intracellular reactive oxygen species (ROS) that functions as a DNA damage signal is mediating responses that promote checkpoint activity. This study will address whether the transcription factor Yap1, a key mediator of the oxidative stress response in yeast, modulates checkpoint responses at the intra-S phase checkpoint when cells are subject to genotoxic/replication stress.

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Background*

Cells are constantly exposed to sources of DNA damage. Maintaining genome stability in the face of damage is a critically important process that involves a wide variety of genome surveillance proteins, collectively known as the DNA damage checkpoints [1]. The conservation of checkpoint mechanisms between single-cell eukaryotes like *Saccharomyces cerevisiae* and higher, more complex eukaryotes like humans underscores the importance of checkpoint function [1-3]. In addition to guarding cells from a dangerous accumulation of DNA damage, the checkpoints are responsible for monitoring cell cycle events and ensuring proper replication of DNA during S-phase [1, 4].

In humans, the malfunctioning of the DNA damage checkpoints has been linked to pathologies such as cancer, ataxia telangiectasia (AT), and Li Fraumeni syndrome [1]. For example, the checkpoint protein and tumor suppressor p53 is implicated in many forms of cancer [1, 5]. Malfunctioning of the central checkpoint protein ATM causes the disease ataxia telangiectasia (AT), which is characterized by chromosomal rearrangements within cells in addition to an immune system deficiency, cerebellar degeneration, and a predisposition to certain types of cancer [6, 7]. Similarly, mutation of Chk2 or p53 is linked to the genetic disorder called Li Fraumeni syndrome that is also

*Abbreviations used: BER (base excision repair), NER (nucleotide excision repair), TLS (translesion synthesis), REC (recombination), dNTP (deoxyribonucleotide triphosphate), RNR (ribonucleotide reductase), ROS (reactive oxygen species), MMS (methyl-methanesulfonate), HU (hydroxyurea).

linked to cancer predisposition [7, 8]. Although many of the links between the checkpoints and these pathologies are not well characterized, it is clear that defects in the DNA damage checkpoints play a central role in their development [1]. In general, cells lacking checkpoint function have genomic instability due to an ineffective response to DNA damage, faulty DNA replication, or abnormal chromosome segregation [9]. How these characteristics lead to oncogenesis is an important topic of research.

The molecular signaling that defines checkpoint responses occurs by means of three classes of checkpoint proteins: sensors, transducers, and effectors [1]. Sensor proteins are responsible for relaying the DNA damage signal to transducer proteins, and the phosphorylation of effector proteins by these protein kinases initiates a variety of checkpoint responses to promote genetic stability [1, 10]. Sensor proteins are thought to directly interact with damaged DNA and serve as points of recruitment for various target proteins; the types of sensors that associate with specific DNA lesions may function to recruit particular proteins for a lesion-specific response [1]. In mammals, several distinct protein complexes are known to sense and signal different types of DNA damage [3].

Specificity also exists within the pathways that respond to DNA lesions. In yeast and other eukaryotes, preferential repair of certain types of lesions occurs via specific DNA damage repair pathways [11]. In most cases, oxidative damage and other base-altering damage is handled by the base excision repair (BER) pathway, while bulky lesions are mainly handled by the nucleotide excision repair (NER) pathway [11]. However, both excision repair pathways and the two DNA damage tolerance pathways (translesion synthesis and homologous recombination) have overlapping specificities,

suggesting a compensatory response by the alternate pathways when one type of repair is absent [12, 13].

The repair capacity of a cell can be overwhelmed by chronic exposure to DNA damage (by exogenous agents or due to a defect in one or more repair pathways). When repair cannot overcome the amount of DNA damage present, cells employ two tolerance pathways (TLS and REC) to handle DNA damage; However, TLS is error-prone and may contribute to mutagenesis [2]. In extreme cases, repair deficiencies can be catastrophic for cells. Chronic, un-repaired oxidative DNA damage due to a lack of BER or both BER and NER can lead to chromosomal instability [14]. In addition, repeated transcriptional blockage by DNA lesions can activate p53-dependent apoptosis [15].

DNA damage is not restricted to any one phase of the cell cycle, but DNA is particularly susceptible to damage during S phase when it is unwound for replication, generating structures that are sensitive to exogenous and endogenous insults [16]. Failure to repair DNA damage before S phase can result in replication fork collapse and resulting chromosomal rearrangements, DNA double strand breaks, and cell death [17, 18]. The checkpoint responses that safeguard the passage of cells through S-phase are therefore particularly important and must serve many functions to protect genome integrity. These tasks include the coordination of replication origin firing, stabilization of replication forks and the continuation of any stalled forks, the transcriptional induction of DNA damage response genes, the regulation of dNTP pools, and the inhibition of mitosis [19-23].

The major regulators of the intra-S phase checkpoint response in yeast are two protein kinases, Mec1 and Rad53, whose human homologues are ATM/ATR and Chk2,

respectively [24]. Mec1 is a sensor protein that plays a role in all checkpoint responses (reviewed in [25]). It can respond to various types of damage, including double strand breaks, base damage, UV-induced damage, and replication stress to initiate a global checkpoint response [16, 26]. Rad53 is an effector protein that functions downstream of Mec1 to activate checkpoint activities that are S-phase specific [16]. During S-phase, these kinases act in concert to slow progression of the cell cycle when DNA is damaged (e.g. by methyl-methanesulfonate) or when replication stress occurs (either by endogenous factors or by an agent such as hydroxyurea) [1, 16, 20, 27, 28]. When DNA damage and/or replication stress occur, both proteins are necessary to prevent the firing of replication origins, to delay the onset of mitosis, and to stabilize replication forks to avoid catastrophic fork collapse [1, 16].

A critical function of Mec1 and Rad53 is the regulation of dNTP pools that is necessary for replication during S phase and for the repair of DNA damage [29-31]. Downstream signals originating from Mec1 and Rad53 influence dNTP pools through the regulation of ribonucleotide reductase (RNR), the four-subunit enzyme that catalyzes the rate-limiting step in the conversion of NTPs to dNTPs [32, 33]. The proper concentration and relative amounts of the four types of dNTPs are tightly controlled for high fidelity replication of the entire genome as well as DNA damage repair [34]. Mec1 and Rad53 regulate dNTP pools in several ways. First, they activate the protein kinase Dun1, which is responsible for the transcriptional regulation of the four *RNR* genes (*RNR1-4*), all of which are DNA damage inducible in a partly Dun1-dependent manner [35-37]. Rad53 is also responsible for de-repression of the RNR genes via Crt1 [19]. Both Mec1 and Rad53

are also necessary for the removal of the Rnr1 inhibitor, Sml1, both during growth and after DNA damage [31]. The multiple levels of regulation of RNR by these checkpoint proteins show the importance of proper dNTP regulation in cell growth and DNA repair. The Mec1-dependent signal transduction cascade leading to RNR regulation is depicted in Figure 1.

