Molecular Biology of the Cell
Vol. 16, 3010–3018, June 2005

The Conserved Mec1/Rad53 Nuclear Checkpoint Pathway Regulates Mitochondrial DNA Copy Number in *Saccharomyces cerevisiae*[D]

Sean D. Taylor,*† Hong Zhang,†‡ Jana S. Eaton,*†§ Matthew S. Rodeheffer,‡‖¶ Maria A. Lebedeva,* Thomas W. O'Rourke,‡ Wolfram Siede,# and Gerald S. Shadel*

*Department of Pathology, Yale University School of Medicine, New Haven, CT 06520-8023; ‡Department of Biochemistry, Emory University School of Medicine, Atlanta, GA 30322; #Department of Cell Biology and Genetics, University of North Texas Health Science Center, Fort Worth, TX 76107; Graduate Programs in ‖Biochemistry and Cell and Developmental Biology and §Genetics and Molecular Biology, Emory University, Atlanta, GA 30322

Submitted January 19, 2005; Revised March 9, 2005; Accepted March 31, 2005
Monitoring Editor: Thomas Fox

How mitochondrial DNA (mtDNA) copy number is determined and modulated according to cellular demands is largely unknown. Our previous investigations of the related DNA helicases Pif1p and Rrm3p uncovered a role for these factors and the conserved Mec1/Rad53 nuclear checkpoint pathway in mtDNA mutagenesis and stability in *Saccharomyces cerevisiae*. Here, we demonstrate another novel function of this pathway in the regulation of mtDNA copy number. Deletion of *RRM3* or *SML1*, or overexpression of *RNR1*, which recapitulates Mec1/Rad53 pathway activation, resulted in an approximately twofold increase in mtDNA content relative to the corresponding wild-type yeast strains. In addition, deletion of *RRM3* or *SML1* fully rescued the ~50% depletion of mtDNA observed in a *pif1* null strain. Furthermore, deletion of *SML1* was shown to be epistatic to both a *rad53* and an *rrm3* null mutation, placing these three genes in the same genetic pathway of mtDNA copy number regulation. Finally, increased mtDNA copy number via the Mec1/Rad53 pathway could occur independently of Abf2p, an mtDNA-binding protein that, like its metazoan homologues, is implicated in mtDNA copy number control. Together, these results indicate that signaling through the Mec1/Rad53 pathway increases mtDNA copy number by altering deoxyribonucleoside triphosphate pools through the activity of ribonucleotide reductase. This comprises the first linkage of a conserved signaling pathway to the regulation of mitochondrial genome copy number and suggests that homologous pathways in humans may likewise regulate mtDNA content under physiological conditions.

INTRODUCTION

Since the discovery that the mitochondrial genome is present at multiple copies per cell (100–10,000 in humans) and is subject to dynamic regulation with regard to tissue type, metabolic signals, and environmental stimuli, an understanding of the pathways and mechanisms that regulate cellular mitochondrial DNA (mtDNA) copy number has been sought (Moraes, 2001). Although nuclear gene products that have direct roles in mtDNA replication or stability have been implicated in copy number regulation (Schultz *et al*., 1998; Zelenaya-Troitskaya *et al*., 1998; Ekstrand *et al*., 2004; Matsushima *et al*., 2004; Tyynismaa *et al*., 2004), signaling pathways involved in modulating cellular mtDNA content have not been fully elucidated. However, important insight into mtDNA copy number regulation and its clinical significance has been gleaned from the study of human mitochondrial disease patients. For example, several mtDNA-depletion syndromes have been characterized, the hallmark of which is decreased mtDNA copy number and/or integrity in certain tissues (Moraes *et al*., 1991; Elpeleg *et al*., 2002). Identification of the nuclear genetic defects underlying several of these diseases has underscored a critical role for cellular deoxyribonucleoside triphosphate (dNTP) pools in mtDNA copy number and stability. For example, mutations in the mitochondrial thymidine kinase gene cause mitochondrial depletion myopathy (Saada *et al*., 2001), mutations in the thymidine phosphorylase gene result in mitochondrial neurogastrointestinal encephalomyopathy (Nishino *et al*., 1999), and mutations in the deoxyguanosine kinase gene cause hepatocerebral mtDNA depletion syndrome (Mandel *et al*., 2001). Although these findings and those of others (Bestwick *et al*., 1982; Tang *et al*., 2000) implicate alterations in cellular dNTP pools in the maintenance

This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E05–01–0053) on April 13, 2005.

[D] The online version of this article contains supplemental material at *MBC Online* (http://www.molbiolcell.org).

† These authors contributed equally to this work.

¶ Present address: Laboratory of Molecular Genetics, The Rockefeller University, 1230 York Ave., Box 305, New York, NY 10021.

Address correspondence to: Gerald S. Shadel (gerald.shadel@yale.edu).

Abbreviations used: dNTP, deoxyribonucleoside triphosphate; mtDNA, mitochondrial DNA; RNR, ribonucleotide reductase.

of mtDNA in humans, how dNTPs are sensed and regulated with regard to mtDNA replication and copy number is currently unknown.

The budding yeast *Saccharomyces cerevisiae* has proven a valuable model system in which to probe mechanisms of mtDNA replication and stability (Shadel, 1999). In this regard, the abundant high mobility group-box, mtDNA-binding protein Abf2p has been shown to be a key player in these processes (Zelenaya-Troitskaya *et al*., 1998). Abf2p has multiple functions in mtDNA metabolism, including stabilization of recombination intermediates (MacAlpine *et al*., 1998), segregation and dynamics of nucleoids (Newman *et al*., 1996; Okamoto *et al*., 1998; Zelenaya-Troitskaya *et al*., 1998), and oxidative DNA damage resistance (O'Rourke *et al*., 2002). It is also one of the first protein factors proposed to have a direct role in regulating mtDNA copy number. Specifically, in glycerol medium, where mitochondrial respiration is required for survival, deletion of the *ABF2* gene results in an ~50% reduction of mtDNA copy number, whereas moderate overexpression of Abf2p increases copy number ~1.5- to 2-fold (Zelenaya-Troitskaya *et al*., 1998). It is worth emphasizing that the ~78-kb mtDNA genome in yeast is present normally at 20–50 copies/cell comprising 10–20% of the total cellular DNA (Williamson and Fennell, 1979). Therefore, a doubling of mtDNA copy number is a substantial and significant increase in total cellular DNA in this organism.

The human homolog of Abf2p is mitochondrial transcription factor A (h-mtTFA or TFAM) (Parisi and Clayton, 1991; Shadel and Clayton, 1993). Interestingly, h-mtTFA has evolved to provide a critical role in transcription initiation that involves a C-terminal tail that is not present in yeast Abf2p (Dairaghi *et al*., 1995) and binds to the transcription factors h-mtTFB1 and h-mtTFB2 to facilitate promoter recognition (McCulloch and Shadel, 2003; Shadel, 2004). Because mtDNA replication in mammals is likely primed by RNA derived from transcription, h-mtTFA has a dual role in gene expression and mtDNA replication (Shadel and Clayton, 1997). Recently, the abundance of h-mtTFA has been reported to be greater than previously thought (Alam *et al*., 2003), and an additional role for vertebrate mtTFA homologues in packaging mtDNA, in much the same way as its yeast counterpart, has been postulated by several groups (Shen and Bogenhagen, 2001; Ekstrand *et al*., 2004; Kanki *et al*., 2004; Matsushima *et al*., 2004). In fact, these reports suggest that h-mtTFA can influence mtDNA copy number primarily, if not exclusively, through its proposed DNA packaging function and therefore independently of its role in transcription. However, if and how the abundance or activity of mtTFA dynamically regulates mtDNA copy number awaits further clarification (Maniura-Weber *et al*., 2004). Furthermore, whether other pathways exist to dynamically regulate mtDNA copy number has not been addressed adequately.

