Autophagy-Dependent Regulation of the DNA Damage Response Protein
Ribonucleotide Reductase 1

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Abstract

Protein synthesis and degradation are post-transcriptional pathways used by cells to regulate protein levels. We have developed a systems biology approach to identify targets of post-transcriptional regulation and we have employed this system in *S. cerevisiae* to study the DNA damage response. We present evidence that 50 – 75% of the transcripts induced by alkylation damage are regulated post-transcriptionally. Significantly, we demonstrate that two transcriptionally-induced DNA damage response genes, *RNR1* and *RNR4*, fail to show soluble protein level increases after DNA damage. To determine one of the associated mechanisms of post-transcriptional regulation, we tracked ribonucleotide reductase 1 (*Rnr1*) protein levels during the DNA damage response. We show that *RNR1* is actively translated after damage, and that a large fraction of the corresponding Rnr1 protein is packaged into a membrane-bound structure and transported to the vacuole for degradation, with these last two steps dependent on autophagy proteins. We found that inhibition of target of rapamycin (TOR) signaling and subsequent induction of autophagy promoted an increase in targeting of Rnr1 to the vacuole and a decrease in soluble Rnr1 protein levels. Additionally, we demonstrate that defects in autophagy result in an increase in soluble Rnr1 protein levels and a DNA damage phenotype. Our results highlight roles for autophagy and TOR signaling in regulating a specific protein and demonstrate the importance of these pathways in optimizing the DNA damage response.
Introduction

Cellular exposure to genotoxic agents can result in DNA damage and promote mutations and cell death. In response to DNA damage, cells activate genome maintenance pathways associated with the DNA damage response. In *S. cerevisiae*, the DNA damage response is activated by Mec1, Rad53 and Dun1 kinases to mediate cell cycle checkpoint activation, to activate DNA repair and to promote the regulation of ribonucleotide reductase (RNR) activity (1, 2). RNR catalyzes the rate-limiting step in the production of dNTPs. RNR activity is high during S-phase and increases after DNA damage to elevate dNTP levels (3, 4). In *S. cerevisiae*, there are four RNR genes encoding two large subunits (*RNR1* and *RNR3*) and two small subunits (*RNR2* and *RNR4*), with the final RNR complex consisting of a tetramer of two large and two small subunits. In order to promote high RNR activity after DNA damage, the transcription of the RNR subunits *RNR1*, *RNR3*, and *RNR4* is induced in a Mec1-dependent fashion (5). Similarly, after DNA damage, RNR activity is increased by Dun1-dependent phosphorylation of the Rnr1 inhibitor Sml1 (6). RNR activity has proved to be a vital regulator of DNA synthesis and cell cycle progression after DNA damage; RNR regulation by transcription and protein kinase cascades can be considered prototypical for components of the DNA damage response.

After DNA damage, transcription and kinase-based signaling are global themes: the DNA damage response works in tandem with these and a broad range of other cellular processes to optimize DNA repair. Transcriptional
profiling studies of *S. cerevisiae* have demonstrated that alkylation damage induces the transcription of hundreds of genes corresponding to proteins associated with DNA repair, cell cycle checkpoints, amino acid metabolism and protein degradation (7-11). Protein degradation has been observed previously for components of the DNA damage response (12, 13) and, in theory, can work to optimize essential enzyme activity. In addition, protein degradation is used to remove damaged or unfolded proteins. Protein degradation can occur using either ubiquitin-proteasome or vacuolar-mediated pathways. Both proteasome and vacuolar-mediated protein degradation have been implicated in stress responses and components of both pathways are associated with the reaction to and recovery from DNA-damaging agents (14-16). Transcriptional profiling and targeted studies have highlighted the role of the proteasome after cellular exposure to alkylating agents and demonstrated that the proteasome-associated factor Rpn4 is involved in the regulation of the DNA damage response (9). Global phenotypic and sub-cellular localization studies have shown that the vacuolar H⁺-ATPase complex modulates the toxicity of the alkylating agent MMS (17, 18). Phenotypic studies also demonstrated that the vacuole plays an important role in promoting cellular viability after *S. cerevisiae* have been exposed to bleomycin (19).

The ability of the vacuole to modulate the toxicity of damaging agents may rest either in its role in the sequestration of toxic compounds or in its degradation activity. The degradation of cellular constituents in the vacuole requires a
functional set of vacuolar ATPases, degradation enzymes and autophagy proteins. Yeast mutants that are compromised for vacuole function are defective in autophagy and display reduced degradation of proteins and organelles (20). Autophagy is a complex catabolic program that uses the vacuole and lysosome in yeast and humans, respectively. In the process of autophagy, parts of the cytoplasm, long-lived proteins, and intracellular organelles are sequestered into a membrane-bound structure termed the autophagosome. The autophagosome is then targeted to the vacuole for degradation (21). TOR, a phosphatidylinositol kinase regulated by nutritional stress, controls autophagy. TOR signaling is active under nutrient-rich conditions and is dormant under starvation conditions to induce autophagy, thereby promoting the breakdown and recycling of cellular macromolecules (22, 23). In addition to controlling autophagy, TOR regulates cell growth, cell cycle, protein synthesis and nutrient import. In mammals, autophagy is regulated by mTOR and this catabolic process has been implicated in carcinogenesis and cancer progression (24, 25). The human Beclin 1 gene, an ortholog of yeast ATG6, is a critical component of the mammalian autophagy pathway and is classified as a haploinsufficient tumor suppressor (26). Beclin 1 is monoallelically deleted in breast, ovarian and prostate cancers, and beclin-1 knockout mice show increased levels of spontaneous tumors (27, 28). Allelic loss of Beclin 1 leads to genome instability (29), but the mechanism connecting autophagy to genome maintenance is not well understood.
In this study, we postulated that many DNA damage response transcripts are regulated post-transcriptionally. To test our hypothesis, we have used transcriptional profiling and proteomic approaches to identify targets of post-transcriptional regulation following DNA damage. We determined that over 50% of the transcripts induced by MMS were regulated after transcription, with \textit{RNR1} and \textit{RNR4} as specific examples. We have used genetic and pharmacological methods to demonstrate increased \textit{RNR1} transcription, increased Rnr1 protein synthesis, increased Rnr1 targeting to a membrane bound structure and increased Rnr1 degradation after DNA damage. Notably, we also demonstrate that Rnr1 targeting and degradation increased upon TOR inhibition, and in all cases the removal of Rnr1 from the soluble fraction required proteins participating in autophagy. We show that cells defective in autophagy have increased Rnr1 protein levels and they display a DNA damage phenotype under conditions of TOR inhibition. Under nutrient limiting conditions we propose the autophagy dependent degradation of Rnr1 serves to optimize the DNA damage response. Ultimately, the following results highlight new regulatory roles for autophagy and TOR signaling during the DNA damage response and further links nutrient sensing to the control of RNR activity.
Results

Matched systems studies identify targets for post-transcriptional regulation

Previous studies in *S. cerevisiae* have reported that MMS induces hundreds of different transcripts, depending on the exposure conditions (7-11). We hypothesized that many of the MMS-induced transcripts would be post-transcriptionally regulated and we have matched transcriptional profiling and protein level data to identify potential targets. Mild exposure to the DNA damaging agent MMS promotes a delay in cell cycle progression, induces minimal cell death, and promotes global reprogramming of the yeast transcriptome (30-32). We have performed transcriptional profiling studies to identify transcripts regulated after MMS exposure. Similar to results reported in the literature, Affymetrix mRNA analysis indicated that MMS exposure induced the levels of 145 transcripts (2.0-fold, \( p < 0.05 \)) and decreased the levels of 44 transcripts (2.0-fold, \( p < 0.05 \)) after DNA damage (Figure 1A, Supplemental Table 1S). Specifically, MMS exposure increased the levels of the DNA damage response transcripts *RNR1*, *RNR3*, *RNR4*, and *MAG1* (Figure 1B). Similar to previous studies (7-11), we determined that the 145 MMS-induced transcripts corresponded to protein activities over-represented (\( p < 0.05 \)) in the following functional categories: DNA damage response, deoxyribonucleotide metabolism, DNA repair, cell cycle arrest, and protein/peptide degradation (Supplemental Table 2S).