Many of the downstream effects of checkpoint proteins have been characterized extensively in the literature. However, much less is known about the mechanisms behind checkpoint activation. It is still uncertain whether direct recognition of DNA damage (e.g. by sensor proteins) is sufficient to initiate checkpoint responses or if the processing of DNA lesions leads to structures or signals that produce a downstream checkpoint response [38]. For example, proteins that are part of the replication fork machinery play a role in checkpoint activation, including DNA helicases Sgs1 and Top3, the DNA polymerase subunit Pol 2, Dpb11, and Drc1 [39-43]. Their proximity to the damage suggests a direct method for sensing damage in situ. In addition, the complex of Mec1 and its cofactor Ddc2 is known to associate with damaged DNA in the absence of other checkpoint proteins and initiate a Mec1-dependent phosphorylation of Ddc2, suggesting that Mec1 directly responds to damage and likely plays a pivotal role in the recruitment of other checkpoint proteins [44-48].

However, there is evidence that processing of DNA damage may be required for the Mec1-Ddc2 complex to recognize it. For example, studies suggest that checkpoint proteins can associate with DNA repair proteins to sense DNA damage [38]. In addition, the Mec1-Ddc2 complex is thought to recognize and associate with stretches of single-

strand DNA (ssDNA) that are coated with replication protein A (RPA) [49]. Since ssDNA is produced during many processes, including DNA replication, repair, recombination, and degradation of DSBs, it is a likely candidate for a structure that can signal multiple types of damage [49-51]. Although the presence of RPA-coated ssDNA is a strong recruiter of the Mec1-Ddc2 complex to areas of DNA damage, it is not the only stimulus to do so, nor is it sufficient to elicit the classic Mec1-Rad53 phosphorylation cascade of the intra-S phase checkpoint [49]. Thus, there are alternate checkpoint stimuli and potential regulators that must be elucidated to gain a fuller understanding of the mechanisms of checkpoint activation.

One candidate that has emerged as a checkpoint activator is reactive oxygen species (ROS) [52]. This class of molecules includes derivatives of molecular oxygen such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet -}$), and the hydroxyl radical ($HO^{\bullet}$). ROS can be beneficial or deleterious to a cell depending on their concentration and cellular localization. At low levels, ROS function in signaling pathways that help maintain cellular redox balance, with H_2O_2 being a key messenger [53-56]. However, their reactive nature makes high ROS concentrations dangerous for cells because ROS can react with and damage DNA, RNA, proteins, and lipids. The study of ROS production and subsequent cellular signaling is a relatively new and expanding field, and there is still much to learn about ROS metabolism and cellular oxidative stress responses. This research has clear implications for human health, as numerous deleterious consequences have been attributed to ROS-mediated responses, including cancer, neurodegenerative diseases, cardiac dysfunction, and the process of aging [57-60].

ROS can be produced by endogenous sources such as the electron transport chain in mitochondria, some cytosolic enzymes (e.g. NADPH oxidases), and peroxisomal metabolism, or from exogenous sources like ionizing radiation and various chemical agents [56, 61, 62]. In addition to this knowledge, recent studies have shown that DNA damage is implicated in ROS production in yeast. Elevated levels of DNA damage from both endogenous and exogenous sources (i.e. by DNA repair deficiencies or the DNA alkylating agent MMS) have been shown to increase intracellular ROS [62, 63]. The role of ROS as a DNA damage signal is a current topic of research that could bring clarity to current models of DNA damage responses in yeast, including those involving the DNA damage checkpoints.

Yeast have developed a highly complex response system to deal with increased levels of ROS and oxidative stress, which is often defined as the imbalance of prooxidants and antioxidants in the cell [64]. Oxidative stress leads to activation of DNA repair pathways and alters transcriptional regulation of target genes involved in ROS scavenging and processing [65, 66]. One transcription factor that plays a major role in this transcriptional response in yeast is Yap1, a member of the *jun* family of transcription factors [67]. Yap1 shares a DNA binding sequence and has functional similarity with its human homolog AP-1 [67]. During oxidative stress, post-translational modification of Yap1 and subsequent nuclear accumulation of Yap1 leads to the regulation of many genes involved in the oxidative stress response [68-72]. These genes code for enzymes (such as catalases, superoxide dismutases, and glutathione peroxidases) and small molecules (such as glutathione and thioredoxins) that contribute to ROS scavenging [73].

Indirect evidence suggests that Yap1 can activate genes involved in DNA damage repair, replication, and checkpoint control, including *MEC1*, *NTG1*, *POL1*, *MAG1*, and *POL3* [74, 75].

A recent study shows evidence that DNA damage-induced ROS mediate signaling processes that activate the transcription factor Yap1; Yap1 acts as a DNA damage responder that is important for cell survival and maintaining genomic stability (Rowe, 2010 submitted data). A model for Yap1 in this role is shown in Figure 2. The ability of DNA damage-induced ROS to activate Yap1 combined with the evidence of a regulatory association between Yap1 and *MEC1* suggests a mechanism of DNA damage checkpoint regulation via Yap1. It has not been investigated whether DNA damage-induced ROS can activate the intra-S phase checkpoint.

The purpose of this project is to investigate whether Yap1 is involved in the intra-S phase checkpoint response. To do this, we examine several aspects of checkpoint control in DNA repair proficient and DNA repair deficient *Saccharomyces cerevisiae* strains that lack Yap1. Checkpoint function is assessed by measuring *MEC1* transcript induction, Mec1 protein production, and checkpoint activation in response to two agents that are known to activate the intra-S phase checkpoint: methyl-methanesulfonate (MMS) and hydroxyurea (HU) [76, 77]. It is hypothesized that yeast lacking Yap1 will have a diminished checkpoint response after treatment with these agents, and that this effect will be exaggerated by compromising base excision repair. A model depicting the hypothesized role for Yap1 in checkpoint regulation is shown in Figure 3.

Materials and Methods

Strains, Media, and Growth conditions

Four haploid *Saccharomyces cerevisiae* strains were used in this study. All are derivatives of hDNP42. For a list of strains and relevant genotypes, see Table 1.

All strains were grown in rich media (YPD – 1% yeast extract, 2% peptone, 2% dextrose, and 2% agar for plates). YPD was supplemented with 0.005% adenine sulfate.

Cell Growth and viability

Frozen stock strains were plated on YPD and grown at 30°C for ~48 hours. Liquid YPD media (5 mL) was inoculated with yeast cells and grown overnight to saturation (>7x10^7 cells/mL) at 30°C. Fresh YPD media was inoculated with overnight culture and grown to a density of ~ .4 OD600 measured by a spectrophotometer (UV-Visible Spectrophotometer, Shimadzu). Cells were treated with DNA damaging/replication blocking agents (see below) and survival was determined by diluting cultures and counting colonies grown for two days on rich media.