Pif1p is a conserved DNA helicase (Zhou *et al*., 2000) that resides both in the nucleus and mitochondria in *S. cerevisiae* (Foury and Lahaye, 1987; Schulz and Zakian, 1994). Our previous analysis of the petite phenotype of *pif1* null strains led us to postulate a role for this unique helicase in the repair or tolerance of oxidative mtDNA damage, perhaps by governing the rate of mtDNA replication or regulating mtDNA copy number (O'Rourke *et al*., 2002). Consistent with this model, we reported recently (O'Rourke *et al*., 2005) that the mtDNA instability (petite-induction) phenotype of a *pif1* null strain can be partially rescued by deletion of the *RRM3* gene, encoding the related DNA helicase Rrm3p (Ivessa *et al*., 2002), or by overexpression of *RNR1*, the gene encoding a large subunit of ribonucleotide reductase. Deletion of *RRM3* or overexpression of *RNR1* activates or partially recapitulates, respectively, signaling through the conserved Mec1/Rad53 intra-S-phase checkpoint pathway (Ivessa *et al*., 2003; O'Rourke *et al*., 2005), which results in increased de novo synthesis of dNTPs as a primary endpoint (Zhao *et al*., 1998). Together, these data led us to postulate that Pif1p, Rrm3p, and dNTP pool regulation by the Mec1/Rad53 pathway are critical for mtDNA stability (O'Rourke *et al*., 2005). In this report, we demonstrate that mtDNA copy number is dynamically regulated by activation of the Mec1/Rad53 pathway, thereby defining the first conserved signaling pathway that controls cellular mtDNA copy number.

MATERIALS AND METHODS

Yeast Strains and Plasmids

The parental strains used in this study were DBY2006 (*MATα his3-Δ200 leu2-3, -112 ura3-52 trp1Δ1 ade2*) and Y300 (*MATa his3-11, 15 leu2-3, -112 ura3-1 trp1-1 ade2-1 can1-100*). Specific gene knockouts to produce the *pif1*, *rrm3*, *rad53*, and *sml1* null mutations in DBY2006 or Y300 were generated by standard recombination-mediated gene replacement of the desired wild-type locus with a nutritional or drug-selectable marker. The *PIF1* gene was replaced by the *KANMX4* (G418 resistance) cassette in DBY2006, the *RRM3* gene was replaced by the *TRP1* gene, the *SML1* gene was replaced by the *HIS3* gene, and the *RAD53* gene was replaced by *URA3*. The plasmids pRS313-ABF2 (*ABF2 CEN HIS3*) and pRS316-ABF2 (*ABF2 CEN URA3*) were used to moderately overexpress Abf2p from its own promoter as described previously (Parisi *et al*., 1993; Zelenaya-Troitskaya *et al*., 1998). The plasmid pBAD70 (2μ *TRP1 RNR1*) used to overexpress the *RNR1* gene has been described previously (Huang and Elledge, 1997). The plasmids pRS313, pRS316, and pRS304 (2μ *TRP1*) were used as a control plasmids with no insert when indicated (Sikorski and Hieter, 1989).

Yeast Growth and Petite-Induction Assays

Yeast strains were grown at 30°C on standard media preparations: rich glucose (YPD), rich glycerol (YPG), or synthetic dextrose (SD) with appropriate nutritional supplements as described previously (O'Rourke *et al*., 2002). Yeast strains were maintained on YPG (if plasmid selection was not required) or appropriate SD (if plasmid selection was required) solid medium. The petite-induction assays were carried out as described previously (O'Rourke *et al*., 2002), except that when strains harboring plasmids were involved the petite-induction step was carried out in SD medium rather than YPD. And, in some cases, petite formation was measured on petite-indicator medium (YPG + 0.1% dextrose) rather than by plating identical samples onto YPD and YPG.

mtDNA Copy Number Analysis

The relative amount of mtDNA and nuclear DNA was determined using a quantitative real-time PCR strategy. We routinely use one of two protocols for this measurement, which differ only in the fluorescence method that is used to measure the progress of the PCR reaction. Protocol 1 involves two sets of standard PCR primers and two fluorescently labeled Taqman probes to quantify the amplification signals from the mtDNA target and nuclear target simultaneously in the same reaction, whereas protocol 2 involves separate PCR reactions for the mtDNA and the nuclear DNA assessment and uses SYBR Green to follow the PCR reaction instead of Taqman probes. These two protocols yield virtually identical results (e.g., *rrm3*Δ in Figure 1 was analyzed using protocol 1 and *rrm3*Δ in Figures 2b and 3a was analyzed using protocol 2) and are described in detail below.

In protocol 1 (Figures 1 and 2a), the yeast strains to be analyzed were grown in 10 ml of liquid YPG medium to an optical density (at 600 nm) of 0.6–0.8. The cultures were harvested by centrifugation, and total cellular nucleic acids were isolated using a "smash and grab" protocol essentially as described previously (Hoffman and Winston, 1987). The nucleic acid pellet was dissolved in 100 μl of TE buffer containing DNase-free RNase A (10 μg/ml) and incubated at 37°C for 1.5 h. This DNA was quantified by absorbance at 260 nm and used in a duplex, quantitative, real-time PCR reaction (25-μl total volume) by using the Taqman PCR core reagent kit (Applied Biosystems, Foster City, CA) as follows: 1× buffer A; 200 nM each of dATP, dGTP, and dCTP; 400 nM dUTP; 5.5 mM $MgCl_2$; 125 nM each primer (four total; see below); and 200 nM each probe (two total; see below). The PCR reaction was performed in 96-well format using a Bio-Rad iCycler and analyzed using and accompanying iCycler IQ version 3.1 software.

Each duplex PCR reaction simultaneously measured amplification of the nuclear *ACT1* gene and the mtDNA-encoded *COX1* gene. For each gene, this involved a set of two PCR primers and an internal probe oligonucleotide that is conjugated to a unique fluorophor at its 5′ end (HEX for *ACT1* and 6-FAM for *COX1*) and a fluorescence quencher moiety (BHQ-1 for *ACT1* and TAMRA for *COX1*) at its 3′ end. Primers and probes were synthesized by Integrated

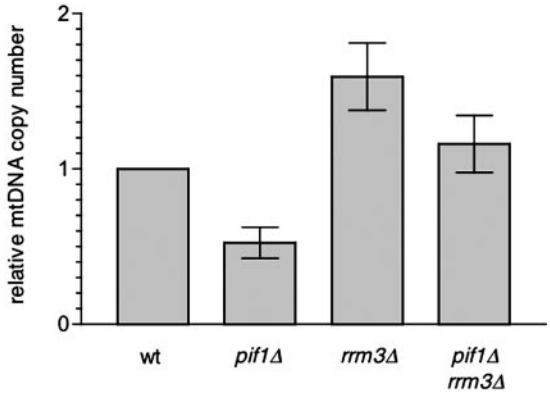

Figure 1. Pif1p and Rrm3p reciprocally affect mtDNA copy number. The relative mtDNA copy number (plotted on the ordinate) of a *pif1* null (*pif1*Δ), *rrm3* null (*rrm3*Δ), and the corresponding double-mutant (*pif1*Δ *rrm3*Δ) strain is shown compared with that of the isogenic wild-type (wt) strain DBY2006. The wt mtDNA copy number was given a value of 1 so that the fold difference in mtDNA copy number [relative to wild type (wt)] is easily compared (see *Materials and Methods* and Supplemental Table 1 for details on the calculations). The mean of three independent measurements of mtDNA copy number for each strain along with the SD of the measurement observed between the three experiments (brackets) is plotted (Supplemental Table 1).

