Rad53 is essential for a mitochondrial DNA inheritance checkpoint regulating G1 to S progression

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The Chk2-mediated deoxyribonucleic acid (DNA) damage checkpoint pathway is important for mitochondrial DNA (mtDNA) maintenance. We show in this paper that mtDNA itself affects cell cycle progression. *Saccharomyces cerevisiae* rho[0] cells, which lack mtDNA, were defective in G1- to S-phase progression. Deletion of subunit Va of cytochrome *c* oxidase, inhibition of F_1F_0 adenosine triphosphatase, or replacement of all mtDNA-encoded genes with noncoding DNA did not affect G1- to S-phase progression. Thus, the cell cycle progression defect in rho[0] cells is caused by loss of DNA within mitochondria and not loss of respiratory activity or mtDNA-encoded genes. Rad53p, the yeast Chk2 homologue, was required for inhibition of G1- to S-phase progression in rho[0] cells. Pif1p, a DNA helicase and Rad53p target, underwent Rad53p-dependent phosphorylation in rho[0] cells. Thus, loss of mtDNA activated an established checkpoint kinase that inhibited G1- to S-phase progression. These findings support the existence of a Rad53p-regulated checkpoint that regulates G1- to S-phase progression in response to loss of mtDNA.

Introduction

Mitochondrial DNA (mtDNA) encodes subunits of the electron transport chain and ATP synthase as well as tRNAs and ribosomal RNAs that are required for mitochondrial protein synthesis. Although mtDNA can be deleted in *Saccharomyces cerevisiae* or in cultured mammalian cells, mtDNA is essential in complex multicellular organisms. Mutations of human mtDNA have clinical manifestations in the brain, heart, skeletal muscle, kidney, and endocrine system and have been linked to aging and to age-associated neurodegenerative diseases (Wallace, 2005; Park and Larsson, 2011). Moreover, changes in mtDNA copy number have been observed in several primary human cancers (Yu, 2011).

The unit of inheritance of mtDNA is the mitochondrial nucleoid, a nucleoprotein complex that localizes to the mitochondrial matrix and interacts with mitochondrial outer and inner membranes. Nucleoids contain mtDNA, proteins that mediate mtDNA packaging, transcription, replication, repair, or recombination, and metabolic enzymes that may serve as sensors that regulate mtDNA nucleoids in response to changes in metabolism (Kucej and Butow, 2007). Although all nucleoids contain mtDNA and Abf2p, a high mobility group box protein that packages mtDNA, only actively replicating nucleoids contain Mgm101p, a protein that mediates DNA recombination and is essential for maintenance of mtDNA (Meeusen and Nunnari, 2003).

Here, we report that deletion of mtDNA triggers a checkpoint that inhibits progression from G1 to S phase of the cell division cycle in *S. cerevisiae*. We also identify a regulatory role for Rad53p in the mtDNA inheritance checkpoint. Rad53p and its mammalian homologue Chk2 are protein kinases that are part of the DNA damage checkpoint. These proteins arrest the cell cycle at G1, S, and G2 phase and activate

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Figure 1. **Cell cycle defects are caused by loss of DNA in mitochondria.** Wild-type (BY4741) cells containing mtDNA (rho⁺) or cells lacking mtDNA (rho⁰, *mgm101Δ* cells) were synchronized and propagated at 30°C. At the times shown, cells were fixed and stained with propidium iodide, and their DNA content was assessed by flow cytometry. (A) Images of DAPI-stained cells are 2D projections of deconvolved 3D volumes of rho⁺, rho⁰, and *mgm101Δ* rho⁰ cells. Cell outlines are shown in white. n, nuclear DNA; m, mtDNA. Bar, 1 µm. (B) Rho⁺ exhibit normal cell cycle progression. Cells lacking mtDNA either by EtBr treatment (rho⁰) or by deletion of a gene required for mtDNA maintenance (*mgm101Δ* rho⁰) fail to progress normally and show a G1 arrest.

