Epigenetic Silencing Mediates Mitochondria Stress-Induced Longevity

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SUMMARY

Reactive oxygen species (ROS) play complex roles in aging, having both damaging effects and signaling functions. Transiently elevating mitochondrial stress, including mitochondrial ROS (mtROS), elicits beneficial responses that extend lifespan. However, these adaptive, longevity-signaling pathways remain poorly understood. We show here that Tel1p and Rad53p, homologs of the mammalian DNA damage response kinases ATM and Chk2, mediate a hormetic mtROS longevity signal that extends yeast chronological lifespan. This pathway senses mtROS in a manner distinct from the nuclear DNA damage response and ultimately imparts longevity by inactivating the histone demethylase Rph1p specifically at subtelomeric heterochromatin, enhancing binding of the silencing protein Sir3p, and repressing subtelomeric transcription. These results demonstrate the existence of conserved mitochondria-to-nucleus stress-signaling pathways that regulate aging through epigenetic modulation of nuclear gene expression.

INTRODUCTION

As multifunctional organelles, mitochondria influence aging, lifespan, and healthspan through a variety of mechanisms. Perhaps the most recognized is the production of reactive oxygen species (ROS), byproducts of mitochondrial respiration that damage macromolecules to promote cell dysfunction, apoptosis, and aging (Balaban et al., 2005). However, ROS also function as signaling molecules that regulate physiological processes (Finkel, 2011; Sena and Chandel, 2012) and participate in conserved signal transduction pathways, including the target of rapamycin (TOR) and insulin-sensing pathways that regulate longevity (Longo et al., 2012; Pan et al., 2011; Raimundo et al., 2012; Zarse et al., 2012). Additionally, studies in model organisms show that increasing ROS levels can positively influence health- and lifespan (Lee et al., 2010; Pan et al., 2011; Schulz et al., 2007; Yang and Hekimi, 2010; Zarse et al., 2012).

Chronological lifespan (CLS) of the budding yeast, Saccharomyces cerevisiae, is measured by assaying the amount of time that cells survive in the late post-diauxic and stationary phases of culturing that follow exponential growth (Longo et al., 2012). Yeast CLS studies, which model postmitotic cellular aging in higher eukaryotes, have led to the identification of conserved pathways and processes involved in lifespan regulation (Fabrizio and Longo, 2007; Kaerberlein, 2010; Longo et al., 2012). We recently demonstrated that elevating mitochondrial ROS (mtROS) during yeast exponential growth elicits an adaptive response that reduces ROS in post-diauxic and stationary phases and extends CLS (Pan et al., 2011). Interventions that elevate mtROS in C. elegans also extend lifespan (Lee et al., 2010; Schulz et al., 2007; Yang and Hekimi, 2010; Zarse et al., 2012), and exercise in mammals is believed to improve health via increasing mtROS (Ristow et al., 2009). These examples indicate that hormetic adaptation to ROS is an emerging paradigm in lifespan and healthspan regulation (Gems and Partridge, 2008; Ristow and Schmeisser, 2011; Tapia, 2006). However, the signaling pathways that sense ROS and coordinate hormetic changes in cellular function to curtail aging and promote longevity remain largely unknown.