DNA Technologies (Coralville, IA), and their sequences were as follows: ACT1-forward, 5′-GTATGTGTAAAGCCGGTTTTG-3′; ACT1-reverse, 5′-CATGATACCTTGGTGTCTTGG-3′; ACT1 probe, 5′-CGCTCCTCGTGCTGTCTTCCCA-5′; COX1-forward, 5′-CTACAGATACAGCATTTCCAAGA-3′; COX1-reverse, 5′-GTGCCTGAATAGATGATAATGGT-3′; and COX1 probe, 5′-AGTTCACCCTGTACCAGCACCTGA-5′.

The PCR protocol was empirically designed around a primer annealing temperature of 58°C, a probe annealing temperature of ~68°C, and 45 cycles of real-time PCR amplification and data collection as described in the iCycler manual. For each strain analyzed, the copy number of the mtDNA and the nuclear DNA is calculated using the threshold cycle number (C_T), making sure the reactions were in a linear range of detection, by running a variety of dilutions. This is done in triplicate to obtain an average value for C_T for *COX1* and *ACT1*, C_T (*COX1*) and C_T (*ACT1*), respectively. The difference (ΔC_T) between these averages C_T (*COX1*) − C_T (*ACT1*) is determined and used to arrive at a value of the relative mtDNA copy number (RCN) of each strain, which is equal to $2^{\Delta CT}$. The fold change in the RCN of each mutant strain is calculated by dividing its RCN by the wild-type RCN value, which yields the fold change compared with wild type, which now has a value of 1. At least three experiments of this type were run for each strain analyzed, and the mean fold change in RCN (labeled in the figures as relative mtDNA copy number) ± 1 SD of these independent trials are plotted in the figures (Supplemental Table 1). In our initial characterization of the *pif1*Δ, *rrm3*Δ and *pif1*Δ *rrm3*Δ strains, we also measured mtDNA copy number by using a quantitative Southern blotting approach with different *ACT1* and *COX1* probes. We observed similar results by this method (Supplemental Figure 1).

In protocol 2 (Figures 2b, 3, and 4), yeast growth and nucleic acid isolation were the same as in protocol 1, except 5-ml cultures were used. Various dilutions (≥80-fold) of the template DNA were used in the final PCR reaction to ensure measurements were within the linear range. Nuclear DNA and mtDNA PCR were done in individual wells for each sample dilution in the following standard 50-μl SYBR Green reaction: 25 μl of Bio-Rad iQ SYBR Green Supermix, 10 μl of diluted template, 3 μl of H_2O, and 1 μl of each nuclear primer (*ACT1* forward and reverse primers, same as protocol 1) or each mtDNA primer (*COX3* forward and reverse primers, same as protocol 1). Each primer was stored separately as a working stock of 6.25 μM, and the final concentration in the above-mentioned reaction for each primer was 125 nM. Analysis of the data to generate the relative mtDNA copy number of the strains was performed as described above for protocol 1.

RESULTS

The Pif1p and Rrm3p Helicases Reciprocally Influence mtDNA Copy Number

We reported previously that the Pif1p DNA helicase has a critical role in mtDNA damage resistance, mutagenesis, and

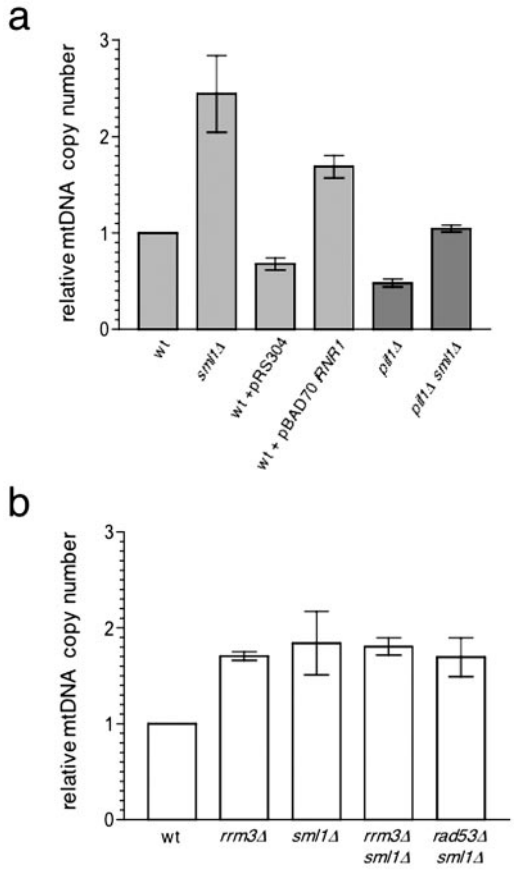

Figure 2. Activity of the Mec1/Rad53 signaling pathway regulates mtDNA copy number. Plotted in the same manner as in Figure 1 is the relative mtDNA copy number of the strains indicated. (a) Strains analyzed were *sml1* null (*sml1*Δ), *pif1* null (*pif1*Δ), *pif1* null and *sml1* null (*pif1*Δ *sml1*Δ), wild type with the plasmid pBAD70 that overexpresses RNR1 (wt + pBAD70/*RNR1*), and wild type with empty vector control for pBAD70 comparison (wt + pRS304). (b) Strains analyzed were wild-type DBY2006 (wt), *rrm3* null (*rrm3*Δ), *sml1* null (*sml1*Δ), *rrm3* and *sml1* null (*rrm3*Δ *sml1*Δ), and *rad53* and *sml1* null (*rad53*Δ *sml1*Δ).

stability that involves its cooperation with the related DNA helicase Rrm3p (O'Rourke *et al*., 2002, 2005). Based on these original observations, we speculated that Pif1p and Rrm3p might have a role in regulating mtDNA replication or copy number (O'Rourke *et al*., 2002, 2005). To test this hypothesis directly, we analyzed mtDNA copy number in *pif1* and *rrm3* single-mutant and *pif1 rrm3* double-mutant strains using a quantitative, real-time PCR assay (Supplemental Table 1). In glycerol medium (YPG), which selects for mitochondrial respiration competence, the mtDNA copy number of a *pif1* null mutant strain was reduced to ~50% of that in the corresponding isogenic wild-type strain DBY2006 (Figure 1). This reduction of copy number in the *pif1* strain was completely restored in the *pif1 rrm3* double mutant strain (Figure 1), which had wild-type levels of mtDNA. These data are consistent with our previously published results that deletion of *rrm3* also rescues the mtDNA instability (petite induction) phenotype of a *pif1* null strain (O'Rourke *et al*.,

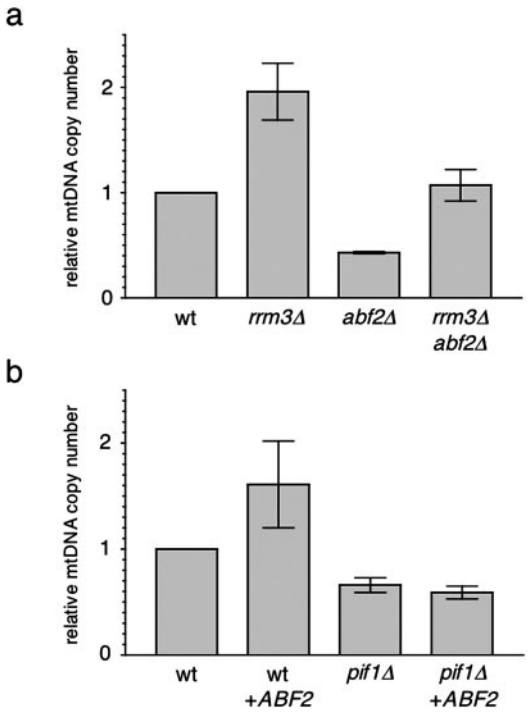

Figure 3. Increased mtDNA copy number via activation of the Mec1/Rad53 pathway can occur independently of Abf2p. Plotted in the same manner as in Figure 1 is the relative mtDNA copy number of the strains indicated. (a) Strains analyzed were wild-type DBY2006 (wt), *rrm3* null (*rrm3*Δ), *abf2* null (*abf2*Δ), and *rrm3* null and *abf2* null (*rrm3*Δ *abf2*Δ). (b) Strains analyzed were wild-type DBY2006 with a control plasmid lacking the *ABF2* gene insert (wt), wild type with a plasmid overexpressing ABF2 from its own promoter (wt + ABF2), *pif1* null with a control plasmid lacking the *ABF2* gene insert (*pif1*Δ), and *pif1* null with a plasmid overexpressing *ABF2* from its own promoter (*pif1*Δ + *ABF2*).