Exposure to DNA damaging/replication blocking agents

Methyl-methanesulfonate (MMS, Sigma) was added directly to cultures for a final concentration of 3mM. Hydroxyurea (HU, US Biosystems) was added to cultures for a final concentration of 300mM. Cultures were incubated with the agents for 2 hours at 30°C.

Whole Cell Extracts

Following exposure to either MMS or HU, all cells were washed twice with dH_2O. Cell pellets were frozen at -80°C overnight. The pellets were thawed over ice, and cells were physically lysed using acid washed glass beads in a buffer containing protease inhibitors (Complete Mini, Roche). Cell walls and other debris were removed by centrifugation at 3000 rpm for 5 minutes. Whole cell extracts were either used immediately for western analysis or stored at -80°C.

Western Blots

Western blots were performed as described previously (Towbin and Gordon, 1984, Towbin et al. 1979). An anti-Rnr4p antibody (1:2500 dilution, Santa Cruz Biosystems) and two anti-Mec1p antibodies (1:200 dilution, Santa Cruz Biosystems) were used for western blots on whole cell extracts (WCEs). The densities of the Rnr4p- and Mec1p-associated bands measured on a fluorometer (Fluor-S MultiImager, BioRad) were analyzed using Quantity One software. Because the anti-Rnr4 antibody also recognizes α tubulin, α tubulin was used as a loading control.

Results

This purpose of this project is to investigate if Yap1 is involved in the intra-S phase checkpoint response. To address this question, we propose three methods to assess Yap1 involvement in checkpoint function. First, we investigate the activation of the intra-S phase checkpoint in yeast lacking Yap1 by measuring the amount of a specific protein whose induction is dependent on checkpoint activation. Second, we measure the production of a key checkpoint protein, Mec1, in cells lacking Yap1. Third, we examine whether Yap1 transcriptionally regulates the genes encoding two critical checkpoint proteins, Mec1 and Rad53, by measuring levels of *MEC1* and *RAD53* RNA transcripts in cells lacking Yap1. To investigate the effect of DNA repair defects on checkpoint activity and potential mediation of this activity by Yap1, we utilize a strain deficient in base excision repair and a mutant that is deficient in base excision repair that also lacks Yap1.

Absence of Yap1 decreases checkpoint activation

To measure the degree of checkpoint activation, we quantify the increase in the protein Rnr4 via western analysis when the intra-S phase checkpoint is triggered. As discussed earlier, the four *RNR* genes are targets of the Mec1/Rad53 cascade that are induced upon activation of this checkpoint. To provoke a checkpoint response, cells were exposed to methyl-methanesulfonate (MMS) or hydroxyurea (HU). MMS produces both DNA damage and a block to replication. MMS can alkylate the DNA bases and cause mispairing during DNA replication as well as the stalling of replication forks [78]. Hydroxyurea is a direct inhibitor of the ribonucleotide reductase subunit Rnr1, leading to

decreased dNTP pools and the stalling of replication forks [1, 79, 80]. Thus, MMS and HU are thought to activate the intra-S phase checkpoint by similar mechanisms [1]. Specifically, the cellular responses to MMS and HU are dependent on the Mec1/Rad53 pathway discussed earlier [20, 21, 23, 77, 81, 82]. Exposure conditions to these agents are described in Materials and Methods. Notably, cells were exposed to agents for 2 hours to allow time for all cells in the unsynchronized culture to reach S-phase.

The degree of checkpoint activation is determined by the fold difference in induction of Rnr4 between exposed and non-exposed conditions for each strain. In wild type cells, treatment with MMS or HU increases Rnr4 by 16 or 35 fold, respectively, over the amount present when cells are not exposed to either agent (Figure 4). This increase in Rnr4 indicates that checkpoint activation occurs and is in agreement with previous studies that MMS and HU cause the activation of the intra-S phase checkpoint.

The basal level of Rnr4 in the *yap1Δ* strain is slightly elevated, with ~2.6 times the basal level observed in wild type cells. Treatment with MMS or HU induced Rnr4 in a partially Yap1-dependent manner. In response to MMS, the wild type strain exhibits a ~16 fold increase in Rnr4 over the level found in untreated wild type cells, while the *yap1Δ* strain shows a ~5.7 fold increase in Rnr4 over the level found in *yap1Δ* untreated cells. However, the absolute level of Rnr4 after treatment with MMS is similar in both strains (approximately 15±3 fold higher than the level present in untreated wild type cells – see Figure 4). In response to HU, the wild type strain exhibits a ~35 fold increase in Rnr4 over the level found in untreated wild type cells, while the *yap1Δ* strain shows only a ~10 fold increase Rnr4 over the level found in *yap1Δ* untreated cells (Figure 4 and

Table 2). Again, the absolute amount of Rnr4 that is produced following HU treatment is similar in both strains. The wild type strain exhibits a ~35±12 fold higher level of Rnr4 compared to untreated wild type cells while the *yap1Δ* strain exhibits a ~26±5 fold higher level of Rnr4 compared to untreated wild type cells (Figure 4). Because we define the degree of checkpoint activation to be the fold increase in Rnr4 between exposed and non-exposed conditions for each strain, we conclude that the strain lacking Yap1 has a diminished checkpoint response compared to the wild type strain after treatment with either MMS or HU.

Cytotoxicity of MMS and HU

A moderately toxic dose of MMS (3mM) was used to provoke checkpoint activation without causing severe cytotoxicity. Cytotoxicity of MMS was not altered by the deletion of Yap1. Both wild type and *yap1Δ* strains had ~60-70% survival after exposure to MMS (Figure 5). Exposure to a moderately toxic dose of HU (300 mM) provoked checkpoint activation with fairly low cytotoxicity. The cytotoxicity of HU was unaffected by the deletion of Yap1. Cells with or without Yap1 had ~70-80% survival after exposure to HU (Figure 6).

Using moderately toxic doses of these agents as opposed to highly toxic doses is important to distinguish the dynamics of checkpoint processes when cells are not under excessive genotoxic stress, resulting in the death of the majority of cells. It is necessary that we measure checkpoint activity in a situation where cells have the capacity to respond to the stress and are not likely to utilize stress tolerance mechanisms that are

atypical of normal cellular responses, such as apoptosis. Because neither MMS nor HU kills the majority of cells, we can be confident that the checkpoint response we measure indicates the cellular response to these agents and not processes caused by cell death.