Hormetic ROS might extend lifespan by increasing stress resistance or decreasing ROS levels late in life (Gems and Partridge, 2008). However, other cellular processes may be targets of mtROS signaling. In particular, aspects of telomere function determine replicative capacity and impact lifespan (Blasco, 2005). In yeast, telomeres consist of a repetitive TG1–3 sequence bound by several highly conserved proteins that regulate telomere length, transcription, and packaging (Grunstein, 1997). The silent information regulator (Sir) proteins maintain telomeric heterochromatin to repress transcription (Rusche et al., 2003). Histone modifications adjacent to telomeres regulate the spread of silencing, termed telomere position effect (TPE), by enhancing or preventing Sir complex binding (Altaf et al., 2007; Dang et al., 2009; Kozak et al., 2010; Park and Lustig, 2000). Yeast impaired in specific histone modifications that support TPE have a shorter replicative lifespan (Dang et al., 2009), and decreased heterochromatin formation at telomeres and subtelomeres in mammals induces telomere dysfunction to trigger apoptosis or senescence (Blasco, 2007). However, the signaling pathways that regulate histone modifications to support subtelomeric silencing are not fully understood. Additionally, although substantial progress has been made toward understanding the relationships between telomere dysfunction and replicative lifespan, how telomere function might influence postmitotic aging remains unknown.
Herein, we focused on the process of hormetic mtROS signaling and adaptation in yeast, reasoning that our studies would reveal conserved mechanisms of ROS sensing and longevity regulation. We show that mtROS signal via Tel1p and Rad53p, homologs of mammalian ATM and Chk2, uncovering a function for the DNA damage response in communicating mitochondria functional status to the nucleus. This signaling pathway inhibits the histone demethylase Rph1p specifically at subtelomeric regions, which enhances transcriptional silencing to extend CLS. In yeast and mammals, telomere dysfunction signals changes in mitochondrial biogenesis and function (Nautiyal et al., 2002; Sahin et al., 2011). Thus, our results demonstrate that two-way communication pathways exist between mitochondria and telomeres that regulate aging and longevity.

**RESULTS**

The H3K36 Demethylase Rph1p Mediates Hormetic mtROS Responses to mtROS

Reduced TOR complex 1 (TORC1) signaling extends lifespan in many model organisms (Kenyon, 2005), and we showed previously that mtROS hormesis contributes to this mode of CLS extension in yeast (Pan et al., 2011). Furthermore, transient treatment with a sublethal dose of the redox-cycling compound menadione during exponential growth generates mtROS (Castro et al., 2008) and extends yeast CLS (Pan et al., 2011) (Figure S1A). Menadione treatment and reduced TORC1 signaling decrease cellular ROS levels in post-diauxic and stationary phases, supporting that hormetic adaptation to ROS occurs under these conditions (Bonawitz et al., 2007; Pan et al., 2011). To identify factors that mediate mtROS longevity signaling, we crossed the results of two published microarrays from conditions that increase mtROS in yeast (1 mM menadione and 100 nm rapamycin, a TORC1 inhibitor) and identified the commonly induced genes (Figure 1Ai and Table S1) (Gasch et al., 2000; Hardwick et al., 1999). Rather than...
systematically analyzing all identified genes, which may not show individual robust contributions to mtROS adaptation, we searched first for transcriptional regulators that globally coordinate these gene expression responses and second for signaling events that determine their activity (Figures 1Aii, 1Aiii, and 1B). We identified a promoter motif, AG5, enriched in these genes (Figure 1B) (Romer et al., 2007) and transcriptional regulators that bind motifs similar to AG5 (Abdulrehman et al., 2011). Candidate transcriptional regulators included Gis1p and Msn2p/Msn4p, which have known roles in oxidative stress resistance, nutrient sensing, and CLS (Wei et al., 2008). Strains lacking Msn2p or Msn4p demonstrated a moderately reduced adaptive CLS response relative to the wild-type strain (72% and 81% of the menadione-dependent CLS extension observed in wild-type; Figures S1C and S1D), indicating nonredundant contributions of each transcription factor. Combined deletion of MSN2 and MSN4 abrogated menadione-induced CLS extension to 53% of that observed in the wild-type strain (Figure S1E). This additive effect suggests that Msn2p and Msn4p have partially redundant functions in mtROS adaptation, consistent with their overlapping regulation of gene expression in response to stress and downstream of TORC1 signaling (Gasch et al., 2000; Wei et al., 2008, 2009). Deletion of GSI1 reduced menadione-dependent CLS extension to 47% of the response seen in the wild-type (Figure S1F), indicating that it also participates in mtROS adaptation. Gis1p and Msn2p/Msn4p cooperatively contribute to yeast CLS extension under conditions of reduced TORC1 signaling and caloric restriction (Wei et al., 2008). However, a gis1Δ/msn2Δ/msn4Δ mutant strain retained an adaptive CLS response that was 47% of wild-type (Figure S1G), identical to that of the gis1Δ single mutant. This epistatic relationship indicates that Gis1p, Msn2p, and Msn4p partially contribute to the mtROS adaptive response in the same genetic pathway. Only deletion of the histone 3 lysine 36 (H3K36) demethylase Rph1p completely abrogated CLS extension (Figure 1C), eliminating the hormetic decrease in ROS at day 1 of CLS (Figure 1D), and impaired expression of mtROS-responsive genes (Figure 1E). Rph1p also contributed to the CLS extension observed in a tor1Δ strain (Figure S1H), consistent with Rph1p participating in TORC1-regulated mtROS hormesis (Pan et al., 2011). Intrigued by the notion that mtROS adaptation might involve epigenetic changes in nuclear gene expression, Rph1p became our primary focus.