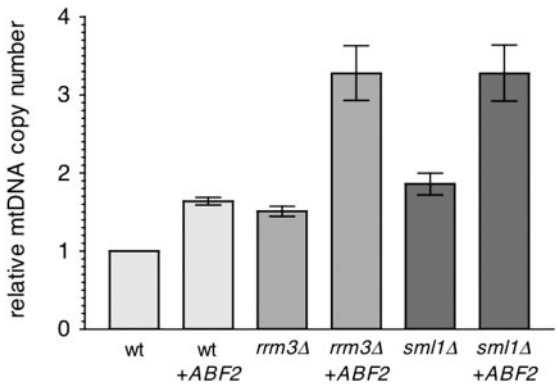

Figure 4. Abf2p and the Mec1/Rad53 pathways for increased mtDNA copy number are additive. Plotted in the same manner as in Figure 1 is the relative mtDNA copy number of the strains indicated. The strains analyzed were wild-type DBY2006 with a control plasmid lacking the *ABF2* gene insert (wt), wild-type with a plasmid overexpressing ABF2 from its own promoter (wt + *ABF2*), *rrm3* null with a control plasmid lacking the *ABF2* gene insert (*rrm3*Δ), *rrm3* null with a plasmid overexpressing *ABF2* from its own promoter (*rrm3*Δ + *ABF2*), *sml1* null with a control plasmid lacking the *ABF2* gene insert (*sml1*Δ), and *sml1* null with a plasmid overexpressing *ABF2* from its own promoter (*sml1*Δ + *ABF2*).

2005). Of particular additional interest was the striking observation that deletion of *RRM3* alone resulted in a near doubling of the mtDNA copy number (Figure 1). To ensure the observed effects on copy number were not strain dependent, we repeated this analysis in a different yeast genetic background (Y300), where virtually identical results were obtained (Zhang and Shadel, unpublished data). Together, these results (Figure 1) are the first to indicate that Pif1p and Rrm3p can influence mtDNA copy number, perhaps in a manner analogous to their cooperative roles in the nucleus.

Signaling through the Mec1/Rad53 Pathway Dynamically Influences mtDNA Copy Number

We showed previously that the kinase Rad53p is phosphorylated in *rrm3* and *pif1 rrm3* strains and that the mtDNA stability phenotype of a *pif1* null mutation is partially rescued by overexpression of *RNR1*, indicating that signaling through the Mec1 checkpoint pathway is responsible for the increased mtDNA stability in these strains (O'Rourke *et al*., 2005). Based on these observations and the fact that mtDNA copy number is increased in *rrm3* null strains (Figure 1), we tested the hypothesis that mtDNA copy number is dynamically regulated by activity of the Mec1/Rad53 pathway (Figure 5). To do this, we measured mtDNA content in wild-type and *pif1* null strains that contain increased activity of ribonucleotide reductase (RNR), the rate-limiting enzyme in dNTP synthesis. Activation of the Mec1 pathway causes transcriptional induction of the *RNR* genes (Huang and Elledge, 1997) and degradation of Sml1p (Zhao *et al*., 1998, 2001), an inhibitor of the RNR complex, which together lead to a well-documented increase in dNTP synthesis necessary for S-phase completion or DNA repair. For example, deletion of the *SML1* gene alone leads to a ~2.5-fold increase in all four dNTPs (Zhao *et al*., 1998). As was the case with *rrm3* null strains (Figure 1), we observed an approximately two-fold increase in mtDNA copy number in strains that have *SML1* deleted or that overexpress *RNR1* from a multicopy plasmid (Figure 2a). In addition, like deletion of *RRM3* (Figure 1), deletion of *SML1* restored mtDNA copy number to normal levels in a *pif1* null strain (Figure 2a). Because overexpression of *RNR1* or deletion of *SML1* recapitulates a primary known endpoint of Mec1/Rad53 pathway activation (increased dNTP synthesis via elevated cellular RNR activity), these results indicate that increased dNTP synthesis is a significant parameter that influences mtDNA copy number via the Mec1/Rad53 signaling pathway.

To ensure that the effects of deleting *SML1* and overexpressing *RNR1* were due to similar endpoints in Mec1/Rad53 pathway activation as predicted (Figure 5), we determined whether the petite-induction phenotype of a *pif1* null strain is rescued by deletion of *SML1*. Accordingly, deletion of *SML1* partially rescued the petite-induction phenotype of a *pif1* null strain (Table 1), in fact, to virtually the same degree as did the overexpression of *RNR1* we reported previously (O'Rourke *et al*., 2005).

To further address the role of signaling through the Mec1/Rad53 pathway on mtDNA copy number, we next determined whether *RRM3*, *RAD53*, and *SML1* are all in the same genetic pathway with regard to mtDNA copy number. To do this, we compared mtDNA copy number in *sml1*Δ *rad53*Δ and *rrm3*Δ *sml1*Δ double-mutant strains to that in the corresponding *sml1*Δ and *rrm3*Δ single mutant strains. Deletion of the *RAD53* gene is lethal, which precludes the analysis of *rad53*Δ single mutant. However, its lethality is suppressed by simultaneous deletion of *SML1*, allowing a *rad53* null background to be analyzed (Zhao *et al*., 1998). We reasoned that if the increase in mtDNA copy number ob-