To determine whether an increase in a specific transcript was matched at the protein level, we used global proteomic and targeted protein analyses. We
matched protein expression changes to our 145 MMS-induced transcripts. A global proteomic analysis of soluble proteins was performed with proteins isolated from both MMS-treated and untreated cells using tandem mass spectrometry of trypsinized total proteins. The corresponding peptides were labeled with isobaric tags for relative and absolute quantitation (iTRAQ) reagents. iTRAQ identified peptides corresponded to ~600 proteins (~10% coverage), with 33 proteins up-regulated (2.0-fold) and 13 proteins down-regulated (2.0-fold) in the MMS-treated samples (Supplemental Table 3S). Up-regulated proteins were over-represented in the functional categories of stress response, translation initiation, and oxidative stress (p < 0.05, Supplemental Table 4S). We paired the 145 damage-induced transcripts with total proteins identified by iTRAQ and identified 16 transcripts with corresponding protein data (Supplemental Table 3S). In this limited overlap, only 25% of the MMS-induced transcripts displayed concordant protein level increases, with the other 75% displaying no change or a decrease at the protein level. The identification of protein level changes corresponding to the 129 other MMS-induced transcripts was not feasible at this point as matching peptides were not identified by mass spectrometry. To help fill in the missing information, we used a high-throughput ECL-based technology from MesoScale Discoveries (Gathersburg, MA) and analyzed 29 specific TAP-tagged strains that corresponded to the transcripts with the highest MMS induction (Figure 1A). These strains are specific to individual TAP-tagged proteins and allow for the measurement of endogenous protein levels using a single antibody. We also analyzed Rnr4-TAP as it was transcriptionally induced
by MMS and two other components of the RNR complex were picked for analysis using the ECL-based method. Our targeted analysis of the 30 TAP-tagged proteins found only 50% of the MMS-induced transcripts to be concordant with protein level increases. This finding further demonstrates that a large number of MMS-induced transcripts or corresponding proteins were regulated post-transcriptionally, translationally or by protein degradation. We note that a schematic detailing these transcript and protein based methodologies and results is provided in Supplemental Figure S1A.

In evaluating the DNA damage response, we have determined that two transcriptionally induced components fail to show a corresponding protein level increase after DNA damage: the large and small ribonucleotide reductase subunits Rnr1 and Rnr4 (Figure 1B). We also analyzed the MMS-induced transcripts ARG3 and PAC11, and corresponding proteins (Supplemental Figure S1B), and determined that both failed to have concurrent protein level increases. This suggests that these transcripts were regulated post-transcriptionally. As both Rnr1 and Rnr4 are recognized components of the DNA damage response, we were intrigued by the discrepancy at the protein level. Thus we keyed in on components of the ribonucleotide reductase complex for the rest of our study and to probe the post-transcriptional regulation of DDR components. We note that our assay only detected levels of soluble Rnr1 and Rnr4 proteins. In addition, we only detected a slight increase in Rad53 protein levels after MMS damage, somewhat matching the transcriptional increase. However, our protein level
analysis may have been distorted by extensive phosphorylation of Rad53 after MMS damage, which we observed as a high molecular weight species on western blots (Supplemental Figure S1C). Thus, we have omitted Rad53 from our list of proteins decoupled from a transcriptional increase. Northern and western blot analysis (Figure 1B) of RNR1 and RNR4 transcripts and corresponding proteins further demonstrated that the MMS-induced transcriptional increases for each do not lead to an increase in soluble protein. In contrast, we detected matched transcript and protein level increases for the DNA damage response genes RNR3 and MAG1 (Figure 1B). These results suggest that post-transcriptional regulation of the RNR1 and RNR4 transcripts or corresponding proteins is occurring.

To further analyze the potential post–transcriptional regulation of ribonucleotide reductase components after DNA damage, we have performed detailed analysis of RNR1 transcript and protein levels over three doses of MMS (0.006, 0.0125 and 0.025% MMS). Cell viability results demonstrated that there was little killing using these MMS doses (Figure 1C). Notably, we observed transcriptional induction of the RNR1 transcript using all three concentrations of MMS, yet there was no observable increase in Rnr1 protein levels in any of the exposed cells (Figure 1D). Thus our observation that the transcriptional increase in RNR1 is de-coupled from protein levels increases holds true for both sub-lethal and mildly lethal MMS exposures. MMS is a classic DNA damaging agent that promotes strand breaks and replication blocks, as well as readily methylating RNA and proteins. In order to rule out MMS-induced protein damage as a cause
for our observed discrepancy, we have also analyzed the levels of the $RNR1$ transcript and Rnr1 protein following bleomycin-induced double strand breaks. Similar to our MMS studies, we found that $RNR1$ transcript levels increased in response to bleomycin treatment; yet, Rnr1 protein levels remained unchanged (Figure 1E). The transcriptional increase in $RNR1$ after bleomycin treatment is more pronounced compared to MMS treatment, suggesting that the decoupling of protein level increases from transcriptional increases is more dramatic after treatment with agents that directly cause double strand breaks. The MMS and bleomycin results support our hypothesis that protein level changes can be decoupled from transcriptional changes during the DNA damage response and that post-transcriptional regulation affects soluble Rnr1 protein levels.

$RNR1$ is transcribed and translated after DNA damage

As a consequence of observing MMS-induced increases in only one of the two homologous large subunits of the ribonucleotide reductase complex (i.e., Rnr3 and not Rnr1), we have focused in-depth studies on the potential post-transcriptional regulation of the $RNR1$ transcript and the regulation of the Rnr1 protein. To further validate our observation that increased $RNR1$ transcription does not lead to increased levels of soluble protein, we monitored $RNR1$ and $RNR1$-TAP transcript and protein levels in both MMS-treated and untreated controls (10 to 60 minutes post-damage) using northern and western blots (Figure 2A). Rnr1 protein levels were also analyzed using two different antibodies: an anti-TAP antibody to examine the levels of endogenously
expressed Rnr1-TAP and an anti-Rnr1 antibody to analyze the native protein. We determined that at 10 and 60 minutes after MMS treatment, RNR1 transcription increased 2.0- to 3.5-fold. Similar MMS-induction results were obtained for the RNR1-TAP transcript. Rnr1 and Rnr1-TAP protein levels in the soluble fraction showed little increase at any time point when compared to untreated controls (Figure 2B). Analogous northern and western blot results were observed at 20, 30, and 45 minute time points (Supplemental Figure S1D). Our time course results using both endogenous and TAP-tagged Rnr1 further demonstrate that levels of soluble Rnr1 protein do not dramatically increase after MMS damage. In addition, the parallel behavior of the endogenous and TAP-tagged Rnr1 proteins supports that the C-terminal TAP tag on Rnr1 does not promote protein degradation. We have performed similar time-course studies using our quantitative ECL approach to track levels of endogenous Rnr1 and in all cases we observed minimal change in soluble Rnr1 protein levels after MMS treatment (data not shown). The observed decoupling of the MMS-induced RNR1 transcriptional increase from a soluble protein level increase suggests that a fraction of the RNR1 transcript or Rnr1 protein pools were affected by post-transcriptional, translational or post-translational regulation.