Checkpoint activation in BER-deficient cells

We also investigated checkpoint activation in cells that lack base excision repair. Because these cells accumulate oxidative DNA damage and have elevated levels of intracellular ROS [83], we are interested to see how checkpoint activation is affected by these potential checkpoint stressors. Additionally, we want to determine if Yap1 mediates the checkpoint response in BER-deficient cells. Western analysis was employed to analyze Rnr4 induction in a BER-deficient strain and a BER-deficient *yap1Δ* strain.

Based on preliminary data, the BER-deficient strain and BER-deficient *yap1Δ* strain induce Rnr4 upon treatment with MMS or HU. However, they have lower checkpoint activation in response to MMS and HU compared to the wild type strain. Furthermore, this diminished response does not appear to be affected by the absence of Yap1. Initial results show that in response to MMS, Rnr4 is induced to a level ~7 times and ~4 times higher than its basal levels in the BER-deficient and BER-deficient *yap1Δ* strains, respectively, compared to a ~17 fold induction in the wild type strain over its basal level (Figure 7 and Table 2). The absolute level of Rnr4 produced in response to MMS is somewhat decreased in both the BER-deficient and BER-deficient *yap1Δ* strains compared to the wild type strain. The BER-deficient strain exhibits an ~11 fold higher

level of Rnr4 compared to the untreated wild type cells, while the BER-deficient *yap1Δ* strain exhibits a ~13 fold higher level of Rnr4 compared to untreated wild type cells (Figure 7). In response to HU, Rnr4 is induced to a level ~7.5 and ~5 times higher than basal levels in the BER-deficient and BER-deficient *yap1Δ* strains, respectively, compared to a ~21 fold induction in the wild type strain (Figure 7 and Table 2). Again, the absolute level of Rnr4 is somewhat decreased in both the BER-deficient and BER-deficient *yap1Δ* strains compared to the wild type strain. The BER-deficient strain exhibits a ~12 fold higher level of Rnr4 compared to the untreated wild type cells, while the BER-deficient *yap1Δ* strain exhibits a ~15 fold higher level of Rnr4 compared to untreated wild type cells (Figure 7). Therefore, the initial results suggest that compromising base excision repair negatively impacts Rnr4 production (and therefore checkpoint activation), but that removal of Yap1 from a BER-deficient strain does not cause an additional effect. The removal of BER, Yap1, or both results in a 3-4 fold decrease in the amount of Rnr4 induced in response to MMS and HU compared to wild type cells.

In the BER-deficient and the BER-deficient *yap1Δ* strains, 3 mM MMS exposure resulted in ~10-15% survival (Figure 5). Cells lacking BER are sensitized to the cytotoxic effects of MMS, and removal of Yap1 had no additional effect on cell survival. At shorter MMS exposure times, Yap1 has been shown to protect BER-deficient cells from cytotoxic effects of MMS. A 30-minute exposure to 5 mM MMS results in 75% survival in the BER-deficient strain and 25% survival in a BER-deficient *yap1Δ* strain (Rowe, 2010 submitted data). In this study, exposure to HU resulted in ~70-80%

survival in both the BER-deficient and BER-deficient *yap1Δ* strains. Cells lacking Yap1 are not sensitized to HU under these exposure conditions (Figure 6).

Putative Yap Response Elements (YREs) are present in the promoters of checkpoint genes

This study investigates whether Yap1 regulates genes involved in checkpoint control. There is indirect evidence that there are regulatory associations between the Yap1 transcription factor and the *MEC1* gene [74, 75, 84]. However, there is not an exact match between the reported YREs and any sequence in the *MEC1* promoter. A one-nucleotide substitution in the YRE sequence yields many potential binding sequences for Yap1 in the *MEC1* promoter, although none are confirmed in the literature. Several other genes involved in the intra-S phase checkpoint response, including *RAD53*, *RNR2*, and *RNR3* contain a YRE in their respective promoter regions. Using YEASTRACT tools, the "classical" YRE (TKASTAA) was compared against the promoters of these genes to find the Yap1-DNA binding sequences. A summary of the matches is shown in Table 3.

Measuring Mec1p dynamics under genotoxic/replication stress

To further investigate the possibility of the transcriptional regulation of checkpoint genes by Yap1, we measured the induction of the protein kinase Mec1 in cells lacking Yap1 that are subject to genotoxic/replication stress by MMS or HU. Multiple attempts were made to analyze Mec1 production via western analysis, but these

experiments were not successful. There are two reasons that could account for this: 1) The large size of the Mec1 protein (240 kDa) is not conducive for western analysis. The size of the protein could impact gel resolution and decrease the efficiency of protein transfer from a gel. 2) The antibodies to Mec1 have a low efficiency of binding the protein. Antibodies raised against a small epitope of the protein may not properly recognize the full-length protein. There are only 2 anti-Mec1 antibodies commercially available, and they have not been employed in any published in vivo studies. A western blot employing one of these antibodies is shown in Figure 8.

Discussion

Yap1 contributes to the activation of the intra-S phase checkpoint in repair-proficient cells

If Yap1 is partly responsible for the transcriptional regulation of genes involved in checkpoint activation, it is reasonable to predict that a disruption in this regulatory association will impact downstream checkpoint responses. Our results show that the absence of the Yap1 transcription factor negatively impacts checkpoint activation in response to MMS and HU. Cells may respond similarly to these agents because they are thought to activate the checkpoint in the same way. The diminished production of Rnr4 in the *yap1Δ* strain following genotoxic/replication stress suggests a role for Yap1 in the intra-S phase checkpoint.

The mechanism of Yap1 activation in response to MMS and HU is not fully characterized. Hydrogen peroxide, a reactive oxygen species, is a well-known activator of

Yap1. It is possible that Yap1 is activated via DNA damage-induced ROS to promote various downstream effects, including the regulation of genes involved in the DNA damage checkpoints. Because both MMS and HU have been shown to increase intracellular ROS, this may be one mechanism by which Yap1 is activated. It will be important to measure any changes in intracellular ROS in each strain under the specific exposure conditions used in this study to predict the likelihood or degree of Yap1 activation in these circumstances. This could be important because of the nature of Yap1 activation; for example, the dynamics of Yap1 nuclear localization vary depending on both the dose and the exposure length to MMS (Rowe, et al. 2010 submitted data).

BER-deficient cells are sensitive to MMS-induced damage

The increased cytotoxicity of MMS in the BER-deficient strains is expected because the BER pathway is the primary pathway that repairs oxidative damage and small, non-bulky base damage [11]. MMS causes both alkylation base damage and an increase in ROS that can damage DNA. Because HU does not produce these kinds of DNA lesions, no increased cytotoxic effect is seen in BER-deficient strains compared to the repair-proficient ones. The loss of Yap1 did not further sensitize the BER-deficient cells to MMS, although this effect has been observed for shorter exposure times, suggesting that Yap1 is a DNA damage responder that is specific for damage repaired by the BER pathway. Although survival data for BER-deficient strains was only collected for one experiment, it suggests that the role of Yap1 as a DNA damage responder may change depending on the length of exposure to alkylating DNA damage or the replication status/growth environment of the cell.