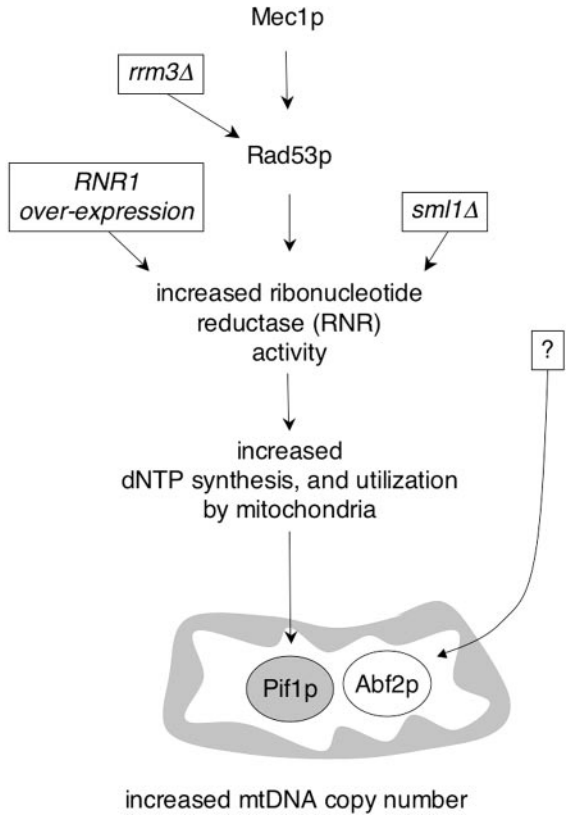

Figure 5. Proposed model for how signaling via the Mec1/Rad53 pathway influences mtDNA copy number. It is established that the Mec1/Rad53 pathway is responsive to nuclear DNA damage, nuclear replication stress, and S-phase signals that result in increased dNTPs needed to allow replication and repair to proceed. Based on the present study, we propose that signaling through this pathway can also regulate mtDNA copy number, primarily (although not necessarily exclusively; see *Discussion*) through changes in RNR activity and up-regulation or alteration of cellular dNTP pools. Arrows denote points in the pathway that are activated by the indicated stimulus (for example, deletion of *SML1* results in increased activity of RNR). Several conditions tested in this work that resulted in increased mtDNA copy number and how they influence this pathway are shown, including deletion of *RRM3* (*rrm3*Δ) or *SML1* (*sml1*Δ) and overexpression of *RNR1*. Mitochondrial Pif1p is drawn at the end of this pathway because its petite-induction phenotype is rescued by overexpression of *RNR1* (O'Rourke *et al.*, 2005), deletion of *RRM3* (O'Rourke *et al.*, 2005), or deletion of *SML1* (Table 1), indicating that a process it is involved in is responsive to alterations in dNTP pools. Finally, Abf2p is shown as a separate pathway that can increase mtDNA copy number. That upstream signals which regulate the abundance (or activity) of Abf2p to regulate mtDNA copy number are currently unknown is indicated by a question mark.

served in an *rrm3*Δ strain (Figure 1) is mediated through activation of the Mec1/Rad53 pathway, then *sml1*Δ should be epistatic to *rrm3*Δ and *rad53*Δ because *SML1* is the most downstream gene in the proposed pathway (Figure 5). As shown in Figure 2b, each double-mutant strain had the same approximate twofold amount of mtDNA (relative to wild type) as the corresponding single mutant strains, indicating that this indeed is the case. We also attempted to analyze mtDNA copy number in a *rad53*Δ *sml1*Δ *rrm3*Δ triple mutant strain, however, because it exhibited a severe growth defect in YPG medium (Lebedeva and Shadel, unpublished observations), we were unable to assess this with any confidence.

Table 1. Results of petite-induction assays on *sml1*Δ and *pif1*Δ single-mutant and *sml1*Δ *pif1*Δ double-mutant strains

Relevant strain genotype[a]	% Respiration competent[b]
Wild-type	98.3 ± 0.6
*sml1*Δ	98.8 ± 0.4
*pif1*Δ	23.3 ± 4.6
*sml1*Δ *pif1*Δ	64.0 ± 4.0

[a] Strains are derivatives of the wild-type strain DBY2006.

[b] Mean percentages of respiration-competent cells (±1 SD from the mean) remaining after 24 h of growth (approximately six generations) in YPD liquid media.

mtDNA Copy Number Regulation by the Mec1/Rad53 Pathway Can Occur Independently of Abf2p

Yeast Abf2p is an abundant mitochondrial DNA-binding protein that, among its many proposed functions, influences mtDNA copy number (Zelenaya-Troitskaya *et al.*, 1998). In fact, as discussed in the Introduction, modulation of Abf2p levels (or its mammalian homologues mtTFA) has been postulated by several investigators to be the major pathway for mtDNA copy number regulation in yeast and in mammalian cells via its DNA packaging function. It was therefore of interest to determine whether the Mec1/Rad53 pathway was influencing mtDNA copy number via the same or a different pathway as Abf2p. To address this we measured mtDNA copy number in *abf2* null strains that simultaneously have an activated Mec1/Rad53 pathway due to deletion of *RRM3*. Consistent with the observations of Butow and colleagues (Zelenaya-Troitskaya *et al.*, 1998), deletion of *ABF2* resulted in an ~50% reduction in mtDNA (Figure 3a). As was the case for a *pif1* null strain (Figure 1), deletion of *RRM3* also restored the mtDNA copy number in the *abf2*Δ strain to normal levels (Figure 3a), demonstrating that modulation of mtDNA via an activated Mec1/Rad53 pathway can occur in the complete absence of Abf2p. The increase in mtDNA copy number in the *rrm3*Δ strain is shown in Figure 3A as a positive control for this experiment. However, in contrast to a *pif1*Δ strain (O'Rourke *et al.*, 2005), deletion of *RRM3* did not significantly rescue the petite-induction phenotype of the *abf2*Δ strain (Table 2), despite the fact that mtDNA copy number was restored to wild-type levels (Figure 3a).

As a second test of the concept that regulation of mtDNA copy number by the Mec1/Rad53 pathway can occur independently of Abf2p, we performed the following experi-

Table 2. Results of petite-induction assays on *abf2*Δ single-mutant and *abf2*Δ *rrm3*Δ double-mutant strains

Relevant strain genotype[a]	% Respiration competent[b]
Wild-type	96.7 ± 18.1
*abf2*Δ	3.8 ± 1.6
*abf2*Δ *rrm3*Δ	6.1 ± 2.5

[a] Strains are derivatives of the wild-type strain DBY2006.

[b] Mean percentages of respiration-competent cells (±1 SD from the mean) remaining after 24 h of growth (approximately six generations) in YPD liquid media.

ment. Abf2p was moderately overexpressed from a *CEN* plasmid in a wild-type and isogenic *pif1* null strain that has decreased mtDNA copy number (Figure 1). As reported by Butow and colleagues (Zelenaya-Troitskaya *et al*., 1998), this resulted in an ~1.5- to 2-fold increase in mtDNA copy number in a wild-type strain (Figure 3b). However, unlike deletion of *RRM3* (Figures 1 and 2b) or *SML1* (Figure 2a), overexpression of Abf2p was unable to increase mtDNA copy number in the *pif1* null strain (Figure 3b). These data indicate that increased mtDNA copy number imparted by overexpression of Abf2p or activation of the Mec1/Rad53 pathway is not achieved via the same mechanism.

Increased Levels of Abf2p and Activation of the Mec1/Rad53 Pathway Additively Increase mtDNA Copy Number

Based on the data presented in the previous section (Figure 3), we concluded that the increase in mtDNA copy number resulting from Abf2p overexpression or by activation of Mec1/Rad53 signaling is occurring largely, if not exclusively, through separate pathways. One prediction of this model is that the two pathways should not be epistatic. That is, if the two pathways are independent, then their effects most likely will be additive rather than one pathway masking the effect of the other. To examine this, we overexpressed Abf2p in *rrm3*Δ or *sml1*Δ strains that recapitulate an activated Mec1/Rad53 pathway. As already shown, overexpression of Abf2p (Figure 3b), deletion of *RRM3* (Figures 1, 2a, and 3b), or deletion of *SML1* (Figure 2a), each result in ~1.5-fold increase in mtDNA copy number (Figure 4). However, when Abf2p was overexpressed in the *rrm3*Δ or *sml1*Δ strains, mtDNA copy number was increased approximately threefold above that of the corresponding wild-type strain (Figure 4). These data show that the Abf2p and Mec1/Rad53 pathways for mtDNA copy number amplification are additive and therefore most likely represent independent mechanisms of mtDNA copy number control.

DISCUSSION

There are two main conclusions that we reach based on the results of this study. The first is that there is a direct link between the conserved Mec1/Rad53 nuclear checkpoint pathway and mtDNA copy number (Figure 5), which to our knowledge is the first conserved signaling pathway to be implicated in this process. The second is that regulation of mtDNA copy number by the Mec1/Rad53 pathway can occur independently of the DNA-packaging protein Abf2p, which is involved in a previously elucidated mechanism of mtDNA copy number control, and therefore represents a novel pathway for mitochondrial genome regulation.