**Horneric Menadione Treatment Induces Mitochondria Superoxide to Extend CLS**

Overexpression of SOD2, which encodes the mitochondria matrix superoxide dismutase, curtails CLS extension in tor1Δ yeast, supporting that elevated mtROS initiates an adaptive signal (Pan et al., 2011). To determine if hormetic menadione treatment elicits adaptive responses via a similar mechanism, we measured CLS in strains with different mtROS detoxification capacities. We utilized a strain in which the strong TORC1-regulated mtROS hormesis (Pan et al., 2011). Intrigued by the notion that mtROS adaptation might involve epigenetic changes in nuclear gene expression, Rph1p became our primary focus.

**Tel1p and Rad53p, Components of the Nuclear DNA Damage Response, Contribute to mtROS Hormesis**

The DNA damage checkpoint kinase Rad53p (Chk2 in mammals) phosphorylates Rph1p in response to DNA damage (Kim et al., 2002) (Figure 3A). Subsequent Rph1p-mediated chromatin modifications permit transcription of the DNA repair enzyme PRR1 (Liang et al., 2011). Mec1p (ATR in mammals) activates and phosphorylates Rad53p (Pellicioli et al., 1999), and phosphorylation of Rph1p in response to DNA damage is Mec1p dependent (Kim et al., 2002); therefore, we investigated
the requirement for the Rad53p and Mec1p kinases in mtROS signaling. Deletion of SML1 suppressed the lethality of rad53Δ and mec1Δ and allowed us to examine their role in mtROS signaling and adaptation (Zhao et al., 1998). Although Rad53p was required for mtROS-mediated CLS extension (Figure 3B) and hormetic reduction of ROS at day 1 of CLS (Figure S2A), Mec1p was not (Figure S2A). Therefore, we investigated the involvement of the related kinase Tel1p, which has partially overlapping functions with Mec1p (Figure 3A) but unique roles in telomere maintenance (Harrison and Haber, 2006). Additionally, the mammalian homolog of Tel1p, ATM, senses ROS (Guo et al., 2010), making Tel1p an attractive candidate to mediate mtROS signaling in yeast. Menadione treatment did not extend CLS in a tel1Δ strain (Figure 3D), yet MMS-induced Rad53p phosphorylation was not affected by tel1Δ (Figure 4A, right). Rad53p contains multiple Tel1p/Mec1p target phosphorylation sites, characterized by an (S/T)Q motif (Kim et al., 1999; Lee et al., 2003). Analysis of (S/T)Q phosphoprotein profiles revealed that hormetic menadione treatment induced phosphorylation of a specific subset of (S/T)Q-containing targets compared to an MMS treatment that had similar effects on cell growth and toxicity (Figure 4B). This distinct profile, combined with partial phosphorylation of Rad53p in response to menadione, indicated to us that hormetic mtROS signaling via Tel1p and Rad53p is distinct from canonical Mec1p-mediated DNA damage signaling. We additionally demonstrated that the transcriptional outcomes of hormetic mtROS signaling are distinct from a DNA damage transcriptional signature (DDTS), which corresponds to genes similarly altered by 0.02% MMS and 170 Gy of ionizing radiation (Gasch et al., 2001) (Figure S3D and Table S2). We further showed that hormetic mtROS signaling via Tel1p and Rad53p is distinct from canonical Mec1p-mediated DNA damage signaling. RT-PCR analysis of three well-characterized DNA damage repair enzyme and is therefore hypersensitive to oxidative DNA damage (Karahalil et al., 1998) (Figure 4D). Finally, mtROS adaptation does not require DNA double-strand break detection or signaling mediated by Mre11p, a component of the MRN/MRX complex (Nakada et al., 2004) (Figure S3F). Together, these data support that mtROS produced during hormetic menadione treatment activates specific DNA damage response kinases without inducing oxidative nuclear DNA damage or a canonical nuclear DNA damage response.