Checkpoint activation in BER-deficient cells

Although there is only preliminary data on checkpoint activation in these strains, the emerging trends could support certain hypotheses regarding the sensing of DNA damage by the checkpoints. If these initial results can be reproduced, they suggest that BER proteins contribute to the activation of the intra-S phase checkpoint. This is in opposition to my original hypothesis that BER-deficient strains would not only have increased checkpoint activation in the absence of endogenous stress, but that the removal of Yap1 would further exaggerate the diminished checkpoint response seen in repair-proficient strains. This hypothesis is based on the observations that BER-deficient cells accumulate oxidative damage and have increased levels of intracellular ROS [63, 83], which are two potential stimulators of the intra-S phase checkpoint. If Yap1 responds to damage normally repaired by the BER pathway and if Yap1 contributes to checkpoint activation via DNA damage-induced ROS, then one could expect that the deletion of Yap1 in the absence of BER would lead to a discernible decrease in checkpoint activation. Additional experiments are needed to determine if this is the case.

Another surprising observation from these studies is that genotoxic/replicative stress by MMS or HU has less of an impact on checkpoint activation in BER-deficient strains. This is demonstrated by a decrease in the induction of Rnr4 after treatment with these agents compared the wild type strain. Again, these data are only preliminary, but repetition of these experiments could show that BER proteins play a role in the activation of the intra-S phase checkpoint in response to these agents. As mentioned previously,

there is evidence that checkpoint proteins may interact with DNA repair proteins to initiate a checkpoint response. This kind of interaction could explain why the absence of BER proteins negatively impacts checkpoint activation and why the removal of Yap1 from BER-deficient strains would have no further effect.

The complex nature of DNA damage checkpoints

It is evident from this study and several others that the mechanisms of damage sensing and checkpoint control are highly complex. Although Yap1 (and possibly BER proteins) seems to contribute to the activation of the intra-S phase checkpoint, there are still other players in this response. The absence of Yap1, BER proteins, or both does not completely inhibit checkpoint activation in response to MMS or HU. Because the DNA damage checkpoints are so vital, it is reasonable that evolution would promote redundancy in the system to overcome a loss of function in particular DNA damage sensors or DNA damage responders. A subtle change in checkpoint response caused by the deletion of Yap1 may therefore be difficult to detect by western analysis alone. Other methods must be employed to further characterize Yap1's involvement.

Limitations of quantitative analysis

As mentioned above, subtle changes in checkpoint response caused by the deletion of Yap1 may be difficult to detect. Our results indicate that the magnitude of the diminished induction of Rnr4 in the *yap1Δ* strain compared to the wild type strain is on the order of ~3 fold. Because the sensitivity of the assay for detecting this level of

change is poor, the results to date cannot support a definitive conclusion about Yap1 involvement in checkpoint activation. It is also not clear what impact this reduction in Rnr4 will have on the proficiency of the checkpoint response, i.e. whether the decrease in Rnr4 will lead to reduced RNR activity or a diminished repair capability due to lower dNTP pools. However, these results are encouraging in that they provide the groundwork for further testing the effect of Yap1 on other components of the intra-S phase checkpoint. These components include the transcriptional regulation of genes such as *MEC1* and *RAD53* and the dynamics of checkpoint protein production in the event of genotoxic/replication stress.

Proposed future experiments

There are several experiments that will be helpful in further characterizing Yap1 involvement in the intra-S phase checkpoint. Several of these involve the continuation of experiments presented in this thesis. The western blot analysis of Rnr4 induction in BER-deficient strains needs to be repeated to confirm the results thus far. Also, the induction of checkpoint proteins (Mec1p, Rad53p) should be measured in all strains following genotoxic/replication stress. Measurement of Mec1p induction was attempted, but there were no usable results due to antibody quality issues. This analysis should be continued and should also include Rad53p. Measuring both the quantity and the phosphorylation status of this protein under genotoxic/replication stress could shed light on Yap1 involvement in the progression of the canonical Mec1-Rad53 cascade.

Another proposed experiment is to employ quantitative real time PCR to measure the amount of RNA transcript produced from several checkpoint-related genes in cells with or without Yap1 that are subject to genotoxic/replication stress. This analysis should include *MEC1, RAD53,* and *RNR1-4.* Yap1 has previously been shown to down-regulate the amount Rnr3 and Rnr4 transcripts in natural conditions and in the presence of H_2O_2 (a well-documented Yap1 activator) via transcriptome profiling [85]. We will address transcriptional regulation of these and other genes by Yap1 following exposure to MMS and HU, which activate Yap1 through mechanisms that are yet to be elucidated.

As mentioned previously, measurements of ROS should be taken after treatment of cells with MMS and HU. Treatment with MMS has been shown to increase intracellular ROS in a dose dependent manner and to increase Yap1 nuclear accumulation in a dose- and time-dependent manner [83], so the particular level of ROS caused by the specific treatment conditions described in this manuscript need to be established. There is also little data on how treatment with HU affects intracellular ROS. Treatment with HU has been shown to cause an increase in ROS in wild type cells (N. Degtyareva. unpublished data) and to cause Yap1 nuclear accumulation [86], but it is still unknown how these dynamics are affected by genotoxic agent exposure time or by the elimination of BER.