(C) Quantitation of progression through G1 phase of rho⁺, rho⁰, and *mgm101Δ* rho⁰ cells was assessed as the fold change in the fraction of cells in G1 phase at the time specified, relative to the fraction of cells that were in G1 at the time of release from pheromone-induced G1 arrest (cells in G1 at t_x/cells in G1 at t_0). The data shown are pooled from multiple experiments (wild type, $n = 5$; rho⁰, $n = 3$; *mgm101Δ* rho⁰, $n = 3$).
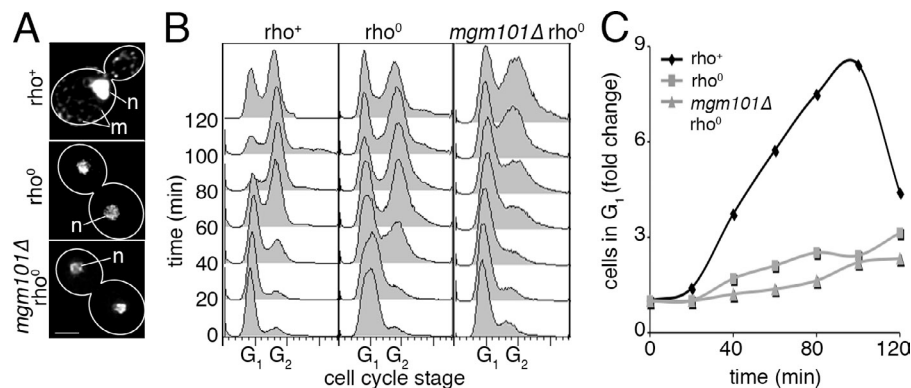
DNA repair pathways in response to nuclear DNA damage or replication interference events, including DNA breaks, adducts, crosslinks, and inhibition of DNA polymerases (Elledge, 1996). Rad53 is activated by autophosphorylation or phosphorylation by Mec1 and has downstream targets, including Swi6p, a regulator of G1 cyclins, Dun1p, a regulator of deoxyribonucleotide triphosphate (dNTP) levels, and Pif1p, a DNA helicase that localizes to nuclear DNA and mtDNA (Sidorova and Breeden, 1997; Lee et al., 2003; Makovets and Blackburn, 2009).

Recent studies also support a functional link between *RAD53* and mtDNA. A genome-wide screen revealed that *RAD53* and/or DNA repair genes, including *RAD6* and *RAD27*, interact genetically with genes that are required for mtDNA maintenance, including *MMM1*, *MMM2*, *MDM10*, *MDM12*, and *MDM32* (Pan et al., 2006). Rad53 also regulates mtDNA copy number by two independent mechanisms. One is through Rad53 effects on dNTP levels. Rad53 regulates dNTP pool size through effects on ribonucleotide reductase, the initial and rate-limiting enzyme in dNTP synthesis (Elledge, 1996; Chabes et al., 2003). Lower ribonucleotide reductase activity or dNTP levels result in an increase in mtDNA loss, whereas higher dNTP levels reduce mtDNA loss and increase mtDNA copy number (Taylor et al., 2005; Lebedeva and Shadel, 2007; Reinhardt and Yaffe, 2009). Rad53p also regulates mtDNA copy number by a dNTP pool–independent mechanism, which may reflect Rad53p function in checkpoint signal transduction (Lebedeva and Shadel, 2007). Interestingly, the function of the DNA damage checkpoint in controlling mtDNA content is conserved. Ataxia telangiectasia is a neurodegenerative disease that is caused by mutation of ataxia telangiectasia mutated (ATM), another DNA damage checkpoint kinase. mtDNA copy number is reduced in cells and tissues from patients with ataxia telangiectasia and from ATM-null mice and in fibroblasts treated with the ATM inhibitor KU-55933 (Eaton et al., 2007).

Previously, we described a checkpoint that inhibits cytokinesis when there are severe defects in inheritance of mitochondrial membranes and/or proteins (García-Rodríguez et al., 2009). Here, we describe a second checkpoint that is associated with mitochondrial inheritance. This newly described checkpoint is triggered by loss of mtDNA.

Results and discussion

Loss of mtDNA induces a G1 arrest in cell cycle progression

Rho⁰ yeast cells, cells with no mtDNA and no mitochondrial respiratory activity, can be produced by treatment with ethidium bromide (EtBr), an agent that inhibits mtDNA synthesis and increases mtDNA degradation (Goldring et al., 1970). Rho⁰ cells are also produced by deletion of *MGM101*, a protein that is homologous to conserved recombination proteins, is required for repeat-mediated DNA recombination, localizes to actively replicating nucleoids, and is required for repair of oxidatively damaged mtDNA (Chen et al., 1993; Meeusen and Nunnari, 2003; Mbantenkhu et al., 2011). As expected, there are no detectable mtDNA nucleoids or mitochondrial respiratory activity in EtBr-treated or *mgm101Δ* cells (Fig. 1 and Fig. S1).