A primary endpoint of activation of the Mec1/Rad53 pathway is an increase in dNTP synthesis (Figure 5), mediated by transcriptional induction of the *RNR* genes, encoding the catalytic and regulatory subunits of the two forms of RNR (Huang and Elledge, 1997), and degradation of the RNR inhibitor protein Sml1p (Zhao *et al*., 1998, 2001; Zhao and Rothstein, 2002). We provide several lines of evidence that it is this increase in size (or utilization) of the cellular dNTP pool via signaling through the Mec1/Rad53 pathway that can dynamically regulate mtDNA copy number. First, we (O'Rourke *et al*., 2005) and others (Ivessa *et al*., 2003) have shown that deletion of the *RRM3* gene results in a nuclear DNA replication checkpoint response and phosphorylation of the Rad53p kinase. Under these conditions, we observe a reproducible ~1.5- to 2-fold increase in mtDNA copy number in otherwise wild-type strains (Figures 1, 2b, 3a, and 4). In addition, deletion of *RRM3* rescues the mtDNA depletion we observe in *pif1* null (Figure 1) and *abf2* null (Figure 3a) strains. Second, overexpression of *RNR1* or deletion of *SML1*, both of which recapitulate an endpoint of an activated Mec1/Rad53 pathway (Figure 5), result in an approximately twofold increase in mtDNA copy number (Figure 2a) similar to that observed in *rrm3*Δ strains. And third, results of epistasis experiments are consistent with the placement of *RRM3*, *RAD53*, and *SML1* in a linear genetic pathway of mtDNA copy number regulation (Figure 2b).

Together, our data strongly indicate that the relative activity of the Mec1/Rad53 pathway is an important mechanism through which cells can regulate mtDNA copy number. In particular, our data point to fluctuations of cellular dNTP pools in general as an important regulatory parameter for mtDNA copy number (Figure 5). This conclusion is supported further by early work with cell division control (CDC) mutants that also revealed a connection between regulation of cellular dNTP pools (via *CDC8* and *CDC21*) and mtDNA replication rates (Newlon and Fangman, 1975). However, given that there is some recent preliminary evidence that Rrm3p is localized in mitochondria (Prokisch *et al*., 2004) as well as in the nucleus, we note here, as we discussed previously (O'Rourke *et al*., 2005), that our data do not discount the possibility that Rrm3p has a direct role in mtDNA metabolism. In addition, our data do not address directly whether signaling through the Mec1/Rad53 pathway can affect mtDNA copy number through mechanisms in addition to increasing dNTP pools via RNR activity or that other pathways might also be involved.

Our assertion that mtDNA copy number and stability are governed by the activity of the Mec1/Rad53 pathway is consistent with several previously published observations. First, mutations in several genes in this pathway, including *RNR* genes (Huang and Elledge, 1997), *DUN1* (Zhao and Rothstein, 2002), and *MEC1* and *RAD53* (Zhao *et al*., 2001), result in increased petite mutant formation due to mtDNA instability in yeast. Second, we have shown previously that increased expression of *RNR1* or deletion of *RRM3* partially rescues the petite-induction phenotype of a *pif1* null mutation (O'Rourke *et al*., 2005). Third, in this study, we have shown that deletion of *PIF1* results in reduced mtDNA copy number (Figures 1, 2a, and 3b) that is restored in an *sml1* null background (Figure 2a). Finally, the petite-induction phenotype of a *pif1* null mutation is partially rescued by deletion of *SML1* (Table 1). Together, these results demonstrate that alterations in mtDNA copy number are a significant component of the mechanism causing the mtDNA instability phenotypes of Mec1/Rad53 pathway mutant strains reported by others and support our main conclusion that this pathway is involved in regulating mtDNA content and stability through modulation of RNR activity and dNTP pools. It is also relevant in this regard that overexpression of *RNR1* rescues the petite-induction phenotype caused by a point mutation in (or haplo-insufficiency of) *MIP1*, encoding mitochondrial DNA polymerase γ (Lecrenier and Foury, 1995), indicating that the mtDNA replication machinery is responsive to alterations in the cellular dNTP pools.

Yeast Abf2p has previously been implicated in mtDNA copy number regulation (Zelenaya-Troitskaya *et al*., 1998). Abf2p is an abundant mtDNA-binding protein that results in increased mtDNA copy number when moderately overexpressed, reduced mtDNA levels in glycerol medium when absent (Figure 3a; Zelenaya-Troitskaya *et al*., 1998), and low mtDNA copy number and increased mtDNA instability when expressed at higher levels (Zelenaya-Troitskaya *et al*., 1998). It is generally thought that Abf2p can stabilize

mtDNA through a DNA-packaging or nucleoid organization function and thereby increase mtDNA copy number when overexpressed moderately. Given that Abf2p is a previously assigned regulator of mtDNA copy number, it was important to determine whether the regulation of mtDNA copy number by the Mec1/Rad53/dNTP pathway elucidated here (Figure 5) can occur independently of Abf2p. We provide several lines of evidence that suggest that it can. First, as is the case in an otherwise wild-type background, deletion of *RRM3* results in an approximately twofold increase in mtDNA copy number in an *abf2* null background (Figure 3a), clearly demonstrating that activation of the Mec1/Rad53 pathway by deletion of *RRM3* results in increased mtDNA copy number in the absence of Abf2p. Second, the increase in mtDNA copy number observed by overexpression of Abf2p is additive to that observed in *RRM3*- or *SML1*-deleted strains (Figure 4), indicating independent contributions of Abf2p and an activated Mec1/Rad53 pathway to increased mtDNA copy number. And third, unlike deletion of *RRM3* and *SML1* (Figures 1 and 2a), increased expression of Abf2p cannot rescue the copy number defect of a *pif1* null strain (Figure 3b), indicating again that an activated Mec1/Rad53 pathway and overexpression of Abf2p are not operating through the same mechanism. However, we acknowledge that the fact that the Mec1/Rad53 pathway can influence mtDNA copy number independently of Abf2p does not necessarily mean that these two pathways are entirely independent. Deciphering if and how these pathways cooperate to establish mtDNA levels in response to changing cellular demands represents an important area for future investigation.

Although there is often a correlation between mtDNA copy number and mtDNA stability (as measured by petite-induction), it is important to emphasize that this is not always the case. For example, deletion of *RRM3* (O'Rourke *et al*., 2005), deletion of *SML1* (Table 1), or overexpression of *RNR1* (O'Rourke *et al*., 2005) each partially rescues the petite-induction phenotype of a *pif1* null mutation. All of these conditions likewise lead to increased mtDNA copy number in *pif1* null and/or wild-type strains (Figures 1 and 2a). However, as reported previously for deletion of *RRM3* and overexpression of *RNR1* (O'Rourke *et al*., 2005), deletion of *SML1* only partially rescues the petite-induction phenotype of a *pif1* null strain (Table 1). Thus, only partial rescue of the *pif1* null phenotype is achieved, despite the fact that mtDNA copy number is restored to normal levels under these conditions (Figure 2a). Clearly, mtDNA instability in a *pif1* null strain is not entirely due to its reduced mtDNA copy number. The same is true of an *abf2* null strain. Although deletion of *RRM3* clearly rescues the mtDNA copy number depletion of an *abf2* null strain (Figure 3a), it has little or no effect on its petite-induction rate (Table 2). This is most likely explained by the fact that Abf2p (and very likely also Pif1p) has multiple functions that impact mtDNA stability and expression, only a subset of which can be overcome by increasing dNTP pools or mtDNA copy number through the Mec1/Rad53 pathway.