Figures

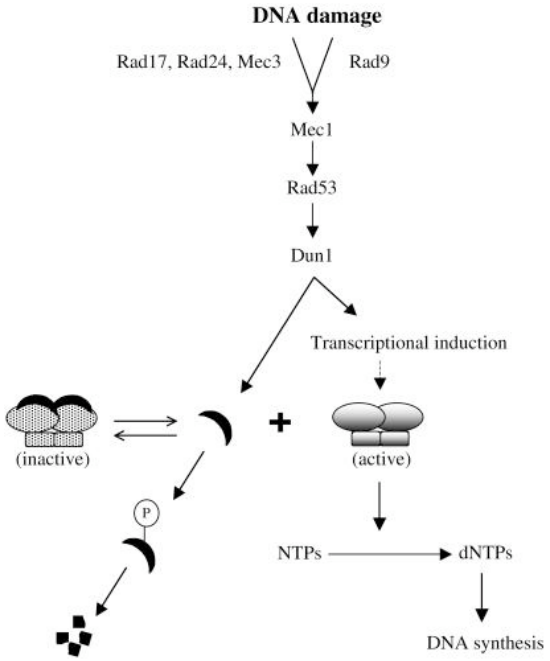


Figure 1

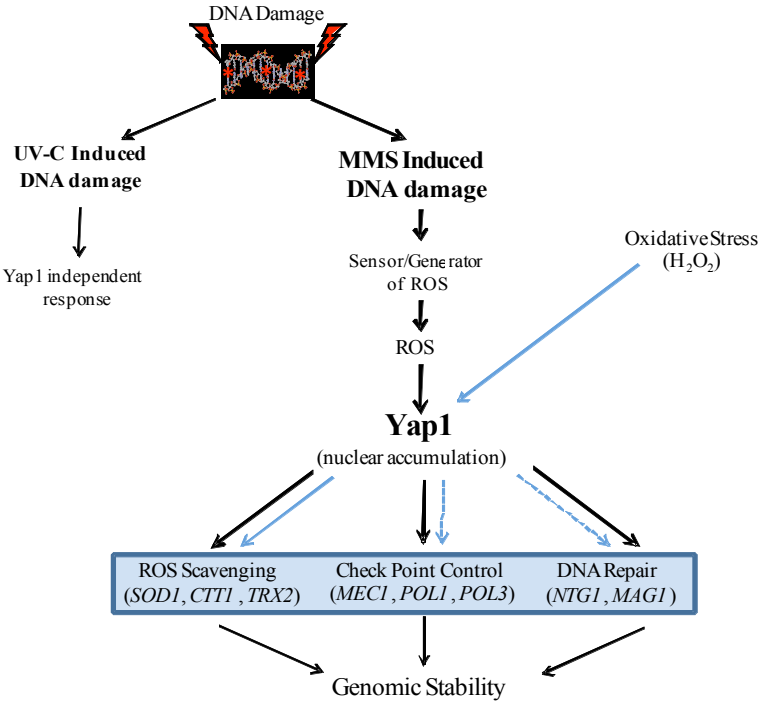


Figure 2

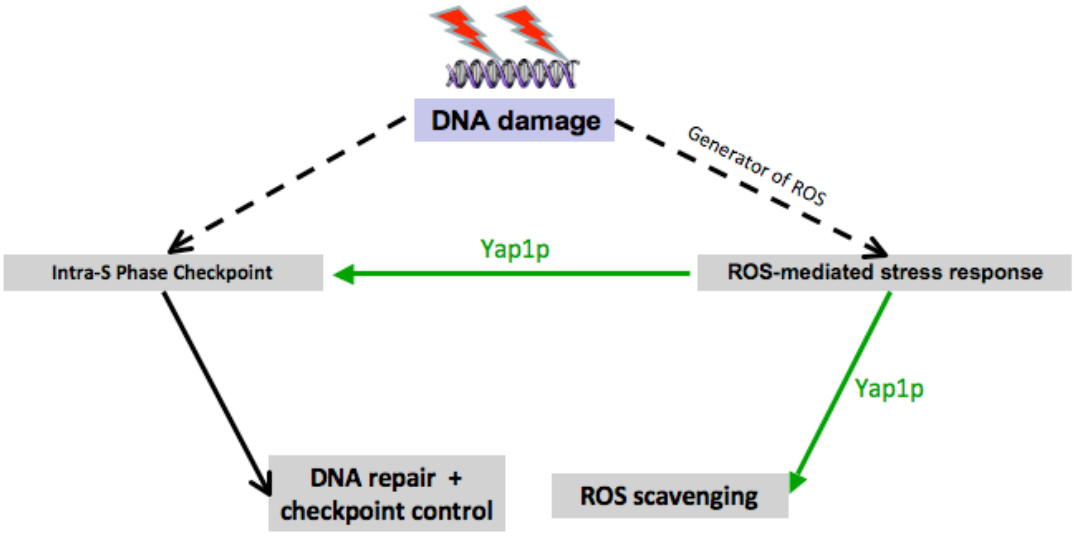
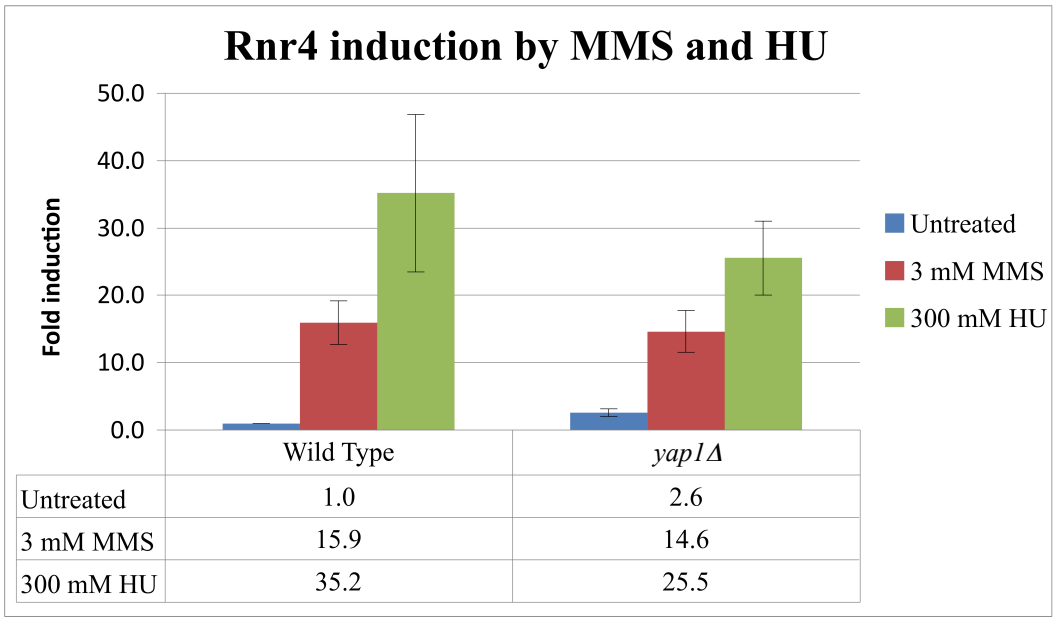