Using flow cytometry, we find that loss of mtDNA results in defects in transition from G1 to S phase of the cell division cycle (Fig. 1). After release from G1 arrest, wild-type rho⁺ cells undergo G1- to S-phase transition within 40–60 min and S- to G2-phase transition within 100–120 min. In contrast, 50% of rho⁰ cells produced by EtBr treatment or deletion of *MGM101* fail to progress from G1 to S phase. Rho⁰ cells that do progress through the cell cycle do so at a lower rate. They undergo G1- to S-phase transition at 60–100 min and S- to G2-phase transition at 150 min, after release from G1 arrest. Thus, loss of mtDNA results in defects in cell cycle progression from G1 to S phase.

mtDNA encodes respiratory chain proteins as well as tRNAs and ribosomal RNAs that are required for synthesis of mtDNA-encoded proteins. In *Drosophila melanogaster*, defects in mitochondrial respiratory activity produced by deletion of cytochrome oxidase complex subunit Va or disruption of complex 1 trigger a checkpoint that inhibits G1 to S progression (Mandal et al., 2005; Owusu-Ansah et al., 2008). Therefore, it is possible that the G1- to S-phase transition defect observed in rho⁰ cells is caused by loss of mitochondrial respiration and energy production. To test this hypothesis, we studied the effect of treatment with oligomycin and deletion of *COX5a* on cell cycle progression. Oligomycin binds to the F_1F_0 ATPase proton pump and inhibits ATP production. *COX5a* encodes subunit Va of cytochrome
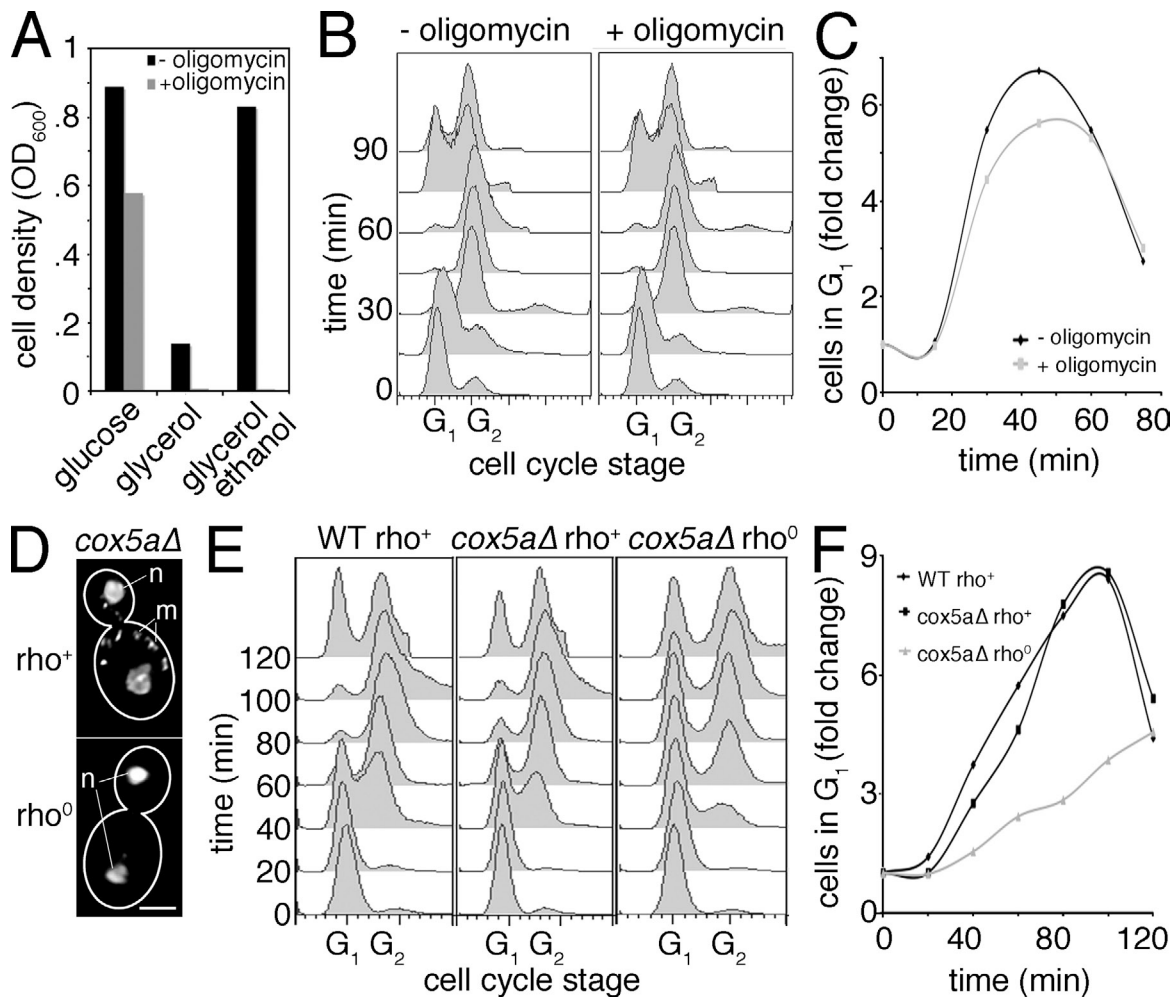
Figure 2. **Inhibition of respiration or of ATP production does not cause the defect in passage through G1.** Cell cycle progression was assessed in cells treated with the F_1F_0 ATPase inhibitor oligomycin and in cells bearing a deletion in a nuclear-encoded mitochondrial respiratory chain component (*COX5A*). (A) Effect of oligomycin on yeast cell growth. Single colonies were incubated in liquid media with or without 1 µg/ml oligomycin and aerated at 30°C. Cell growth was assessed by measuring the OD_{600} of the culture. Oligomycin-dependent inhibition of yeast cell growth on a nonfermentable carbon source was observed on solid media in three independent experiments. Quantitation of cell density in liquid cultures in the presence and absence of oligomycin was performed once. (B) Cell cycle progression of cells in the absence or presence of oligomycin was performed as for Fig. 1. (C) Quantitation of progression from G1 to S in oligomycin-treated and control cells was performed as for Fig. 1. The data shown are pooled from three experiments. (D) Imaging of DAPI-stained rho+, *cox5aΔ* rho+, and *cox5aΔ* rho0 cells was performed as for Fig. 1. Cell outlines are shown in white. n, nuclear DNA; m, mtDNA. Bar, 1 µm. (E) Cell cycle progressions of rho+, *cox5aΔ* rho+, and *cox5aΔ* rho0 cells were determined as for Fig. 1. (F) Quantitation of progression from G1 to S in rho+, *cox5aΔ* rho+, and *cox5aΔ* rho0 cells was performed as for Fig. 1. The data shown are pooled from multiple experiments (wild type [WT], n = 8; *cox5aΔ* rho+, n = 3; *cox5aΔ* rho0, n = 4).