Protein levels can be regulated during translation or by protein degradation pathways. Translation occurs in the ribosomes and polysome profiles are typically used to identify transcripts engaged with the translation machinery. We performed a polysome profile to determine if the RNR1 transcript
is actively translated after MMS damage (Figure 2C). Analysis of the profile for RNR1 from the untreated sample indicated that a portion of the transcript was found in the polysomes, supporting active translation of the transcript under basal conditions. After MMS treatment, we observed that a majority of the RNR1 transcripts were contained in the polysome portion of our profile, indicating that RNR1 is actively translated after damage. Taken together, our RNR1 polysome profile results for untreated and MMS-treated cells suggest that Rnr1 is regulated post-translationally.

**Rnr1 protein degradation is vacuole-dependent and increased after DNA damage**

We reasoned that our failure to observe an increase in soluble Rnr1 protein levels after MMS damage was most likely caused by the degradation of newly synthesized or existing Rnr1 protein. Protein degradation occurs via proteasome- or vacuole-dependent pathways, with the later pathway using membrane bound structures to remove protein from the soluble fraction found in the cytoplasm and deliver it to the vacuole. To determine if either proteasome- or vacuole-dependent pathways promoted the degradation of Rnr1 after MMS damage, we analyzed MMS-induced Rnr1 protein levels in the presence of the proteasome inhibitor MG132 or the vacuolar inhibitor PMSF (14, 33). Pre-incubation of cells with PMSF followed by MMS treatment promoted a notable increase in the soluble levels of Rnr1 protein at 60 minutes (Figure 3A), while the proteasome inhibitor MG132 did not alter the levels of observed protein. We have
also analyzed Rnr4 protein levels after treatment with PMSF and found that inhibition of vacuole-dependent degradation did not affect soluble Rnr4 protein levels (Supplemental Figure S2A). This negative result suggests that regulation of Rnr4 occurs outside of vacuole-associated protein degradation pathways. In contrast our positive results showing an increase in Rnr1 after PMSF treatment lead us to propose that Rnr1 is ultimately degraded in a vacuolar-dependent fashion.

To explore the potential role of the vacuole in Rnr1 protein degradation, we used a panel of yeast mutants specific to a component of the Golgi-associated retrograde protein complex involved in vacuolar protein sorting (vps54Δ) and five VMA gene mutants (vma2Δ, vma4Δ, vma6Δ, vma7Δ and vma21Δ). VMA genes code for vacuolar H+-ATPases that acidify vacuoles and promote proteolysis by vacuolar peptidases (34). Rnr1 protein levels were measured in untreated and MMS-treated cells for the panel of mutants, as well as for wild-type controls. We observed an ~1.5-fold increase in basal Rnr1 protein levels in the vma4Δ, vma6Δ, vma7Δ and vma21Δ cells relative to wild-type cells (Supplemental Figure S2B), suggesting that some vacuolar-mediated degradation of Rnr1 occurs under normal growth conditions. We observed MMS-induced increases in Rnr1 protein levels in the vps54Δ, vma2Δ, vma4Δ, vma6Δ, vma7Δ and vma21Δ cells, relative to each mutant-specific untreated control (Figures 3B & C). In all cases, the MMS-induced increase of Rnr1 in the vacuole-compromised mutants, relative to untreated mutants, was contrasted by
little MMS-induced change in soluble Rnr1 protein levels in wild-type cells after damage (Figure 3C). Our results with the pharmacological inhibitors PMSF and MG132 as well as data using vacuole compromised mutants support the hypothesis that vacuole-dependent degradation of Rnr1 is occurring.

The Rnr1 protein is actively targeted to the vacuole after DNA damage

We reasoned that for vacuole-dependent degradation of Rnr1 to occur, a portion of the Rnr1 protein pool must be re-localized to the vacuole. It has previously been demonstrated that sub-cellular re-localization of the ribonucleotide reductase small subunits from the nucleus to the cytoplasm occurs, and this supports the argument that RNR activity can be regulated by subunit location (35). We have demonstrated that a fraction of Rnr1 is degraded in a vacuole-dependent fashion (Figure 3). We used endogenously expressed Rnr1-GFP and Rnr3-GFP to study the location of the large subunits pre- and post-MMS damage. Fluorescence analysis under basal conditions demonstrated that Rnr1 was diffuse and cytoplasmic with some discrete foci (38% ± 7% of total cells, N = 3) close to the vacuole in the cell (Figure 4A-B). We note that these foci can also be observed in the Rnr1 specific data found in the Yeast GFP Fusion Localization Database (yeastgfp.yeastgenome.org)(36). In addition, we note that even when cells have discrete foci, we also detected a diffuse cytoplasmic signal for Rnr1-GFP. We determined that MMS treatment induced the transcription of *RNR1* and significantly increased the formation of discrete foci (73% ± 8%, N = 3) (Figure 4B), thus indicating a re-localization of a portion of
Rnr1-GFP after MMS damage. In contrast, Rnr3-GFP remained diffuse and predominantly cytoplasmic under basal and MMS-treated conditions (Supplemental Figure S3A). We have also analyzed the growth rate and viability of the Rnr1-GFP strain, before and after MMS treatment, and demonstrated that it behaves like wild-type cells (Supplemental Figure S3B-C). If the GFP tag destabilized the essential protein Rnr1, growth rate and MMS phenotypes would be expected, and this was not observed. Further, data from half-life experiments indicates that both native Rnr1 and Rnr1-GFP are long lived proteins with half-lives greater than eight hours (Supplemental Figure S3D). Together, these results support that the C-terminal GFP tag on Rnr1 is not hindering RNR activity or destabilizing the Rnr1 protein. Further they demonstrate that the Rnr1 protein is long lived under basal conditions. We note that for Rnr1-GFP, DNA damage appeared to induce an increase in focus size, a finding that we confirmed using quantitative image analysis. When compared to the untreated cells (478 ± 290 arbitrary units), the focus area was increased ~2.2-fold in MMS-treated cells (1030 ± 478 arbitrary units). The increase in the number and area of Rnr1-GFP foci in the MMS-treated cells, relative to untreated cells, reveals that a large fraction of the Rnr1 protein pool is localized to foci following DNA damage.