The discovery of a conserved pathway (the Mec1/Rad53 pathway; Figure 5) for mtDNA copy number regulation in yeast that can operate independently of Abf2p is likely relevant to the mechanism of mtDNA copy number regulation in metazoans. For example, it has been known for some time that the mammalian homologues of Abf2p, mtTFA/Tfam, can influence mtDNA copy number. Reduced levels of h-mtTFA are observed in patients with mtDNA depletion (Larsson *et al*., 1994) and in cultured cells that are artificially depleted of mtDNA (Seidel-Rogol and Shadel, 2002). In addition, moderate overexpression of mtTFA in certain cultured cells results in increased mtDNA copy number (Ekstrand *et al*., 2004; Kanki *et al*., 2004; Matsushima *et al*., 2004) and decreased mtDNA copy number is observed in tissues of homozygous and heterozygous mtTFA knockout mice (Larsson *et al*., 1998). These and other observations have led many investigators to conclude that mtTFA is the key player in mtDNA copy number regulation (Shen and Bogenhagen, 2001; Ekstrand *et al*., 2004; Kanki *et al*., 2004; Matsushima *et al*., 2004). However, based on the results presented herein, we propose that the activity of mtTFA is likely not the only mechanism, and may not even be the primary mechanism, through which mtDNA copy number is regulated in mammalian cells.

The multicopy nature of the mitochondrial genome contributes significantly to the modulation of mitochondrial function in different tissue types (Moraes *et al*., 1991). In addition, mitochondrial function is dynamic, responding to internal and external stimuli such as developmental signals (Shen and Bogenhagen, 2001), metabolic state (Wu *et al*., 1999; Zong *et al*., 2002), and exercise (Wu *et al*., 2002), indicating that signaling pathways exist to alter mtDNA content in response to cellular and environmental cues. Finally, depleted mtDNA levels lead to human diseases (Elpeleg *et al*., 2002), many of which presumably involve tissue-specific alterations in the cellular dNTP pool for nuclear and mtDNA replication and repair (Marti *et al*., 2003). Our finding that the conserved Mec1/Rad53 signaling pathway, which regulates cellular dNTP pools, controls mtDNA copy number in yeast (Figure 5) may shed new light onto why dNTP pool perturbations cause mtDNA-depletion syndromes in humans and strongly suggests that signaling pathways that involve the analogous protein kinases in human cells (i.e., ATM, ATR, Chk1, or Chk2) will likewise influence mtDNA copy number. Differential activity of these pathways in specific cell types may provide a mechanism underlying the natural variation in mtDNA copy number observed in various tissues or allow dynamic responses of mtDNA to environmental and metabolic cues, such as those observed during development or exposure to genotoxic stress. Our findings open new avenues of investigation toward understanding mtDNA dynamics and the role of mitochondrial dysfunction and altered mtDNA copy number in human disease and aging.

ACKNOWLEDGMENTS

We thank Dr. Steve Elledge for the pBAD70 plasmid. G.S.S. thanks Drs. Susan Kaech and David Stern and the Shadel laboratory group for critical insight, interpretation, and helpful comments on the manuscript. This work was supported by Program Project Grant P01 ES011163. Portions of this work also were supported by grant DAAD19-00-1-0560 from the Army Research Office awarded to G.S.S.

REFERENCES

Alam, T. I., Kanki, T., Muta, T., Ukaji, K., Abe, Y., Nakayama, H., Takio, K., Hamasaki, N., and Kang, D. (2003). Mitochondrial DNA is packaged with TFAM. Nucleic Acids Res. *31*, 1640–1645.

Bestwick, R. K., Moffett, G. L., and Mathews, C. K. (1982). Selective expansion of mitochondrial nucleoside triphosphate pools in antimetabolite-treated HeLa cells. J. Biol. Chem. *257*, 9300–9304.

Dairaghi, D. J., Shadel, G. S., and Clayton, D. A. (1995). Addition of a 29 residue carboxyl-terminal tail converts a simple HMG box-containing protein into a transcriptional activator. J. Mol. Biol. *249*, 11–28.

Elpeleg, O., Mandel, H., and Saada, A. (2002). Depletion of the other genome-mitochondrial DNA depletion syndromes in humans. J. Mol. Med. *80*, 389–396.

Ekstrand, M. I., Falkenberg, M., Rantanen, A., Park, C. B., Gaspari, M., Hultenby, K., Rustin, P., Gustafsson, C. M., and Larsson, N. G. (2004). Mitochondrial transcription factor A regulates mtDNA copy number in mammals. Hum. Mol. Genet. *13*, 935–944.

Foury, F., and Lahaye, A. (1987). Cloning and sequencing of the PIF gene involved in repair and recombination of yeast mitochondrial DNA. EMBO J. *6*, 1441–1449.

Hoffman, C. S., and Winston, F. (1987). A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. Gene *57*, 267–272.

Huang, M., and Elledge, S. J. (1997). Identification of RNR4, encoding a second essential small subunit of ribonucleotide reductase in *Saccharomyces cerevisiae*. Mol. Cell. Biol. *17*, 6105–6113.

Ivessa, A. S., Lenzmeier, B. A., Bessler, J. B., Goudsouzian, L. K., Schnakenberg, S. L., and Zakian, V. A. (2003). The *Saccharomyces cerevisiae* helicase Rrm3p facilitates replication past nonhistone protein-DNA complexes. Mol. Cell *12*, 1525–1536.

Ivessa, A. S., Zhou, J. Q., Schulz, V. P., Monson, E. K., and Zakian, V. A. (2002). *Saccharomyces* Rrm3p, a 5′ to 3′ DNA helicase that promotes replication fork progression through telomeric and subtelomeric DNA. Genes Dev. *16*, 1383–1396.

Kanki, T., Ohgaki, K., Gaspari, M., Gustafsson, C. M., Fukuoh, A., Sasaki, N., Hamasaki, N., and Kang, D. (2004). Architectural role of mitochondrial transcription factor A in maintenance of human mitochondrial DNA. Mol. Cell. Biol. *24*, 9823–9834.

Larsson, N. G., Oldfors, A., Holme, E., and Clayton, D. A. (1994). Low levels of mitochondrial transcription factor A in mitochondrial DNA depletion. Biochem. Biophys. Res. Commun. *200*, 1374–1381.

Larsson, N. G., Wang, J., Wilhelmsson, H., Oldfors, A., Rustin, P., Lewandoski, M., Barsh, G. S., and Clayton, D. A. (1998). Mitochondrial transcription factor A is necessary for mtDNA maintenance and embryogenesis in mice. Nat. Genet. *18*, 231–236.

Lecrenier, N., and Foury, F. (1995). Overexpression of the RNR1 gene rescues Saccharomyces cerevisiae mutants in the mitochondrial DNA polymerase-encoding MIP1 gene. Mol. Gen. Genet. *249*, 1–7.

MacAlpine, D. M., Perlman, P. S., and Butow, R. A. (1998). The high mobility group protein Abf2p influences the level of yeast mitochondrial DNA recombination intermediates in vivo. Proc. Natl. Acad. Sci. USA *95*, 6739–6743.

Mandel, H., *et al*. (2001). The deoxyguanosine kinase gene is mutated in individuals with depleted hepatocerebral mitochondrial DNA. Nat. Genet. *29*, 337–341.

Maniura-Weber, K., Goffart, S., Garstka, H. L., Montoya, J., and Wiesner, R. J. (2004). Transient overexpression of mitochondrial transcription factor A (TFAM) is sufficient to stimulate mitochondrial DNA transcription, but not sufficient to increase mtDNA copy number in cultured cells. Nucleic Acids Res. *32*, 6015–6027.

Marti, R., Nishigaki, Y., Vila, M. R., and Hirano, M. (2003). Alteration of nucleotide metabolism: a new mechanism for mitochondrial disorders. Clin. Chem. Lab. Med. *41*, 845–851.

Matsushima, Y., Garesse, R., and Kaguni, L. S. (2004). Drosophila mitochondrial transcription factor B2 regulates mitochondrial DNA copy number and transcription in Schneider cells. J. Biol. Chem. *279*, 26900–26905.