c oxidase. Oligomycin treatment and deletion of COX5a inhibit respiration-driven yeast cell growth (Fig. 2 and Fig. S1) but do not result in defects in progression from G1 to S phase (Fig. 2). Thus, the defect in cell cycle progression observed in rho0 cells is not a consequence of loss of mitochondrial respiration.

To determine whether the observed cell cycle delay is caused by loss of DNA in mitochondria, we studied cell cycle progression in a cell (N24 rho−) in which all mtDNA-encoded genes are replaced with noncoding DNA. The noncoding DNA in N24 rho− consists of tandem repeats of a 171–base pair fragment of the cytochrome b gene (Tzagoloff et al., 1979). The N24 rho− cell contains mtDNA nucleoids (Fig. 3); however, because the mtDNA has no expressed genes, the cell is respiratory incompetent and cannot grow on a nonfermentable carbon source (Fig. S1).

The N24 rho− does not display a cell cycle progression defect (Fig. 3). In contrast, a rho0 cell generated in the same genetic background as N24 (D273-10B) exhibits G1- to S-phase progression defects that are similar to that observed in other rho0 cells. Moreover, we observe that two other rho− cells, one in the same genetic background as the N24 rho− and another that we generated in the BY4741 genetic background, also progress through the cell cycle like rho+ cells (Fig. S2). Therefore, the defect in G1- to S-phase transition observed in rho0 cells is a result of loss of DNA within mitochondria and not caused by loss of genes encoded by mtDNA.