Figure 3



	Wild Type	*yap1Δ*
Untreated	1.0	2.6
3 mM MMS	15.9	14.6
300 mM HU	35.2	25.5

Figure 4

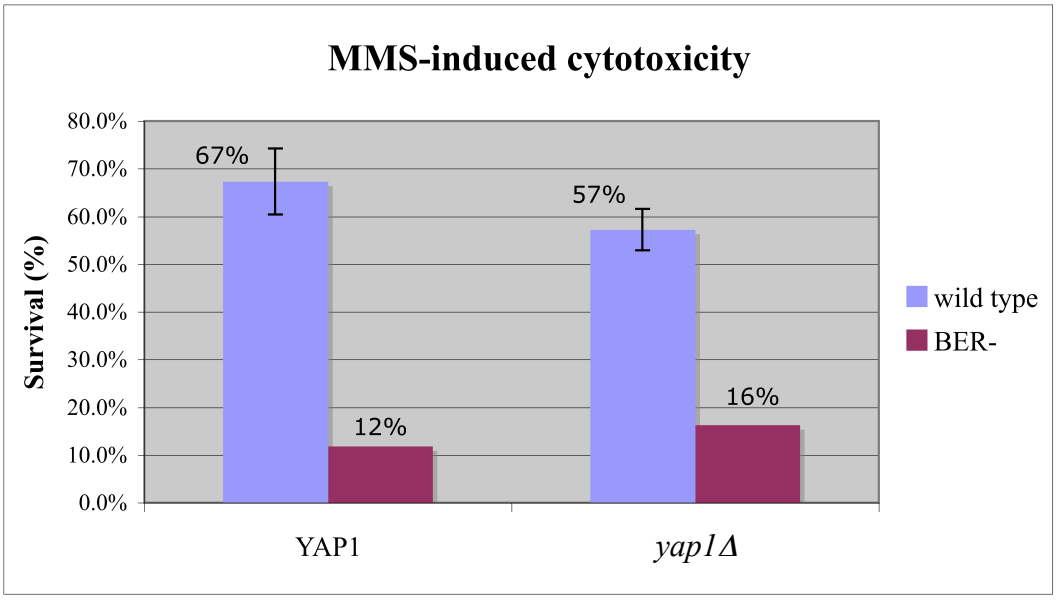


Figure 5

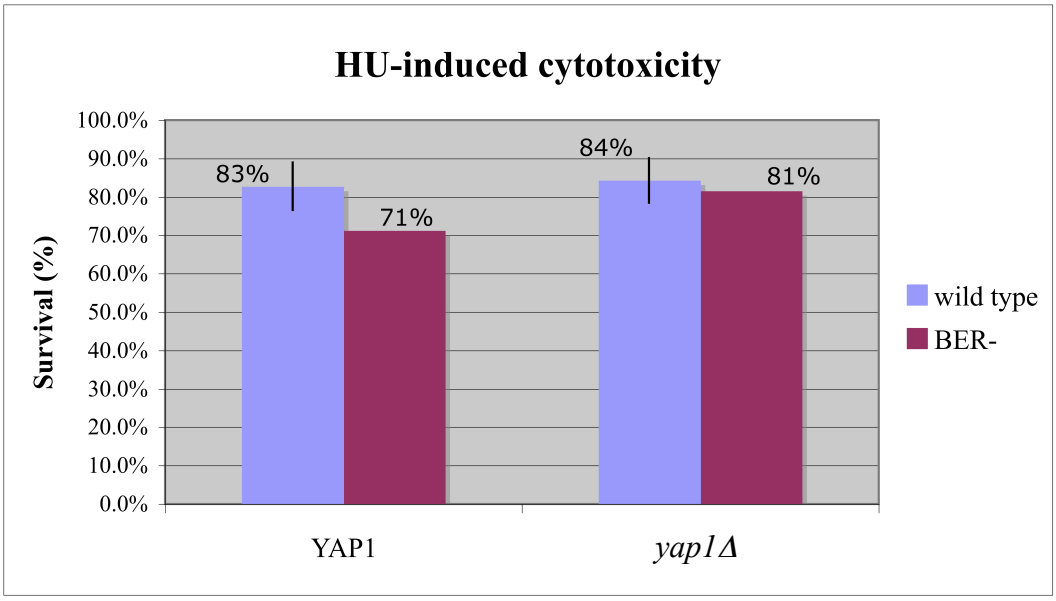
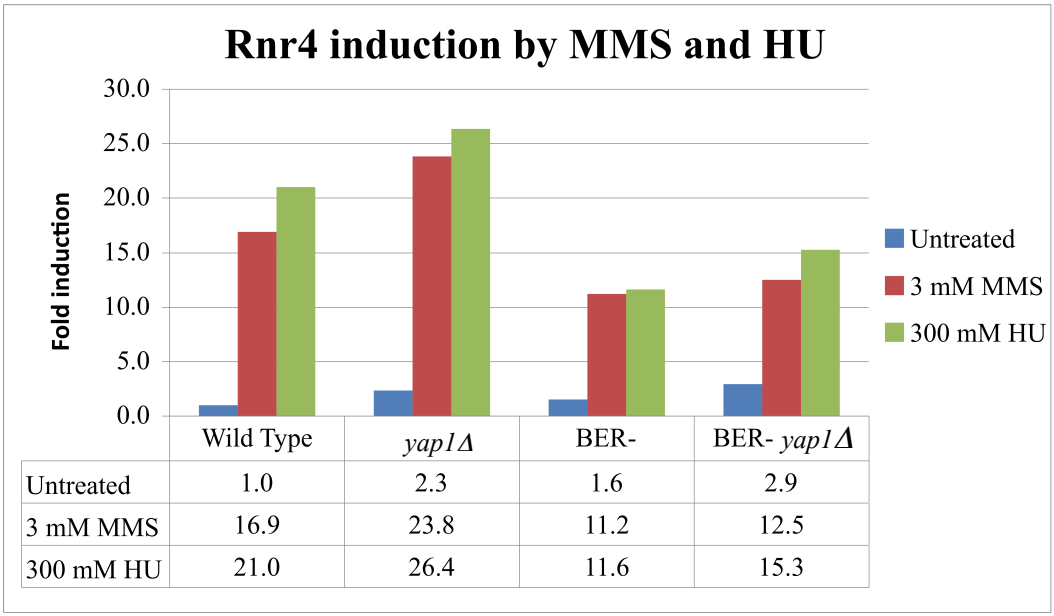


Figure 6



	Wild Type	*yap1Δ*	BER-	BER- *yap1Δ*
Untreated	1.0	2.3	1.6	2.9
3 mM MMS	16.9	23.8	11.2	12.5
300 mM HU	21.0	26.4	11.6	15.3