Figure 3. Hormetic mtROS Longevity Signaling Requires the Tel1p and Rad53p DNA Damage Response Kinases
(A) Schematic of DNA damage signaling in Saccharomyces cerevisiae.
(B) CLS of sml1Δ and rad53Δ/sml1Δ strains treated with 50 μM menadione (MD) or vehicle (nt) during exponential growth.
(C) ROS levels in sml1Δ and rad53Δ/sml1Δ strains treated with menadione (+) or vehicle (−) during exponential growth as in Figure 1D. Samples were collected at day 1 of CLS.
(D) CLS of wild-type (DBY2006) and tel1Δ strains as in (B). See also Figure S2.
Hormetic mtROS Signaling Inactivates Rph1p to Alter Subtelomeric Chromatin

Rad53p-dependent phosphorylation of Rph1p causes it to dissociate from chromatin, elevating H3K36me3 levels to regulate transcription (Figure 5A) (Liang et al., 2011). We examined Rph1p phosphorylation in response to mtROS and found that hormetic menadione treatment induces a partial Rph1p upward mobility shift relative to treatment with MMS (Figures 5B, left, and S4A), mirroring the partial activation of Rad53p under these conditions (Figure 4A). Rph1p phosphorylation in response to menadione, but not MMS, also required Tel1p and Rad53p (Figure 5B). These data show that mtROS signaling via Tel1p and Rad53p induces phosphorylation of Rph1p, which may subsequently alter the chromatin binding capacity of Rph1p and H3K36me3 levels.

In yeast, histone methylation and acetylation contribute to heterochromatin formation at the ribosomal DNA (rDNA) gene cluster, mating loci, and subtelomeric regions (defined as within 25 kb of telomeric repeat DNA) (Barton and Kaback, 2006; Dang et al., 2009; Millar and Grunstein, 2006; Santos-Rosa et al., 2004). Yeast deficient in heterochromatin formation at these silenced loci exhibit decreased replicative lifespan (Dang et al., 2009; Kaeberlein et al., 1999), yet it is unknown how silencing at these regions influences chronological aging. H3K36me3 is implicated in both transcriptional repression and activation within euchromatic regions (Wagner and Carpenter, 2012), but the role of this modification at silenced loci has not been described. Since Rph1p phosphorylation regulates its chromatin binding capacity (Liang et al., 2011) and Rph1p is phosphorylated in response to mtROS (Figure 5B), we investigated whether mtROS signaling altered the association of Rph1p with silenced regions of the yeast genome. Chromatin immunoprecipitation (ChIP) of Rph1-HA at day 1 of CLS (when adaptive response to menadione would be evident; Figure S4B) revealed that, in the absence of hormetic menadione treatment, Rph1p binding at all examined silenced genes was enhanced relative to ACT1 (Figures 5C and S4C). However, Rph1p binding at subtelomeric regions was specifically altered in response to hormetic mtROS signaling in a manner dependent on Rad53p (Figures 5C and S4C). Consistent with loss of Rph1p, H3K36me3 levels increased at subtelomeric genes in an mtROS- and RPH1-dependent manner, suggesting that mtROS signaling regulates the H3K36me3 demethylase activity of Rph1p (Figures 5D and S4D). The requirement for H3K36 demethylation in adaptive CLS extension is specific to Rph1p, as deletion of Jhd2p, which also demethylates di- and trimethyl H3K36 (Tu et al., 2007), did not affect mtROS adaptation (Figure S4E). These data suggest that, although Rph1p may function at several silenced regions in chronologically aged yeast, hormetic mtROS signaling via Rad53p specifically targets Rph1p activity at subtelomeric regions, eliciting epigenetic changes that promote lifespan extension.