Role for Rad53 checkpoint signaling in regulating cell cycle progression in cells lacking mtDNA

We investigated whether Rad53, a nuclear DNA checkpoint regulator (Elledge, 1996; Chabes et al., 2003), might also regulate cell cycle progression at the G1- to S-phase transition in response

Figure 3. **Cell cycle defects are caused by loss of DNA in mitochondria.** (A) Imaging of DAPI-stained rho+, rho0, and N24 rho− cells was performed as for Fig. 1. All cells used were in the D273-10B genetic background. Cell outlines are shown in white. n, nuclear DNA; m, mtDNA. Bar, 1 μm. (B) Cell cycle progression of rho+, rho0, or N24 rho− cells was performed as for Fig. 1. (C) Quantitation of cell cycle progression of rho+, rho0, and N24 rho− cells was performed as for Fig. 1 C. The data shown are pooled from three experiments.
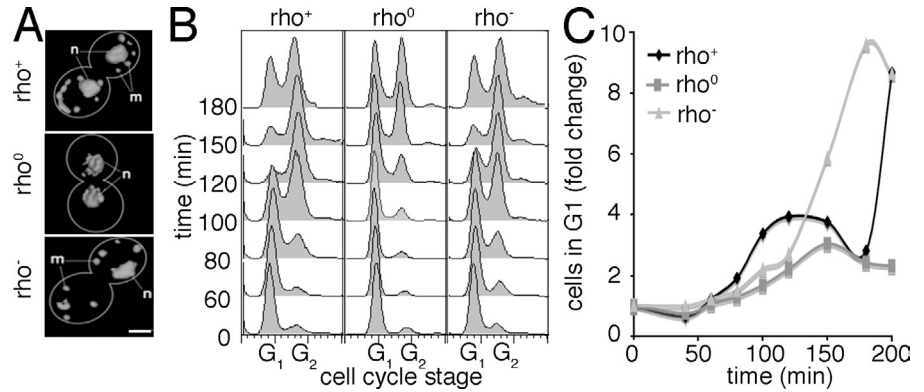
to loss of mtDNA. Deletion of Rad53 impairs DNA checkpoint activation. Unlike many checkpoint proteins, Rad53p is essential; its essential function in budding yeast is regulation of dNTP pool size through effects on ribonucleotide reductase (Rnr1p; Zhao et al., 1998).

To determine whether dNTP pool size is responsible for cell cycle regulation in response to loss of mtDNA, we studied the effect of deletion of *SML1* on cell cycle progression in rho+ and rho0 cells. Sml1p is a negative regulator of Rnr1p (Zhao et al., 1998). As a control, we studied the effect of deletion of mtDNA in W303 cells, which are congenic with our *sml1Δ* and *sml1Δ rad53Δ* cells. W303 rho0 cells exhibit progression from G1 to S phase (Fig. S3). Our finding that arrest of rho0 cells occurs in three genetic backgrounds (W303, D273-10B, and BY4741) indicates that it is not a consequence of genetic background. Moreover, because W303 rho0 cells exhibit robust growth (Fig. S1), the findings indicate that the arrest observed is not a consequence of severe mitochondrial damage that compromises cell growth rates.

Equally important, deletion of *SML1*, as well as the associated increase in dNTP pool size, does not suppress the cell cycle progression defect observed upon loss of mtDNA (Fig. 4). In contrast, deletion of *RAD53* suppresses the G1 to S progression defect observed in rho0 cells: *rad53Δ sml1Δ* rho0 cells exhibit wild-type progression from G1 to S phase (Fig. 4). Previous studies indicate that treatment with low levels of hydroxyurea (HU) can alter dNTP pool size (Julias and Pathak, 1998; Koç et al., 2004). We find that modulating dNTP pool size by HU treatment does not affect the cell cycle progression observed in *rad53Δ sml1Δ* rho0 cells (Fig. S3). Together, these findings indicate that Rad53p is required for regulation of cell cycle progression in response to loss of mtDNA and that it does so by a mechanism that is independent of its function in dNTP pool size control.