To further analyze the location of Rnr1-GFP foci, we used fluorescence and confocal microscopy with two different vacuole-specific dyes: FM4-64 stains the vacuolar membrane and CellTracker Blue selectively stains the vacuolar
lumen. Rnr1-GFP expressing cells were treated with MMS and counterstained with each of the vacuole markers. Merged images specific to Rnr1-GFP foci and the vacuole membrane stained with FM4-64 indicated that the Rnr1-GFP foci were docked at the vacuole membrane and poised to enter the vacuole (Figure 4C). We obtained a similar result using CellTracker Blue: the Rnr1-GFP foci were observed overlapping the periphery of the vacuole (Figure 4D). The fluorescence and confocal microscopy results support the hypothesis that Rnr1-GFP foci are targeted to the vacuole and that Rnr1 degradation occurs in the vacuole.

Our observations from experiments using both native Rnr1 and a GFP tagged version predicted that a portion of the Rnr1 protein pool was being packaged into a membrane-bound vesicle and targeted for degradation. To test this prediction, we isolated the supernatant and membrane fraction of cells from mock- and MMS-treated cells. As expected the levels of native Rnr1 in the supernatant from untreated and MMS treated cells was similar. In contrast, we have determined that there is an ~2-fold increase in native Rnr1 in the membrane fraction after MMS damage (P < 0.0135), relative to the amount of Rnr1 found in the membrane fraction of untreated cells (Figures 4E & F). This finding supports that Rnr1 is transported to the vacuole in membrane-bound autophagosomes for degradation. Ribonucleotide reductase activity is regulated during the cell cycle. We reasoned that the removal Rnr1 from the soluble fraction could help optimize dNTP levels or promote cell cycle transitions, and in either case Rnr1 targeting to the autophagosome should be cell cycle dependent. In addition, the
autophagosome results, when combined with previous data, predict that Rnr1 foci formation and transport to the vacuole should also be dependent on autophagy and TOR.

**Rnr1-GFP foci levels peak during late S and early G2 of the cell cycle**

MMS promotes an S phase arrest and induces the DNA damage response. During this response, cells will optimize enzyme activities to promote efficient DNA replication. We reasoned that if Rnr1 targeting to the vacuole is used to optimize or control DNA synthesis, then Rnr1-GFP foci should increase during S phase. To test this prediction, cells were synchronized, released from G1, and analyzed for Rnr1-GFP foci formation as a function of time (0, 15, 30, 45, 60, 90 and 120 minutes). Cell cycle characteristics were also quantitated by FACS analysis at each of the seven time points. We observed (Figure 5A) foci at increasing levels after cells were released from G1. Peak foci formation occurred at 60 minutes (Figure 5B), a time that coincides with a majority of the cells being in late S or early G2 phases, with approximately half in each phase. As this is a population of cells, this point represents the S to G2 transition point. Peak mRNA expression for *RNR1* occurs during S, while the foci were present during both S and early G2; this most likely occurs because of the high translation level of *RNR1* throughout S-phase and its increased targeting to the vacuole during late S and into early G2. Our results using synchronized and released cells confirm our prediction that foci formation is cell cycle dependent.
Autophagy associated proteins target Rnr1 to the vacuole for degradation

Targeting macromolecules or organelles to the vacuole requires Atg proteins that participate in autophagy. Yeast atg mutants are defective in autophagy and have been reported to be corrupted in vacuole-dependent protein degradation (37). Based on our western blot and microscopy results, we postulated that Rnr1 degradation would be dependent on Atg proteins. To test this hypothesis, we focused our study on native Rnr1 protein levels in atg2Δ, atg5Δ, atg8Δ, atg9Δ and atg14Δ cells. Cultures of atg mutants were either left untreated or treated with 0.0125% MMS for 1 hour, after which Rnr1 protein levels were measured by western blot. Relative to each untreated mutant, we observed MMS-induced increases in Rnr1 protein levels in our panel of atg mutants (Figure 6A), with 2.0 to 3.5-fold increases for Rnr1 identified in atg2Δ, atg5Δ, atg8Δ, atg9Δ, and atg14Δ (Figure 6B). In contrast, wild-type cells demonstrated little change in soluble Rnr1 protein levels after MMS damage. Atg2, Atg5, Atg8, Atg9 and Atg14 are proteins involved in autophagosome assembly or vacuolar docking, and deficiencies in any of these proteins increased soluble Rnr1 protein levels. Western blot results reveal that proteins participating in autophagy are involved in the removal of Rnr1 from the soluble fraction. In addition, they demonstrate that deficiencies in either autophagosome assembly or vacuolar docking result in increased levels of soluble Rnr1 protein after MMS damage.
Four lines of evidence support that Rnr1 is sequestered into autophagosomes and subsequently released into the vacuole for degradation. Included among these were the observations that Rnr1-GFP foci localized at the vacuole and that Rnr1 protein levels were increased in membrane fractions after MMS treatment, relative to untreated cells (Figure 4E-F). In addition, we also observed MMS-induced increases in soluble Rnr1 protein levels in vacuole compromised and \textit{atg} mutants (Figures 3B-C & 6A-B). Consequently, we postulated that defects in the early stages of the autophagy pathway would prevent the formation of autophagosomes, which we propose are represented as discrete Rnr1-GFP foci. We deleted specific \textit{atg} genes (\textit{atg2Δ}, \textit{atg5Δ}, \textit{atg8Δ}, \textit{atg9Δ}, and \textit{atg14Δ}) in the Rnr1-GFP strain and analyzed Rnr1-GFP levels and their corresponding foci, both before (data not shown) and after MMS-exposure (Figure 6C). Rnr1-GFP was found throughout the cytoplasm before and after MMS exposure in \textit{atg2Δ}, \textit{atg5Δ}, \textit{atg8Δ}, \textit{atg9Δ}, and \textit{atg14Δ}; in contrast, we observed MMS-induced foci in wild-type cells. We note that in figure 6, experiments with \textit{atg} mutants were performed using both native Rnr1 (Figure 6A-B) and a C-terminal GFP-tagged version (Figure 6C), and results associated with each form of Rnr1 implicate a role for autophagy proteins in regulating soluble protein levels.

\textbf{TOR inhibition and activation of autophagy promotes Rnr1 degradation}

Autophagy is increased when TOR activity is inhibited by nutrient starvation or rapamycin. We reasoned that if Rnr1 is degraded via autophagy,
then Rnr1 protein levels should be sensitive to TOR activity. Rnr1 protein and 
\textit{RNR1} transcript levels were analyzed in wild-type cells, both before and after 
treatment with rapamycin for 60 minutes (Figure 7A). While the \textit{RNR1} transcript 
was detected under all conditions and induced after MMS treatment, there was a 
large decrease in Rnr1 protein levels in cells treated with rapamycin, when 
compared to those left untreated. Notably, \textit{RNR1} transcript levels in the 
rapamycin + MMS samples are higher than in the untreated sample, yet the Rnr1 
protein levels are much lower in this treated sample. Further, we found that this 
rapamycin-induced decrease in Rnr1 protein levels is abrogated in cells 
corrupted in autophagy (\textit{atg9}\Delta)(Figure 7B). The rapamycin induced decrease in 
Rnr1 protein levels suggest that foci form quickly in response to treatment. To 
test our prediction we analyzed Rnr1-GFP foci levels 15 minutes after cells had 
been subjected to rapamycin treatment (Figures 7C & D). We found a significant 
increase (~35%, \( P < 0.007 \)) in Rnr1-GFP foci levels in rapamycin treated cells, 
when compared with those left untreated. In addition, we analyzed native Rnr1 
protein and transcript levels in cells where TOR was inhibited by nutritional stress 
(Figure 7E). We found that similar to our results with rapamycin, nutritional 
stress promoted a substantial decrease in soluble Rnr1 protein levels in wild-type 
cells. This decrease in Rnr1 protein levels was abrogated in the autophagy 
mutant's \textit{atg9}\Delta and \textit{atg14}\Delta. Our observation of high Rnr1 protein levels in \textit{atg9}\Delta 
and \textit{atg14}\Delta cells, relative to wild-type cells, was a striking result because the 
\textit{RNR1} transcripts were at higher levels in wild-type cells. Ultimately, these
results provide evidence that TOR inhibition and the induction of autophagy will promote the removal of Rnr1 from the soluble protein fraction.