Signaling by mtROS Represses Transcription of Subtelomeric Genes

We next investigated the adaptive transcriptional consequences of Rph1p-mediated epigenetic changes in response to mtROS signaling. We performed gene expression microarrays of wild-type and rph1Δ yeast, that had been treated with menadione or a vehicle control during exponential growth, at day 1 of CLS. To
focus the analysis on Rph1p-dependent transcriptional changes, we excluded transcripts that were altered in an rph1Δ strain treated with menadione. Additionally, we compared the remaining transcripts with those altered in an untreated rph1Δ strain. Since mtROS signaling to Rad53p essentially inactivates Rph1p demethylase activity, we reasoned that although Rph1p has mtROS-independent functions (Orzechowski Westholm et al., 2012), a null strain might reveal a subset of the transcriptional outcomes relevant to mtROS-mediated CLS extension. Gene list comparisons (Table S3) are summarized in Figure 6A. We observed 3-fold enrichment for the Rph1p binding motif (WAG4) among transcripts induced by both mtROS signaling and Rph1p deletion (sector C; Figure S5A), consistent with the involvement of Rph1p in regulating gene induction following hormetic mtROS signaling. mtROS signaling specifically affects Rph1p binding at subtelomeric regions (Figure 5C), and subtelomeric transcripts were enriched only among genes repressed by mtROS signaling and RPH1 deletion (sectors A and C; Figures 6B, S5B, and S5C and Table S4). Using a subtelomeric URA3 reporter and RT-PCR, we confirmed that adaptive mtROS signaling represses transcription from subtelomeric regions (Figures S5D and S5E), but not at other silenced regions (Figure S5F and Table S5). Together, these results indicate that hormetic mtROS signaling to Rph1p selectively silences subtelomeric transcription.

Hormetic mtROS Signaling Modifies Higher-Order Chromatin Structure to Extend CLS

Histone modifications at subtelomeric regions can influence higher-order chromatin formation, which determines gene expression (Schoeftner and Blasco, 2009). The Sir complex is initially recruited to silenced regions via Sir2p-mediated histone deacetylation (Rusche et al., 2003). This enhances the binding of Sir3p, which recruits additional Sir2p to nucleate heterochromatin spreading (Norris and Boeke, 2010). Histone methylation can enhance Sir3p binding, thereby influencing transcriptional silencing (Ng et al., 2002). Sir3p binding was not similarly affected at other silenced loci (Figure S6A). Additionally, Sir3p was required for subtelomeric silencing following mtROS signaling (Figure 7B); however, at other silenced loci, menadione-induced transcriptional changes were mainly Sir3p independent (Figure S6B). Finally, hormetic mtROS signaling failed to extend CLS of a sir3Δ strain (Figures 7C and S6C). These findings demonstrate that Rph1p-regulated subtelomeric silencing, mediated by Sir3p, is a key outcome of hormetic mtROS signaling that extends lifespan.