If Rad53p is a physiologically relevant regulator of cell cycle progression in response to loss of mtDNA, Rad53p should be activated by loss of mtDNA. To test this hypothesis, we studied the phosphorylation state of Pif1p. Pif1p is a DNA helicase

Figure 4. **Rad53 is required for regulation of cell cycle progression in response to loss of mtDNA.** (A) 2D projections of 3D volumes of DAPI-stained *sml1Δ* rho+ and *sml1Δ* rho0 cells. (B) Cell cycle progression of *sml1Δ* rho+ and *sml1Δ* rho0 cells was assessed as for Fig. 1. (C) Quantitation of cell cycle progression of *sml1Δ* rho+ and *sml1Δ* rho0 cells was performed as for Fig. 1. The data shown are pooled from three experiments. (D) 2D projections of a 3D volume of DAPI-stained *rad53Δ sml1Δ* rho+ and *rad53Δ sml1Δ* rho0 cells. (E) Cell cycle progression of *rad53Δ sml1Δ* rho+ and *rad53Δ sml1Δ* rho0 cells was assessed as for Fig. 1. (F) Quantitation of cell cycle progression of *rad53Δ sml1Δ* rho+ and *rad53Δ sml1Δ* rho0 cells was performed as for Fig. 1. All cells used are from the W303 genetic background. The data shown are pooled from three experiments. Cell outlines are shown in white. n, nucleus; m, mtDNA; WT, wild type. Bars, 1 μm.
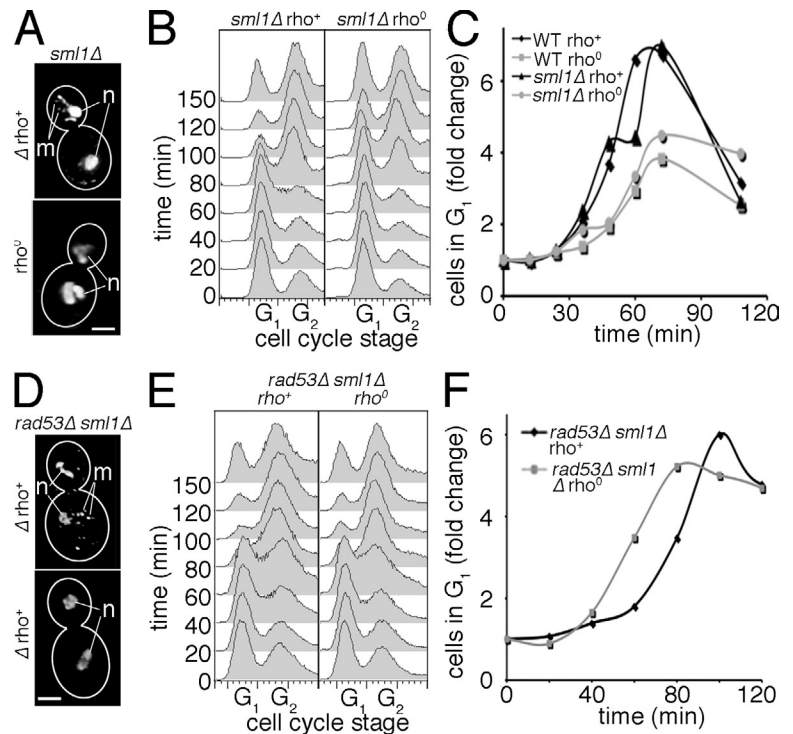
that is encoded by a single nonessential gene that gives rise to proteins that are targeted to the nucleus and to mitochondria (Bochman et al., 2010). The nuclear form of Pif1p is involved in processes including telomere elongation and formation, replication of recombinant DNA, formation of Okazaki fragments, prevention of chromosome rearrangement, and repair of double-stranded DNA. The mitochondrial form of Pif1p localizes to mtDNA nucleoids and is required for mtDNA maintenance. Nuclear Pif1p undergoes phosphorylation upon DNA damage–induced activation of Rad53p (Makovets and Blackburn, 2009). Thus, nuclear Pif1p is a downstream target for Rad53.

We tagged Pif1p with the myc epitope and confirmed that treatment with HU, which induces stalled replication forks, leads to phosphorylation of Pif1p. HU treatment results in a shift in the electrophoretic mobility of Pif1p that is eliminated by treatment with calf intestinal alkaline phosphatase (Fig. 5). In addition, we found that loss of mtDNA results in increased phosphorylation of Pif1p. Moreover, Pif1p is not phosphorylated in *rad53Δ sml1Δ rho⁰* cells (Fig. 5). Thus, Rad53p is activated by loss of mtDNA. This finding, together with our finding that Rad53p is required for inhibition of G1 to S transition in rho⁰ cells, supports a role for Rad53p in cell cycle regulation in response to loss of mtDNA.