In wild-type cells, the degradation of Rnr1 after TOR inhibition most likely prevents DNA synthesis after DNA damage, and we reasoned this should promote an efficient response to DNA damage. Conversely, we postulated that a failure to degrade Rnr1 levels after TOR inhibition should lead to a DNA damage phenotype in \textit{atg} mutants. To test our prediction, we exposed cells to rapamycin in the absence or presence of a DNA damaging agent (Figure 7F). We used rapamycin in plate-based experiments to induce autophagy, as \textit{atg} mutants can grow in the presence of this compound but fail to grow on nutritional stress media. Thus, the use of rapamycin provided us with a methodology to test for a DDR-associated growth phenotype in cells that fail to degrade Rnr1 via autophagy. Conditions were chosen so neither wild-type or \textit{atg} cells exhibited a growth defect in the presence of rapamycin or MMS alone. Plate based killing assays utilizing rapamycin demonstrate that \textit{atg} (\textit{atg2Δ, atg5Δ, atg8Δ, atg9Δ, atg12Δ, and atg14Δ}) mutants are more sensitive to MMS, relative to wild-type cells. Taken together, our results indicate that TOR inhibition and the activation of autophagy plays an important role in promoting the degradation of Rnr1, and our phenotypic studies demonstrate that defects in this process lead to defects in the DNA damage response.
Discussion

Targeted gene- and protein-specific studies report protein regulation at the levels of protein synthesis or protein degradation (15, 38); yet, few global techniques have been developed to efficiently identify multiple targets of post-transcriptional regulation. Our developed methodology uses two standard high-throughput approaches tethered to computational analysis in order to systematically identify targets of post-transcriptional regulation. Our approach has the potential to identify a plethora of novel regulatory strategies because it can be applied to other perturbations and model systems. What we have demonstrated is that matched transcript-protein level studies can be used to identify discrepancies in standard gene regulatory models (i.e., transcript is induced to make more protein). The identification of any discrepancy or unusual regulatory pattern can be challenging to comprehend but detailed study ultimately leads to a better understanding of biology. Here we focused our studies on the large subunit of the RNR complex to elucidate a novel pathway for the post-translational regulation of Rnr1 protein levels.

Because dNTP levels play an important role in checkpoint arrest, mutagenesis, and cell viability after DNA damage, RNR activity is extensively regulated in eukaryotic organisms (3). In mammals, RNR activity is regulated by S phase transcription, the p53-inducible subunit p53R2, proteasome-dependent degradation of the small subunit R2, and allosteric mechanisms (40). In yeast, transcriptional activation of the subunits comprising the RNR complex requires
de-repression of Rfx1 by Dun1-dependent phosphorylation (41), with phosphorylation also being used to inactivate the RNR inhibitor Sml1. Recently, translation of the \textit{RNR1} and \textit{RNR3} transcripts has been reported to be dependent on Trm9-catalyzed tRNA modifications (42) and our current study demonstrates that the soluble levels of the large Rnr1 subunit can be regulated via autophagy. Protein degradation of RNR subunits has now been observed in both yeast and human systems, thus supporting the hypothesis that subunit degradation is a conserved regulatory mechanism controlling dNTP levels \textit{in vivo}.

In general, proteasome-dependent degradation of specific proteins uses ubiquitination and is associated with the removal of short-lived proteins, while vacuolar-dependent degradation is considered a non-specific mechanism to remove long-lived proteins via autophagy. Our results run contrary to this dogma: we observed a very specific removal of Rnr1 from the soluble protein pool by autophagosome transport to the vacuole. Although autophagy is generally considered to be a bulk degradation process, examples of specific targeting have been reported for the cytosolic cysteine protease Lap3 (43), the gluconeogenic enzyme fructose-1,6-bisphosphatase (FBPase) (44), and the metabolic enzyme acetaldehyde dehydrogenase (Ald6) (45). Over-expression of Ald6 has been shown to be cytotoxic, implying that the targeted degradation of this protein is advantageous. Removal of proteins that produce dominant
negative phenotypes may be a theme associated with the specific targeting of proteins via autophagy.

A major question arising from our findings is: Why do cells transcribe and translate Rnr1 when it is unnecessary? In answering this question, we note that our results detailing Rnr1 protein levels involved two distinct yet overlapping responses: DNA damage and nutrient stress (via TOR inhibition). In theory, each response has a different objective and thus a different endpoint for Rnr1 protein levels. The DNA damage response requires a precise amount of Rnr1 to promote DNA synthesis under damaging conditions and to control cell cycle transitions. The degradation of excess Rnr1 protein during DNA damage may be required to promote the formation of an Rnr1-Rnr3 assembly in the final RNR complex, as opposed to an Rnr1-Rnr1 assembly. The Rnr1-Rnr3 assembly is reported to be the most catalytically active form (46). Ghaemmaghami and colleagues have reported that Rnr1 protein levels are ~200-fold higher than Rnr3 levels (47). Thus, a low level of Rnr1 protein localization to autophagosomes, as seen in our cell cycle data for early to mid S-phase, and its subsequent degradation may be part of the mechanism driving subunit formation and optimizing catalytic activity of the RNR tetramer. Because high dNTP levels are needed to drive translesion and replicative polymerases during the DNA damage response, optimal RNR activity is essential to cellular proliferation (3, 4, 39). In addition, our cell cycle data demonstrating peak Rnr1-GFP foci at the S-G2 border suggests that increased Rnr1 degradation could also be used to facilitate
transition into G2. There is a decrease in dNTP levels during G2, relative to S, and Rnr1 degradation could be one of the mechanisms used to promote this reduction. The autophagy-dependent degradation of Rnr1 could play multiple roles, ultimately we believe at low levels it acts to optimize RNR activity during S-phase and at high levels it promotes a transition to G2.