DISCUSSION

Mitochondria, epigenetics, and telomere function have all been independently implicated in lifespan regulation. Our investigation of mitochondrial stress signaling in yeast demonstrates coordination among these processes to promote lifespan extension. Specifically, we have elucidated a mitochondria-to-nucleus signaling pathway (Figure 7D) in which the DNA damage kinase Tel1p transduces an mtROS signal to activate Rad53p, which in turn regulates the ability of the histone demethylase Rph1p to bind subtelomeric chromatin. The loss of Rph1p from subtelomeric chromatin elevates H3K36me3 and enhances binding of the silencing protein Sir3p to repress subtelomeric transcription. Sir3p-mediated subtelomeric silencing is an essential outcome
of mtROS signaling and adaptation that ultimately extendsCLS. Although heterochromatin maintenance at several loci influences yeast lifespan (Dang et al., 2009; Kaeberlein et al., 1999), mtROS signaling specifically targets subtelomeric heterochromatin. We propose that this specificity is a key component of the mtROS hormetic response and corroborates previous studies in which Sir proteins target distinct genomic regions under different conditions, with unique consequences for chronological and replicative aging (Ruault et al., 2011; Salvi et al., 2013). For example, sir2Δ enhances CLS extension under caloric restriction or in sch9Δ strains by inducing stress response and metabolic genes that promote longevity (Fabrizio et al., 2005) but shortens RLS due to a failure to maintain heterochromatin at the RNA cluster (Kaeberlein et al., 1999).

Subtelomeric and telomeric heterochromatin maintenance preserves telomere length and genomic stability in yeast and mammals (Schoeftner and Blasco, 2009). Telomere function is intimately connected with health- and lifespan but is most often associated with replicative senescence. Our finding that altered subtelomeric silencing supports CLS extension implies that telomere functional status and supports recent findings that these processes likely do not regulate aging in isolation (Sahin et al., 2011). The hormetic mtROS signaling pathway we describe should provide a platform for more integrated network theories of aging and lifespan regulation.

Although epigenetic outcomes are essential to the adaptive response, our data also indicate that transcriptional changes mediated by Msn2p/Msn4p, Gis1p, and Rph1p support mtROS adaptation. Menadione treatment induces a number of oxidative stress response genes in a manner dependent on Rph1p, which may account for the hormetic decrease in ROS during post-diauxic and stationary phases (Figure S3E and Table S2D). All of the candidate mtROS-responsive proteins we investigated bind a similar DNA motif (Figure 1B), and since all proteins are at least partially required for mtROS adaptation (Figure S1), we speculate that Rph1p-dependent chromatin remodeling might facilitate Msn2p/Msn4p- and/or Gis1p-mediated transcription at mtROS-responsive genes. Future studies to explore the relationship between Rph1p, Msn2p/Msn4p, and Gis1p in transcriptional regulation and mtROS hormesis may uncover additional cooperation among these proteins in lifespan regulation.

Our results also indicate that Tel1p propagates an mtROS signal in the absence of nuclear DNA damage (Figures 4C, 4D, 5A, 5E, and S3F), which suggests that certain DNA damage response kinases function as both ROS and DNA damage sensors, and can regulate distinct cellular processes in a stimulus-specific manner. Recent studies show that ATM (the mammalian homolog of Tel1p) acts as a redox sensor (Guo et al., 2010). Our finding that Tel1p mediates the hormetic mtROS response in yeast suggests that this function is evolutionarily conserved.

Hormetic mtROS signaling ultimately inhibits the chromatin binding capacity of the histone demethylase Rph1p at subtelomeres, thereby increasing H3K36me3 at these regions (Figures 5C and 5D). Although the role of histone acetylation in transcriptional silencing at subtelomeres is well established (Dang et al., 2009; Kozak et al., 2010; Santos-Rosa et al., 2004), it is increasingly evident that histone methylation can also contribute to this process in both yeast and mammals (Blasco, 2007; Casilini et al., 2009; Dang et al., 2009; Kozak et al., 2010; Santos-Rosa et al., 2004). Thus, it will be important to determine which methyltransferases and demethylases function at subtelomeres during mammalian aging. Our study would support members of the Jumonji-domain demethylases related to yeast Rph1p as prime candidates.