Many laboratories have described DNA damage checkpoints and checkpoint-activated repair of damage, replication defects, or segregation defects in nuclear DNA. We provide the first evidence for a checkpoint that inhibits progression from G1 to S phase of the cell division cycle in response to loss of mtDNA. For nuclear DNA damage checkpoints, the damage detected is not associated with a specific gene or set of genes. Rather, these checkpoints monitor broader processes, including stalled DNA replication forks and DNA alkylation. Similarly, the mtDNA checkpoint does not monitor specific genes or function of genes encoded by mtDNA. Instead, it monitors mtDNA content.

Our experiments also reveal a role for Rad53p, a known checkpoint signaling molecule that regulates G1 to S transition in response to DNA damage, in the mtDNA inheritance checkpoint. Thus, our observations reveal a broader role for Rad53p in DNA surveillance than was previously appreciated. Because Rad53p is conserved and the human DNA damage checkpoint has known effects on mtDNA content, it is possible that mechanisms similar to the yeast mtDNA inheritance checkpoint exist in other eukaryotes. Finally, because changes in mtDNA content have been linked to multiple forms of cancer and DNA damage checkpoint proteins are commonly altered in human tumors, it is possible that alterations in tumor cell metabolism, such as the Warburg effect, in which mitochondrial respiration decreases and aerobic glycolysis increases, could arise from mtDNA checkpoint dysregulation.

Methods and materials

Yeast strains, plasmids, and growth conditions

Yeast strains used in this work are listed in Table S1. Strains W1588-4C, DCY025, W2017-7C, and DCY027 are derivatives of W303. DCY015, N24, PSY036, and PSY037 are derivatives of D273-10B. All other strains are derived from the S288C strain used for the yeast deletion library. rho⁰ derivatives were generated from wild type by two consecutive 2-d treatments of 25 µM EtBr (Goldring et al., 1970). rho⁰ cells were confirmed by lack of growth on plates containing a nonfermentable carbon source and absence of mtDNA with DAPI staining. rho⁻ derivatives were generated from wild-type
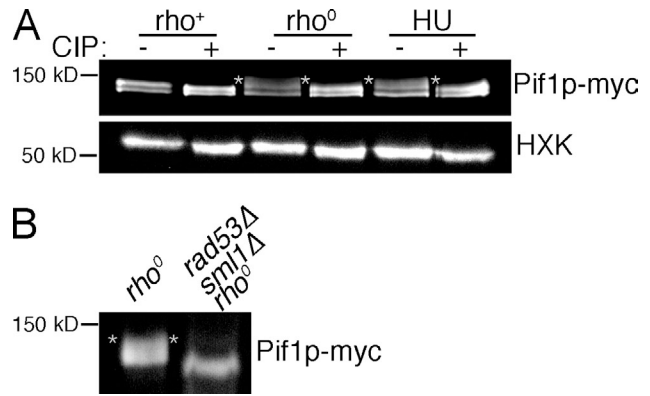

Figure 5. **Pif1p undergoes Rad53-dependent phosphorylation in response to loss of mtDNA.** (A) Western blot showing myc-tagged Pif1p in rho⁺, rho⁰, and HU-treated rho⁺ cells incubated in the presence (+) or absence (−) of 400 U/ml calf intestinal phosphatase (CIP) for 2 h at 37°C. Hexokinase (HXK) was used as a loading control. (B) Western blots showing myc-tagged Pif1p in rho⁰ cells and in rho⁰ cells bearing deletions in RAD53 and SML1. Asterisks mark phosphorylated Pif1p, which is present in rho⁰ cells and HU-treated rho⁺ cells but not in rad53Δ sml1Δ rho⁰ cells.

BY4741 strain by two consecutive 2-d and 1-d treatments of 10 µm EtBr. rho⁻ cells were confirmed by lack of growth on plates containing a nonfermentable carbon source and presence of mtDNA with DAPI staining. Other yeast methods were performed according to Sherman (2002).