Nutrient stress, which is also mimicked by rapamycin treatment, is the other distinct condition used in our study and promotes the removal of all Rnr1 protein. The Rnr1 protein was not detected 60 minutes after TOR inhibition, and its absence should lead to a decrease in dNTP levels and a halt to DNA synthesis. Increased autophagy drives this degradation of Rnr1, and that may be a mechanism to quickly align DNA synthesis with current nutrient conditions. TOR inhibition has also been demonstrated to lead to a reduction in RNR1 transcription (48), and we have observed this transcriptional control in our studies. Our report supports and demonstrates that the cell uses two mechanisms to decrease Rnr1 protein levels after TOR inhibition, an RNR1 transcriptional decrease and a removal of existing Rnr1 from the soluble pool, followed by degradation. We note that we have observed a long half life for Rnr1 protein. The two TOR-based strategies to control Rnr1 protein levels limit the synthesis of new protein and ensure that any existing protein is removed from the soluble fraction. In situations of nutrient stress, cell division decreases and autophagy ensures that the necessary precursors are available to maintain the cell. In this context, Rnr1 degradation might represent one of the control
mechanisms used to arrest cells and prevent DNA synthesis during times of nutritional stress. Our phenotypic studies demonstrate the importance of this immediate response to DNA damage under conditions of TOR inhibition, as atg mutants fail to grow under these conditions. Ultimately, our report links defects in autophagy to a DNA damage phenotype and highlights the importance of protein degradation during the DNA damage response.

In conclusion, we have used systems biology and targeted studies to identify post-transcriptional regulatory targets and to demonstrate that autophagy plays an important role in regulating soluble Rnr1 protein levels. A remaining question deals with how Rnr1 is targeted by the autophagy machinery. It is possible that MMS induced protein damage, aggregation or a post-translational modification provides the signal, but this has yet to be determined and is the focus of future work. Previous studies in human cell systems report that defects in autophagy promote genome instability, arising from increased metabolic stress that generates increased levels of endogenous damaging agents (49). Additionally, mTOR inhibition and the induction of autophagy have been linked to p53 and Ataxia-telangiectasia mutated (ATM) signaling after DNA damage, with ATM activation also occurring by reactive oxygen species in the cytoplasm (50, 51). Nonetheless, these above examples all demonstrate links between autophagy and known components of the DNA damage response. Our findings in yeast further demonstrate that autophagy and DNA damage signaling are intricately coordinated, and lead us to speculate that human cells defective in
autophagy may have altered levels of DNA damage response proteins. Coupled with the increase in DNA damage, a misregulation of DNA damage response proteins could dramatically increase genome instability and carcinogenesis in autophagy deficient cells.
Materials and Methods

Yeast and growth conditions

Supplemental Table 5S lists the strains and oligonucleotides used in this study. All mutants were made using their corresponding G418 knockout cassette from the *Saccharomyces* Gene Deletion Project and were selected on YPD plates supplemented with G418 (200 μg/ml). Mutants were confirmed by PCR. Media preparation and other yeast manipulations were performed using standard methods. The nutritional stress medium consisted of 0.17% yeast nitrogen base without amino acids, ammonium sulfate and 2% glucose.

RNA analysis

Total RNA was isolated using the RNeasy Mini Kit (Qiagen; Chatsworth, CA) with 250 U of Zymolyase (Associates of Cape Cod; Falmouth, MA). RNA was isolated from the spheroplasts and examined spectroscopically, by agarose gel electrophoresis and Bioanalyzer analysis (Agilent; Santa Clara CA). Northern blot analysis was performed using 10-12 μg of RNA with detection facilitated using the Chemiluminescent Nucleic Acid Detection Module (Pierce; Rockford, IL). Affymetrix Gene Chip (Yeast 2.0) analysis was performed as previously described (52). RNA from untreated and MMS-treated cells (N=2) was labeled, applied to chips and the resulting data analyzed using CyberT software (53). Polysome profiles were performed as previously described (54).
Protein analysis

Pelleted cells were lysed with 50 µL of a boiling SDS solution (50mM Tris-HCl, pH 7.5, 5% SDS, 5% glycerol, 50mM DTT, 5mM EDTA, 2µg/mL Leupeptin, 2µg/mL Pepstatin A, 1µg/mL Chymostatin, 0.15 mg/mL Benzamidine, 0.1 mg/mL Pefabloc, 8.8 µg/mL Aprotanin, and 3 µg/mL Anitpain). Lysed cells were centrifuged for 5 minutes at 13,000 rpm and the resulting supernatant was used as protein extract. Membrane protein was extracted using Mem-PER Eukaryotic Membrane Protein Extraction Kit (Pierce; Rockford, IL). Protein concentrations were measured by NanoDrop 1000 (Thermo Scientific; Wilmington, DE) using BSA as a standard. Western blots were performed as described (58) with an anti-TAP antibody (Open Biosystems; Huntsville, AL), anti-Rnr1 antibody (Santa Cruz Biotechnology; Santa Cruz, CA) and anti-β-tubulin antibody (Abcam; Cambridge, MA). Individual bands were quantitated using AlphaEaseFC software (Imgen Technologies, New City, NY). iTRAQ analysis was performed using a methodology previously reported (55, 56).

Protein degradation analysis

Rnr1 protein degradation was measured in the presence of either the proteasome inhibitor MG132 (Sigma; St. Louis, MO) or the vacuolar inhibitor PMSF (Pierce; Rockford, IL) in erg6Δ cells. Cultures were grown at 30 °C to 5 X10^6 cells/ml in YPD media and were pre-incubated with either 100µM MG132 or 1mM PMSF as described (14).
**High-throughput ECL analysis of TAP-tagged proteins**

Lysates from MMS-treated and untreated cells were added to the High Bind Plate from Meso Scale Discovery (Gaithersburge, MD). Plates were incubated at room temperature for 1 hour, blocked, and washed according to manufacturer instructions. A 1:500 dilution of Anti-tap antibody was added followed by an MSD Sulfo-tag labeled secondary antibody. The plate was washed with TBST and read immediately after adding MSD Read Buffer using the SECTOR 2400 from MSD.

**Fluorescence and confocal microscopy and flow cytometry**

Fluorescence microscopy was performed using an endogenously expressed Rnr1-GFP fusion protein (Invitrogen; Carlsbad, CA). Cells were grown to mid-log phase in YPD medium, MMS-treated as indicated, and examined on a Nikon Eclipse TS100 fluorescence microscope. Images were taken with a RT3 camera (Diagnostic Instruments; Sterling Heights, MI) and SPOT Basic and ImageJ software were used to capture images and quantitate the focus area, respectively. Statistical significance was determined using a t-test. The vacuolar membrane was stained using FM4-64 (Invitrogen; Carlsbad, CA) following the manufacturers protocol. For confocal microscopy, the vacuolar lumen was stained using CellTracker Blue (Invitrogen; Carlsbad, CA) and observed under a Leica Confocal microscope using GFP and DAPI channels. The DNA content of
untreated, alpha factor synchronized, and MMS-treated cells was measured by flow cytometry after PI staining, as described (57).
Acknowledgments

This work was supported by grants awarded to TJB from the NIH (ES01225101 and ES015037) and a James D. Watson Award through NYSTAR.
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Figure Legends

Figure 1. Some protein level changes are decoupled from MMS-induced transcriptional increases.

*S. cerevisiae* (BY4741) cultures were grown at 30° C to 5 X 10^6 cells/ml in YPD and were either left untreated or exposed to 0.0125% MMS, or other concentrations where indicated, for 1 hour. A. Up-regulated transcripts (> 2.0-fold, p < 0.05) were matched to protein level changes as measured by ECL analysis of TAP-tagged proteins. B. Northern blot analysis of four DNA damage-induced transcripts and western blots to corresponding TAP-tagged proteins were performed as described in Materials and Methods. C. Cell viability analysis of wild-type BY4741 cells after 1-hour treatment with MMS. D. Northern and western blot analysis of *RNR1* and Rnr1 one hour after treatment with MMS. E. Northern and western blot analysis of *RNR1* and Rnr1-TAP before (UNT) and after MMS or bleomycin (BLM) treatment were performed as described above.