In conclusion, our results provide insight into multiple aspects of aging by integrating mtROS, DNA damage signaling, telomeres, and epigenetics into a prolongevity pathway. The mtROS signaling pathway identified herein connects mitochondria and telomere functional status and supports recent findings that these processes likely do not regulate aging in isolation (Sahin et al., 2011). The hormetic mtROS signaling pathway we describe should provide a platform for more integrated network theories of aging and lifespan regulation.
Cell Metabolism
Mitochondrial ROS Signal Epigenetic Adaptation

EXPERIMENTAL PROCEDURES

Yeast Strains
All strains used in this study are derivatives of DBY2006 (MATα his3Δ200 leu2-3,112 ura3-52 trp1-901 ade2-1) and are listed in Table S7. Deletion strains were generated via gene replacement with URA3, KanMX6, or hphMX3 cassettes and transformed using the lithium acetate method (Gietz and Schiestl, 2007). Endogenous RPH1 was tagged at the C terminus with a HA-KanMX6 cassette (Longtine et al., 1998). To generate the SOD2 overexpression strain (Figure S3D), the endogenous SOD2 promoter (–600 to –1) was replaced with the TDI3 promoter (Kötter et al., 2009). All strains were verified by PCR.

Chronological Lifespan Measurement
Yeast were grown in minimal dextrose medium supplemented with essential amino acids (Sherman, 1991). For lifespan assays, saturated cultures from single colonies were diluted to an optical density 600 (OD600) of 0.01 in 50 ml fresh media and grown at 30°C, 200 rpm until the OD reached 0.5. Either menadione (50 μM final concentration; Sigma-Aldrich) or an equivalent volume of ethanol (no treatment control) was added, and cultures were grown until the OD reached 2.0. At this point, cells were pelleted and resuspended in media from an untreated parallel culture. CLS was determined by trypan blue staining.

Chemical Toxicity
Wild-type yeast were grown to an OD600 of 0.5 and treated with 50 μM menadione, 200 μM menadione, 0.01% MMS, 0.02% MMS, or ethanol (vehicle control). Cell growth was monitored for 24 hr of treatment, and viability was assessed via trypan blue staining.

Flow Cytometry
Measurement of cellular superoxide using dihydroethidium (DHE) was performed as described (Bonawitz et al., 2007; Pan et al., 2011).

Immunoblotting
Yeast whole-cell extracts were prepared from cells in exponential growth phase treated with menadione, MMS (0.01% final concentration), or ethanol for 30 min (Keogh et al., 2006). Proteins were separated in 6% SDS-PAGE gels (to resolve Rad53p and Rph1-HA phosphorylation) or 12% SDS-PAGE gels and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore). Blocking and antibody incubations were carried out in Tris-buffered saline and Tween (TBST; 10 mM Tris [pH 8.0], 150 mM NaCl, 0.05% Tween 20) containing 5% nonfat dry milk. Membranes were incubated with anti-Rad53 (1:300; Santa Cruz Biotechnology), anti-phospho-(S/T)Q (1:1000; Cell Signaling), anti-MnSOD (1:500; Assay Designs), anti-HA (1:1000; Covance), anti-actin (1:1000; Chemicon), or anti-GAPDH-HRP (horseradish peroxidase) (1:1000; Abcam). Immunoblotting was performed on protein isolated from at least two independent experiments. Band density traces and quantification were determined using ImageJ (National Institutes of Health).

8-Hydroxyguanosine Quantification
Yeast cultures in exponential growth phase were treated with ethanol, 50 μM menadione, or 200 μM menadione, or MMS (0.01% final concentration), or vehicle during exponential growth.

RNA Preparation and Microarray Analysis
RNA extraction and purification were performed as described (Bonawitz et al., 2008) from wild-type DBY2006 and rph1Δ cultures grown for 2 or 24 hr after addition of 50 μM menadione or ethanol. RNA quality bioanalysis