Tagging of Pif1

The carboxy terminus of *PIF1* was tagged with 13 copies of the myc epitope using PCR-based insertion into the chromosomal copies of the *PIF1* loci (Longtine et al., 1998). Primers used to tag these genes were forward primer, 5′-CGAACCTCGTGGTCAGGATACCGAAGACCAC-ATCTTA-GAACGGATC-CCCGGGTTAATTAA-3′; and reverse primer, 5′-GCAGTTTGTATTCTATATAACTATGTGTATTAATATGTACGAATTCGAGC-TCGTTTAAAC-3′.

Synchronization

For cell cycle synchronization, cells were incubated with 10–100 µM α-factor for 2.5 h. Cells were released from arrest by washing and were transferred to pheromone-free media. N24 unbudded cells were isolated from mid–log phase cultures by centrifugation through a 10–35% sorbitol gradient for 12 min at 56 g and transferred to fresh media.

Flow cytometry

Analysis of DNA content in propidium iodide–stained, synchronized cell cultures was determined according to Paulovich and Hartwell (1995) using a fluorescence-activated cell analyzer (LSR II; BD). The percentage of cells in G1, S, and G2 phase was determined using the FlowJo program (Tree Star). The time of each phase was defined as the time when the maximum percentage of cells was in a given phase relative to the time when cells were released from pheromone-induced G1 arrest.

Fluorescence microscopy, image analysis, and cytology

All samples were fixed by addition of paraformaldehyde solution (Electron Microscopy Sciences) to the cell culture medium to a final concentration of 3.7% and incubation for 1 h in a shaking incubator (New Brunswick Scientific) with aeration at 30°C. Cells were collected by centrifugation, and fixative was removed by three washes with wash solution (25 mM KPO₄, pH 7.5, and 0.4 M KCl) and mounted on microscope slides using mounting solution (1 mg/ml p-phenylenediamine, 90% [wt/vol] glycerol, and PBS) with 0.5 µg/ml DAPI to detect DNA.

Images were collected with a microscope (Axioplan 2; Carl Zeiss) using a Plan-Apochromat 100×, 1.4 NA oil objective lens and a cooled charge-coupled device camera (ORCA-1; Hamamatsu Photonics) at 25°C. Illumination with a 100-W mercury arc lamp was controlled with a shutter (Uniblitz D122; Vincent Associates). Openlab 3.1.5 software (PerkinElmer) was used to acquire images. Z stacks were obtained at 0.2-µm intervals, and the out of focus light was removed using an iterative deconvolution algorithm in Volocity 5.5 (PerkinElmer). All z sections were assembled and 3D projections were generated with comparable parameters and thresholds.

Protein and immunological techniques

Protein extracts of mid–log phase yeast cells for Western blot analysis were obtained as previously described (Boldogh et al., 1998). Cells were collected by centrifugation, washed with water, and resuspended in lysis buffer (50 mM imidazole, pH 7.4, 10 mM EDTA, 1% Triton X-100, 2 mM PMSF, and protease inhibitor cocktail [10 mM benzamidine, 1 mg/ml 1, 10-phenanthroline, 0.5 mg/ml antipain, 0.5 mg/ml chymostatin, 0.5 mg/ml leupeptin, 0.5 mg/ml pepstatin, and 0.5 mg/ml aprotinin]). Cell suspension was added to glass beads prewashed with 0.8 ml lysis buffer. Cells were disrupted by bead beating for 6 min in cold using Vortex Turbo Mix (Thermo Fisher Scientific). The bicinchoninic acid assay (Thermo Fisher Scientific) was used to determine protein concentration in cell extracts. Immunoblot analysis of the total amount of Pif1p was performed with antibodies specific for myc. HRP-conjugated secondary antibodies and chemiluminescent substrate detection (SuperSignal; Thermo Fisher Scientific) were used to visualize bands.

Online supplemental material

Fig. S1 shows growth of all strains used in this study on fermentable and nonfermentable carbons sources. Fig. S2 shows the growth on different carbon sources, mtDNA content, and cell cycle progression of rho^+, rho^-, and rho^0 cells from two yeast genetic backgrounds (D27310B and BY4741). Fig. S3 shows cell cycle progression of rho^+ and rho^0 cells from the W303 genetic background and of $sml1\Delta\ rad53\Delta\ rho^0$ cells in the presence of different concentrations of HU. Table S1 shows the yeast strains used for this study. Online supplemental material is available at http://www.jcb .org/cgi/content/full/jcb.201205193/DC1.

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