Figure 2. The MMS-induced increase in *RNR1* transcription and corresponding polysome occupancy do not lead to increased Rnr1 protein levels.

A. Cultures of wild-type cells (BY4741 or ATCC201388) were divided equally and either left untreated or exposed to 0.0125% MMS for 10 to 60 minutes. Total RNA and protein were extracted from all samples. Endogenous *RNR1, RNR1-TAP* and *ACT1* transcripts were analyzed by northern blot. Rnr1 and Rnr1-TAP protein levels were measured by western blot using anti-Rnr1 and anti-TAP antibodies.
antibodies. As a loading control, tubulin levels were also analyzed by western blot. **B.** The bar graph represents the fold change in protein and transcript levels after MMS damage in treated samples, as compared to untreated samples. Individual bands were quantitated by densitometry and compared to the loading controls, *ACT1* and tubulin. **C.** Cell lysates of MMS-treated (60 minutes) and untreated cells were separated on a 10% - 50% sucrose gradient; RNA was extracted from each fraction and *ACT1* and endogenous *RNR1* were probed by northern blot. Ethidium bromide staining was used to indicate the relative amounts of 28S and 18S rRNA in each fraction. Note the middle peak in the profile corresponds to the monosome.

**Figure 3. Compromised vacuolar function promotes increased Rnr1 levels after MMS treatment.**

**A.** *S. cerevisiae* (BY4741) *erg6∆* cells were grown at 30°C to 5 X 10⁶ cells/ml in YPD medium. The cells deficient in Erg6 are considered leaky and were used to facilitate entry of the inhibitors. A culture of cells was divided equally and pre-incubated with either 1 mM PMSF or 100 µM MG132 for 90 minutes, and then treated with MMS (0.0125%) for 60 minutes. **B.** Rnr1 protein levels in wild-type and vacuolar mutants were measured by western blot before and after 0.0125% MMS exposure, using an Rnr1-specific antibody. **C.** Quantification of Rnr1 protein levels demonstrated that there is an MMS-induced increase in soluble Rnr1 protein in vacuolar mutants (N = 3), when compared to corresponding
untreated cells; this is contrasted in wild type cells by little change in soluble Rnr1 protein levels after MMS treatment.

**Figure 4. Increased Rnr1-GFP localization to the vacuole after MMS damage.**

**A.** Rnr1-GFP expression in untreated and MMS-treated cells (N = 100) was examined by bright field and fluorescence microscopy. **B.** Foci were quantitated and the number of cells with foci (p < 0.05) was significantly increased in MMS-treated cells. **C.** Cells were stained with FM4-64 to localize the vacuolar membrane; images for green fluorescence, red fluorescence, and bright field microscopy were merged as indicated. **D.** Cells that were stained with Cell Tracker Blue, to localize the vacuolar lumen, were observed in green and DAPI channels, and then merged. **E.** Rnr1 levels in wild-type cells left untreated or exposed to 0.0125% MMS were measured as described in Figure 3. Rnr1 protein levels were analyzed in the supernatant and in the protein isolated from the membrane fraction. **F.** Rnr1 protein levels were normalized to the loading control tubulin and relative levels were compared using Student’s t-test. As expected, tubulin levels were different in the supernatant and membrane extract. This data, therefore, should not be used to compare absolute levels between the supernatant and membrane fractions.

**Figure 5. Rnr1-GFP foci increase during S and peaks at the S-G2 border.**
A. Yeast Rnr1-GFP cultures were grown at 30°C to 5 X 10^6 cells/ml in YPD, treated with alpha factor, and then released into YPD for 60 minutes. Samples were analyzed by flow cytometry and fluorescence microscopy to quantitate cell cycle progression and foci formation. For reference, asynchronous cells were treated with MMS for 60 minutes and analyzed as described above. B. Quantitative analysis of cell cycle stage and Rnr1-GFP foci levels in cells released from G1 (N = 2) was performed as described above. Green, blue and red bars represent the percentage of cells with foci, in S-phase or in G2-phase, respectively.

**Figure 6.** Autophagy mutants promote increased Rnr1 levels and mis-localization of Rnr1.

A. Rnr1 and tubulin levels in wild-type and autophagy mutants, from both untreated and MMS-treated cells, were measured as described above. 50 µg of total protein was loaded for each sample. B. Rnr1 protein levels in MMS and untreated cells were quantitated relative to tubulin, and these values were used to generate fold changes after MMS treatment. C. Wild-type and autophagy deletion mutants (atg2Δ, atg5Δ, atg8Δ, atg9Δ, and atg14Δ) endogenously expressing Rnr1-GFP were grown to 5 X 10^6 cells/ml in YPD and exposed to 0.0125% MMS for 1 hour. Cells were observed under the fluorescence microscope both in bright field and green fluorescence mode, after which the images were merged.
Figure 7. Autophagy-dependent degradation of Rnr1 after TOR inhibition.

A. Wild-type cells were exposed for 60 minutes to either 0.0125% MMS, 50 ng/ml rapamycin, or both. Native Rnr1 and tubulin protein levels, as well as RNR1 and ACT1 transcript levels, from untreated and treated cells were measured as described in Figure 2. B. Wild-type and atg9Δ cells were grown in YPD and treated with rapamycin for 45 minutes. Native Rnr1 and tubulin levels from untreated and treated cells were measured as described above. C. Rnr1-GFP expression in cells left untreated or treated with rapamycin for 15 minutes was examined by bright field and fluorescence microscopy. D. Foci in Panel C were quantitated and out of 100 cells the number of cells with Rnr1-GFP foci (p < 0.007), was significantly increased in rapamycin-treated cells. E. Wild-type, atg9Δ, and atg14Δ cells were grown in YPD to 5 X 10^6 cells/ml and then further grown in starvation media for 12-hours, followed by treatment with 0.0125% MMS for 1 hour. Native Rnr1 and tubulin protein levels, as well as RNR1 and ACT1 transcript levels, from untreated and treated cells were measured as described in Figure 2. F. Overnight cultures of cells were serially diluted and plated on YPD or YPD supplemented with 0.0125% MMS, 25 ng/ml rapamycin (RAP), or 0.0125% MMS and 25 ng/ml RAP.
Supplemental Tables

**Table 1S.** Genes induced more than 2.0-fold after DNA damage.

**Table 2S.** Functional categories over-represented with MMS-induced genes, as determined by FunSpec.

**Table 3S.** Proteins induced more than 2.0-fold after DNA damage.

**Table 4S.** Functional categories over-represented with induced proteins after MMS treatment, as determined by FunSpec.

**Table 5S.** Yeast strains and oligonucleotides used in this study.

Supplemental Figures

**Figure S1.** Systems biology overview and targeted transcript and protein data.

- **A.** Schematic detailing experimental approaches and outcomes for groups of transcripts and proteins.  
  - **B.** Northern blot analysis of DNA damage-induced transcripts and western blots to corresponding TAP-tagged proteins were performed as described in Materials and Methods.  
  - **C.** *S. cerevisiae* (BY4741) *erg6Δ* cells were grown at 30° C to 5 X 10^6 cells/ml in YPD medium. A culture of cells was divided and pre-incubated with either 1 mM PMSF for 90 minutes, 0.0125% MMS or PMSF followed by MMS treatment. Rad53-TAP protein levels were measured by western blot, as described above.  
  - **D.** Full time course corresponding to figure 2A.
**Figure S2.** Rnr4 is not degraded after MMS-damage and Rnr1 protein levels are basally higher in vacuolar mutants.