Figure 7. mtROS Signaling via Rph1p Promotes Sir3p Binding at Subtelomeres to Repress Gene Expression and Extend CLS
(A) ChIP of Sir3p in wild-type (DBY2006) and rph1Δ at a control region (ACT1) and two subtelomeric genes (YP6 and YEL077C). Cells were analyzed at day 1 of CLS following treatment with 50 μM menadione or vehicle during exponential growth. (B) Quantitative RT-PCR of subtelomeric transcripts relative to ACT1 at day 1 of CLS in wild-type or sir3Δ yeast following 50 μM menadione or vehicle during exponential growth. (C) CLS of wild-type and sir3Δ treated with 50 μM menadione (MD) or vehicle (nt) during logarithmic growth. (D) Model for mtROS signaling via DNA damage response kinases and Rph1p to regulate subtelomeric heterochromatin formation and CLS.
complementary DNA (cDNA) synthesis, and hybridization to a Roche NimbleGen 12 x 135 K array were performed in collaboration with the Yale University W.M. Keck Foundation Biotechnology Resource Laboratory according to the manufacturer’s protocol. Two independent biological replicates were prepared for each sample, and hybridization was performed in duplicate to generate technical replicates. Raw data were normalized according to the Robust Multi-array Average (RMA) algorithm using NimbleScan software (Irizarry et al., 2003). Probe expression values were used to determine fold change and filtered to significantly induced and repressed probe lists (p ≤ 0.01 as determined by Student’s t test). Gene lists were generated based on the occurrence of at least two probes for a particular gene present exclusively in induced or repressed probe lists. To map the chromosomal location of genes in sectors A, B, and C, the distance from the transcription start site to the nearest telomere was determined. Gene lists are described in the Tables S2 and S3.

Normalized expression data were downloaded from the Gene Expression Omnibus at NCBI (GSM1102, record GDS108 [Gasch et al., 2000]) and from the supplemental online material associated with Hardwick et al. (1999). Gene lists were ranked according to fold change, and the top 5% induced genes were selected for further analysis to identify genes induced in both data sets. The coinduced genes were further analyzed using WebMOTIFS (Romer et al., 2007) and YEASTRACT (yeast search for transcriptional regulators and consensus tracking; Abdulrehman et al., 2011) to generate the data shown in Figure 1C.

Quantitative RT-PCR
RNA was isolated as described above and converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Agilent) according to the manufacturer’s instructions. cDNA was diluted 1:10, and each 20 μl reaction contained 10 μl diluted cDNA, 0.4 μl dilutions of each primer (from a 25 μM stock), and 9.2 μl Bio-Rad iQ SYBR Mix. PCR conditions were as described (Cotney et al., 2007).

Chromatin Immunoprecipitation
Yeast cultures were treated with 50 μM menadione or ethanol from OD 0.5 to OD 2.0. At 24 hr after the initiation of menadione treatment at OD 0.5, cells were processed for ChiP analysis, which was performed essentially as described (Aparicio et al., 2004), with the following modifications. DNA was sheared in a Bioruptor UCD-200 Sonicator at 4°C five times for 20 s each. Chromatin was precleared by incubating with protein A agarose beads (Millipore) for 30 min at room temperature. Antibodies used were anti-HA (Covance), anti-trimethyl H3K36 (Epigentek), anti-H3 (Abcam), and anti-Sir3 (Santa Cruz Biotechnology). Quantitative PCR of three technical replicates per sample was performed using the same conditions described for RT-PCR, except the reaction proceeded for 40 cycles. Primers are listed in Table S8.

Statistical Analysis
For CLS, fluorescence-activated cell sorting (FACS), 8-hydroxyguanosine, and RT-PCR analysis, all data points represent the mean of biological triplicate samples inoculated from isolated single colonies. Error bars represent SEM. p values were determined using Student’s unpaired t test, and values for relevant comparisons are indicated within figures. Statistical analysis for enrichment of subtelomeric transcripts was determined using a hypergeometric probability distribution (Martin et al., 2004). For ChiP analysis, data points represent the mean of two biological replicate samples inoculated from isolated single colonies. Error bars represent the range.

ACCESSION NUMBERS
Raw microarray data were deposited in the Gene Expression Omnibus repository under the number GSE45383.

SUPPLEMENTAL INFORMATION
Supplemental information includes six figures and eight tables and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2013.04.003.

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