McCulloch, V., and Shadel, G. S. (2003). Human mitochondrial transcription factor B1 interacts with the C-terminal activation region of h-mtTFA and stimulates transcription independently of its RNA methyltransferase activity. Mol. Cell. Biol. *23*, 5816–5824.

Moraes, C. T. (2001). What regulates mitochondrial DNA copy number in animal cells? Trends Genet. *17*, 199–205.

Moraes, C. T., Shanske, S., Tritschler, H. J., Aprille, J. R., Andreetta, F., Bonilla, E., Schon, E. A., and DiMauro, S. (1991). mtDNA depletion with variable tissue expression: a novel genetic abnormality in mitochondrial diseases. Am. J. Hum. Genet. *48*, 492–501.

Newlon, C. S., and Fangman, W. L. (1975). Mitochondrial DNA synthesis in cell cycle mutants of *Saccharomyces cerevisiae*. Cell *5*, 423–428.

Newman, S. M., Zelenaya-Troitskaya, O., Perlman, P. S., and Butow, R. A. (1996). Analysis of mitochondrial DNA nucleoids in wild-type and a mutant strain of Saccharomyces cerevisiae that lacks the mitochondrial HMG box protein Abf2p. Nucleic Acids Res. *24*, 386–393.

Nishino, I., Spinazzola, A., and Hirano, M. (1999). Thymidine phosphorylase gene mutations in MNGIE, a human mitochondrial disorder. Science *283*, 689–692.

Okamoto, K., Perlman, P. S., and Butow, R. A. (1998). The sorting of mitochondrial DNA and mitochondrial proteins in zygotes: preferential transmission of mitochondrial DNA to the medial bud. J. Cell Biol. *142*, 613–623.

O'Rourke, T. W., Doudican, N. A., Mackereth, M. D., Doetsch, P. W., and Shadel, G. S. (2002). Mitochondrial dysfunction due to oxidative mitochondrial DNA damage is reduced through cooperative actions of diverse proteins. Mol. Cell. Biol. *22*, 4086–4093.

O'Rourke, T. W., Doudican, N. A., Zhang, H., Eaton, J. S., Doetsch, P. W., and Shadel, G. S. (2005). Differential involvement of the related DNA helicases Pif1p and Rrm3p in mtDNA mutagenesis and stability. Gene (*in press*).

Parisi, M. A., and Clayton, D. A. (1991). Similarity of human mitochondrial transcription factor 1 to high mobility group proteins. Science *252*, 965–969.

Parisi, M. A., Xu, B., and Clayton, D. A. (1993). A human mitochondrial transcriptional activator can functionally replace a yeast mitochondrial HMG-box protein both in vivo and in vitro. Mol. Cell. Biol. *13*, 1951–1961.

Prokisch, H., *et al*. (2004). Integrative analysis of the mitochondrial proteome in yeast. PLoS Biol. *2*, 795–804.

Saada, A., Shaag, A., Mandel, H., Nevo, Y., Eriksson, S., and Elpeleg, O. (2001). Mutant mitochondrial thymidine kinase in mitochondrial DNA depletion myopathy. Nat. Genet. *29*, 342–344.

Schultz, R. A., Swoap, S. J., McDaniel, L. D., Zhang, B., Koon, E. C., Garry, D. J., Li, K., and Williams, R. S. (1998). Differential expression of mitochondrial DNA replication factors in mammalian tissues. J. Biol. Chem. *273*, 3447–3451.

Schulz, V. P., and Zakian, V. A. (1994). The saccharomyces PIF1 DNA helicase inhibits telomere elongation and de novo telomere formation. Cell *76*, 145–155.

Seidel-Rogol, B. L., and Shadel, G. S. (2002). Modulation of mitochondrial transcription in response to mtDNA depletion and repletion in HeLa cells. Nucleic Acids Res. *30*, 1929–1934.

Shadel, G. S. (1999). Yeast as a model for human mtDNA replication. Am. J. Hum. Genet. *65*, 1230–1237.

Shadel, G. S. (2004). Coupling the human transcriptional machinery to human disease. Trends Genet. *20*, 513–519.

Shadel, G. S., and Clayton, D. A. (1993). Mitochondrial transcription initiation: variation and conservation. J. Biol. Chem. *268*, 16083–16086.

Shadel, G. S., and Clayton, D. A. (1997). Mitochondrial DNA maintenance in vertebrates. Annu. Rev. Biochem. *66*, 409–435.

Shen, E. L., and Bogenhagen, D. F. (2001). Developmentally-regulated packaging of mitochondrial DNA by the HMG-box protein mtTFA during *Xenopus* oogenesis. Nucleic Acids Res. *29*, 2822–2828.

Sikorski, R. S., and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics *122*, 19–27.

Tang, Y., Schon, E. A., Wilichowski, E., Vazquez-Memije, M. E., Davidson, E., and King, M. P. (2000). Rearrangements of human mitochondrial DNA (mtDNA): new insights into the regulation of mtDNA copy number and gene expression. Mol. Biol. Cell *11*, 1471–1485.

Tyynismaa, H., Sembongi, H., Bokori-Brown, M., Granycome, C., Ashley, N., Poulton, J., Jalanko, A., Spelbrink, J. N., Holt, I. J., and Suomalainen, A. (2004). Twinkle helicase is essential for mtDNA maintenance and regulates mtDNA copy number. Hum. Mol. Genet. *13*, 3219–3227.

Williamson, D. H., and Fennell, D. J. (1979). Visualization of yeast mitochondrial DNA with the fluorescent stain 'DAPI'. Methods Enzymol. *56*, 728–733.

Wu, H., Kanatous, S. B., Thurmond, F. A., Gallardo, T., Isotani, E., Bassel-Duby, R., and Williams, R. S. (2002). Regulation of mitochondrial biogenesis in skeletal muscle by CaMK. Science *296*, 349–352.

Wu, Z., Puigserver, P., Andersson, U., Zhang, C., Adelmant, G., Mootha, V., Troy, A., Cinti, S., Lowell, B., Scarpulla, R. C., and Spiegelman, B. M. (1999). Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. Cell *98*, 115–124.

Zelenaya-Troitskaya, O., Newman, S. M., Okamoto, K., Perlman, P. S., and Butow, R. A. (1998). Functions of the high mobility group protein, Abf2p, in mitochondrial DNA segregation, recombination and copy number in *Saccharomyces cerevisiae*. Genetics *148*, 1763–1776.

Zhao, X., Chabes, A., Domkin, V., Thelander, L., and Rothstein, R. (2001). The ribonucleotide reductase inhibitor Sml1 is a new target of the Mec1/Rad53 kinase cascade during growth and in response to DNA damage. EMBO J. *20*, 3544–3553.

Zhao, X., Muller, E. G., and Rothstein, R. (1998). A suppressor of two essential checkpoint genes identifies a novel protein that negatively affects dNTP pools. Mol. Cell *2*, 329–340.

Zhao, X., and Rothstein, R. (2002). The Dun1 checkpoint kinase phosphorylates and regulates the ribonucleotide reductase inhibitor Sml1. Proc. Natl. Acad. Sci. USA *99*, 3746–3751.

Zhou, J., Monson, E. K., Teng, S., Schulz, V. P., and Zakian, V. A. (2000). Pif1p helicase, a catalytic inhibitor of telomerase in yeast. Science *289*, 771–774.

Zong, H., Ren, J. M., Young, L. H., Pypaert, M., Mu, J., Birnbaum, M. J., and Shulman, G. I. (2002). AMP kinase is required for mitochondrial biogenesis in skeletal muscle in response to chronic energy deprivation. Proc. Natl. Acad. Sci. USA *99*, 15983–15987.