**A.** *S. cerevisiae* (BY4741) erg6Δ cells were grown at 30° C to 5 X 10^6 cells/ml in YPD medium. A culture of cells was divided equally and pre-incubated with either 1 mM PMSF for 90 minutes, 0.0125% MMS, or PMSF followed by MMS treatment for 30 and 60 minutes. Rnr4-TAP protein levels were measured by western blot as described in Figure 1. **B.** Rnr1 protein levels in wild-type and vacuolar mutants were determined in untreated samples by western blot using an Rnr1-specific antibody, as described in figure 1. The fold change in intensity values for each Rnr1 band was measured relative to wild-type untreated.

**Figure S3.** Localization of Rnr3-GFP and behavior of the Rnr1-GFP strain.

**A.** The *S. cerevisiae* Rnr3-GFP strain was grown at 30° C to 5 X 10^6 cells/ml in YPD. Cells were either left untreated or exposed to 0.0125% MMS for 1 hour. Cells were observed under the fluorescence microscope in bright field and green fluorescence modes. **B.** Wildtype BY4741 and Rnr1-GFP strains were grown over night, plated on YPD or YPD + MMS containing media and the corresponding plates were imaged after two days of growth at 30° C. **C.** The *S. cerevisiae* Rnr1-GFP and BY4741 strains were grown at 30° C to 5 X 10^6 cells/ml in YPD and left untreated or treated with 0.0125% MMS. The concentration of cells in wildtype (blue), wildtype + MMS (red), Rnr1-GFP (green) and Rnr1-GFP + MMS (purple) strains was determined over five hours. **D.** The *S. cerevisiae*
Rnr1-GFP and BY4741 strains were grown at 30° C to 5 X 10^6 cells/ml in YPD and left untreated or treated with 50 ug/ml of cycloheximide. Cells were collected after indicated time points and total protein was extracted. Rnr1 and Rnr1-GFP were analyzed using an anti-Rnr1 antibody, and western blots were performed as described above.
Figure 2

A

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B

Fold change (MMS/untreated)

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C

Untreated MMS treated

Polysome

Relative OD_{254}

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**Figure 3**

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### C

**Fold change (MMS/untreated)**

- WT: 1.26 ± 0.12
- vps54Δ: 1.34 ± 0.09
- vma2Δ: 1.30 ± 0.07
- vma4Δ: 1.40 ± 0.08
- vma6Δ: 1.50 ± 0.10
- vma7Δ: 1.20 ± 0.06
- vma21Δ: 1.45 ± 0.05

- Fold change relative to untreated control.
**Dyavaiah_Figure 4**

**A**

- **Untreated**
  - Bright field
  - Rnr1-GFP
  - Merge

- **MMS**
  - Bright field
  - Rnr1-GFP
  - Merge

**B**

- **Number of cells with foci**
  - Untreated
  - MMS
  - p < 0.005

**C**

- **Untreated**
  - Bright field
  - Rnr1-GFP
  - FM4-64
  - Merge

- **MMS**
  - Bright field
  - Rnr1-GFP
  - FM4-64
  - Merge

**D**

- **Untreated**
  - Bright field
  - Rnr1-GFP
  - Cell tracker blue
  - Merge

- **MMS**
  - Bright field
  - Rnr1-GFP
  - Cell tracker blue
  - Merge

**E**

- **Supernatant**
  - Untreated
  - MMS
  - Rnr1
  - Tubulin

- **Membrane extract**
  - Untreated
  - MMS
  - Rnr1
  - Tubulin

**F**

- **Relative Rnr1 protein level**
  - Untreated
  - MMS
  - Supernatant
  - Membrane extract
  - p = 0.0135
Figure 6

(A) Western blot analysis of Rnr1 and Tubulin levels in WT, atg2Δ, atg5Δ, atg8Δ, atg9Δ, and atg14Δ strains treated with MMS. 

(B) Graph showing the fold change in Rnr1 expression levels in MMS-treated compared to untreated conditions. 

(C) Confocal microscopy images showing Bright Field, Rnr1-GFP, and Merge for WT, atg2Δ, atg5Δ, atg8Δ, atg9Δ, and atg14Δ strains treated with MMS.
A RAP+ UNT MMS RAP MMS

Rnr1 Tubulin RNR1 ACT1

D

P < 0.007

40 60 80 100

Number of cells with foci

WT RAP

B

RAP - + - +

WT atg9Δ

Rnr1 Tubulin

C

Bright Field Rnr1-GFP Merge

UNT

RAP

E

Nutritional stress

WT atg9Δ atg14Δ

MMS Rnr1 Tubulin RNR1 ACT1

F

YPD MMS RAP MMS+RAP

WT atg2Δ atg8Δ atg9Δ atg5Δ atg12Δ atg14Δ
A. Macromolecules from Untreated and MMS treated *S. cerevisiae* cells

- Total RNA
- Total protein
- Microarray
- ITRAQ

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- 5714 Transcripts
- 603 Proteins
- Paired

- 16 Transcripts paired with corresponding proteins
- 29 Transcripts (Most Up-regulated)

- TAP-tagged proteins (High-throughput ECL-based technology)

- 15 Transcript increase paired with protein increase
- 14 Protein decrease or no change with transcript increase

- Also analyze Rnr4-TAP

B. MMS

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<td>Tubulin</td>
<td></td>
</tr>
<tr>
<td>Rnr1</td>
<td>Tubulin</td>
<td></td>
</tr>
</tbody>
</table>
A

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>MMS</th>
<th>PMSF+MMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rnr4-TAP</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td></td>
</tr>
<tr>
<td>Tubulin</td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
<td></td>
</tr>
</tbody>
</table>

B

![Bar graph](image5.png)

Fold Change (mutant / wild-type)

WT vps54Δ vma2Δ vma4Δ vma6Δ vma7Δ vma21Δ
Dyavaiah_Supp_Fig 3

A

Bright field  Rnr3-GFP  Merge

Untreated

MMS

B

C

WT  RNR1-GFP  WT  RNR1-GFP  WT  RNR1-GFP  WT  RNR1-GFP

Untreated  0.015% MMS  0.0175% MMS  0.02% MMS

Cell concentration (X10^6 cells/ml)

Time (hours)

D

Cycloheximde

Rnr1

Tubulin

Rnr1-GFP

Tubulin
Molecular Cancer Research

Autophagy-Dependent Regulation of the DNA Damage Response Protein Ribonucleotide Reductase 1

Madhu Dyavaiah, John Rooney, Sridar V. Chittur, et al.

Mol Cancer Res  Published OnlineFirst February 22, 2011.

Updated version: Access the most recent version of this article at: doi:10.1158/1541-7786.MCR-10-0473

Supplementary Material: Access the most recent supplemental material at: http://mcr.aacrjournals.org/content/suppl/2011/04/18/1541-7786.MCR-10-0473.DC1

Author Manuscript: Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

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