Figure 7

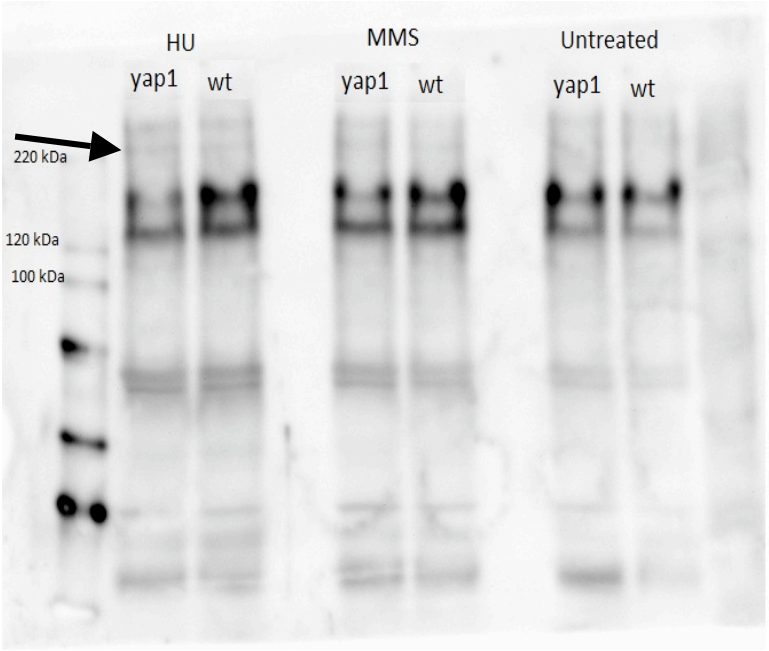


Figure 8

Tables

Table 1. Strain Genotypes

Strains	Relevant genotype	Source
hDNP42	*Mat α/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*	Rowe, 2010 (submitted)
LAR025 (wild type)	*MAT αhis7 -1 lys2 Δ5'::LEU-lys2Δ3' ade 5-1 trp1-289 ura 3-52*	Rowe, 2010 (submitted)
LAR026 (*yap1Δ*)	*MAT αhis7 -1 lys2 Δ5'::LEU-lys2Δ3' ade 5-1 trp1-289 ura 3-52 yap1::natNT*	Rowe, 2010 (submitted)
LAR029 (BER-)	*MAT αhis7 -1 lys2 Δ5'::LEU-lys2Δ3' ade 5-1 trp1-289 ura 3-52 ntg1::hphMX4 ntg2::BSD apn1::TRP1*	Rowe, 2010 (submitted)
LAR030 (BER-*yap1Δ*)	*MAT αhis7 -1 lys2 Δ5'::LEU-lys2Δ3' ade 5-1 trp1-289 ura 3-52 ntg1::hphMX4 ntg2::BSD apn1::TRP1 yap1::natNT*	Rowe, 2010 (submitted)

Table 2. Fold increase in Rnr4 after treatment with MMS or HU compared to untreated levels

Strain	Fold increase in Rnr4 after treatment	
	3 mM MMS	300 mM HU
Wild Type	15.9	35.2
yap1Δ	5.7	9.9
BER-	7.2	7.5
BER- *yap1Δ*	4.3	5.2

Table 3. Yap Response Elements in the promoters of genes involved in the intra-S phase checkpoint

Gene	YRE sequence	Strand	Position
RAD53	TGACAAA	non-coding	-348
	TTACTCA	coding	-692
RNR2	TGACAAA	coding	-312
RNR3	TTACAAA	coding	-864

Figure and Table Legends

Figure 1. **Model of the role of Mec1, Rad53, and Dun1 in the regulation of ribonucleotide reductase**. DNA lesions activate the cascade and lead to phosphorylation of Dun1 and subsequent transcriptional activation of the RNR genes and degradation of the RNR inhibitor Sml1. Upon degradation, RNR is active and can convert NTPs into dNTPs that are necessary for DNA synthesis (Figure from [87]).

Figure 2. **Model of the role of Yap1 in the DNA damage response.** Upon induction of DNA damage by UV-C there is an increase in intracellular ROS levels that activates a Yap1 independent cellular response (left side). Upon induction of DNA damage by MMS (center) there is also an increase in ROS levels via unidentified sensor of DNA damage/Generator of ROS. The increase in ROS following exposure to MMS leads the nuclear accumulation of Yap1 and therefore Yap1 activation. Upon activation Yap1 functions as a transcription factor activating a number of genes involved in ROS scavenging, DNA damage checkpoint control, and DNA repair. The upregulation of these helps maintain genomic stability. The activation of Yap1 following oxidative stress (H_2O_2) (right side) is well documented and known to upregulate genes involved in ROS scavenging, DNA damage check point control, and DNA repair. It also leads to an increase in genomic stability and redox homeostasis.

Text and figure from Rowe, 2010 (submitted)

Figure 3. **Model of the role of Yap1 in the oxidative stress response and intra-S phase checkpoint activation**. Yap1 is a key mediator of the oxidative stress response in yeast. Yap1 is activated by oxidative stress resulting from increased levels of ROS. These ROS are produced in response to DNA damage. Yap1 activation leads to regulation of genes involved in ROS scavenging (right side). A second role for Yap1 is investigated in this study. Yap1 activation by ROS could impact activity of the intra-S

phase checkpoint, resulting in DNA repair and other aspects of checkpoint control (left side).

Figure 4. **Rnr4 induction by MMS and HU in strains with or without Yap1**. Fold induction of Rnr4 in each strain in response to MMS and HU measured by western analysis (see Materials and Methods). Numbers are normalized to the amount of Rnr4 in the wild type strain with no treatment. Data points represent an average of at least 5 independent experiments. Error bars represent standard error.

Figure 5. **MMS-induced cytotoxicity.** A 2 hour exposure to 3 mM MMS in rich media results in ~60-70% survival in repair proficient strains and a ~10-15% survival in BER-deficient strains. Data points for repair proficient strains represent an average of 6 independent experiments. Data points for BER-deficient strains represent data from 1 experiment. Error bars represent standard error. Survival assay protocol is described in Materials and Methods.

Figure 6. **HU-induced cytotoxicity.** A 2 hour exposure to 300 mM HU in rich media results in ~70-80% survival in both repair proficient and BER-deficient strains with or without functional Yap1. Data points for repair proficient strains represent an average of 6 independent experiments. Data points for BER-deficient strains represent data from 1 experiment. Error bars represent standard error. Survival assay protocol is described in Materials and Methods.

Figure 7. **Rnr4 induction by MMS and HU in BER-deficient strains with or without Yap1.** Fold induction of Rnr4 in each strain in response to MMS and HU measured by western analysis (see Materials and Methods). Numbers are normalized to the amount of Rnr4 in the wild type strain with no treatment. Data comes from one experiment.

Figure 8. **Identifying and analyzing Mec1 via western analysis.** An image from attempted western analysis showing an unsuccessful attempt to measure Mec1 protein. Very faint bands appear near the correct apparent molecular weight of the protein (240

kDa), shown by the black arrow. Darker bands have not been identified and may be breakdown products of Mec1 or nonspecific proteins bound by the antibody. Analysis was performed as described in Materials and Methods.

Table 1. **Strain Genotypes**. The relevant genotype of each strain used in this study is listed.

Table 2. **Summary of Rnr4 induction.** The data table shows the fold increase in Rnr4 caused by MMS or HU compared to the untreated condition within each strain. Data for repair proficient strains are an average of at least 5 independent experiments. Data for BER-deficient strains are taken from one experiment.

Table 3. **Yap Response Elements**. All known YREs were compared with the DNA sequence of the promoter regions of several genes involved in the intra-S phase checkpoint. The DNA binding sequences and their positions within the promoters are shown for each gene.

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