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Crosstalk between mitochondrial stress signals regulates yeast chronological lifespan

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ABSTRACT

Mitochondrial DNA (mtDNA) exists in multiple copies per cell and is essential for oxidative phosphorylation. Depleted or mutated mtDNA promotes numerous human diseases and may contribute to aging. Reduced TORC1 signaling in the budding yeast, *Saccharomyces cerevisiae*, extends chronological lifespan (CLS) in part by generating a mitochondrial ROS (mtROS) signal that epigenetically alters nuclear gene expression. To address the potential requirement for mtDNA maintenance in this response, we analyzed strains lacking the mitochondrial base-excision repair enzyme Ntg1p. Extension of CLS by mtROS signaling and reduced TORC1 activity, but not caloric restriction, was abrogated in *ntg1Δ* strains that exhibited mtDNA depletion without defects in respiration. The DNA damage response (DDR) kinase Rad53p, which transduces pro-longevity mtROS signals, is also activated in *ntg1Δ* strains. Restoring mtDNA copy number alleviated Rad53p activation and re-established CLS extension following mtROS signaling, indicating that Rad53p senses mtDNA depletion directly. Finally, DDR kinases regulate nucleus-mitochondria localization dynamics of Ntg1p. From these results, we conclude that the DDR pathway senses and may regulate Ntg1p-dependent mtDNA stability. Furthermore, Rad53p senses multiple mitochondrial stresses in a hierarchical manner to elicit specific physiological outcomes, exemplified by mtDNA depletion overriding the ability of Rad53p to transduce an adaptive mtROS longevity signal.

1. Introduction

Mitochondrial DNA (mtDNA) encodes essential proteins of the oxidative phosphorylation system and the non-coding RNAs needed for their translation in the mitochondrial matrix, making it critical for cellular energy production, redox homeostasis, and signaling (Greaves et al., 2012; Wallace, 2005). The mitochondrial genome is present in multiple copies in most eukaryotic cells, with some human cells containing up to 10,000 mtDNA molecules (Robin and Wong, 1988). The importance of this component of the human genome is manifest by the numerous diseases resulting from mtDNA point mutations, deletions, or depletion (Greaves et al., 2012; Wallace, 2005). Furthermore, single cells can harbor mixtures of wild-type and mutant mtDNA, and the relative amount of each (degree of heteroplasmy) influences disease phenotypes (Schon et al., 2012). The accumulation of mtDNA mutations and reduced copy number is also strongly associated with age and often precedes functional decline in cells and tissues that accompanies aging (Clay Montier et al., 2009; Cortopassi and Arnheim, 1990; Trifunovic et al., 2004). Cellular mechanisms that maintain mtDNA integrity may therefore represent key regulators of longevity and healthspan.

Maintenance of mtDNA represents a balance between replication, repair, segregation to daughter cells, and degradation. Mitochondria of the budding yeast, *S. cerevisiae*, have a base-excision repair (BER) pathway and other overlapping systems for mtDNA maintenance (O'Rourke et al., 2002). Full yeast chronological lifespan, defined as viability in post-diauxic and stationary phases of growth (Longo et al., 2012), requires an intact mitochondrial BER system under stress conditions, highlighting the importance of mtDNA repair during yeast aging (Maclean et al., 2003). Several modes of yeast mtDNA replication have been proposed (Lipinski et al., 2010). During transcription-dependent replication, short transcripts derived from replication origins serve as primers for initiation by mtDNA polymerase γ, or Mip1p (Baldacci and Bernardi, 1982). Alternately, formation of double-strand breaks (DSB) and subsequent DNA resection generates short single-stranded tails, which can hybridize within double-stranded regions and have been proposed to initiate rolling circle or recombination-mediated replication (Ling et al., 2013; Maleszka

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et al., 1991). Importantly, these latter mechanisms faithfully duplicate the parental mtDNA into concatamers, promoting propagation of mtDNA molecules with identical sequence to decrease heteroplasmy (Ling et al., 2013). Although all of the above modes of replication likely contribute to maintenance of normal mtDNA copy number, conditions or signaling pathways that might promote one form of replication over others and consequences of this selection on mtDNA stability, mitochondrial function, and aging remain largely unknown.

Several studies indicate that proteins involved in the nuclear DNA damage response (DDR) sense mtDNA stability and regulate mtDNA maintenance. During a canonical DDR in *S. cerevisiae*, the kinases Tel1p and Mec1p (homologs of ATM and ATR in mammals) initially sense DNA double-strand breaks or stalled replication forks and activate the effector kinase Rad53p (Chk2 in mammals) (Pellicioli and Foiani, 2005). Rad53p then initiates cell cycle delay, increased dNTP production, and expression of enzymes involved in DNA repair (Branzei and Foiani, 2006). In both budding yeast and mammalian cells, DDR kinases regulate mtDNA copy number and stability. For example, cultured fibroblasts from patients with Ataxia-Telangiectasia, a disease caused by mutations in the *ATM* gene, have reduced mtDNA copy number (Eaton et al., 2007). In contrast, activation of an ATM/Chk2 checkpoint increases mtDNA copy number but also increases the frequency of a common mtDNA deletion (Niu et al., 2012). In yeast, both cell cycle progression and dNTP levels, factors regulated by Rad53p, determine mtDNA copy number (Lebedeva and Shadel, 2007; Taylor et al., 2005). Furthermore, Mec1p regulates sumoylation of many proteins involved in DNA repair, which may influence their nuclear versus mitochondrial localization and repair activity (Cremona et al., 2012; Psakhye and Jentsch, 2012). Finally, loss of mtDNA activates a Rad53p-dependent cell cycle checkpoint and phosphorylation of Rad53p target proteins (Crider et al., 2012), indicating that communication between the mitochondrial genome and the DDR pathway is bi-directional.

In addition to sensing nuclear DNA damage and mtDNA maintenance, Rad53p transduces a mitochondrial ROS (mtROS) signal that can extend yeast chronological lifespan (CLS) (Schroeder et al., 2013). CLS measures viability in post-diauxic and stationary phases of yeast growth and models post-mitotic cellular aging in higher eukaryotes (Longo et al., 2012). Mitochondrial ROS adaptation is also a key aspect by which reduced signaling through the conserved Target of Rapamycin (TOR) pathway extends yeast CLS (Pan et al., 2011). Treatment with a sub-lethal dose of the redox-cycling compound menadione during the exponential growth phase generates mitochondrial matrix superoxide that initiates mtROS signaling and mimics the effects of *tor1Δ* on lifespan and ROS adaptation (Pan et al., 2011; Schroeder et al., 2013). One outcome of mtROS pro-longevity signaling is repression of subtelomeric gene expression mediated by the histone 3 lysine 36 (H3K36) demethylase Rph1p, which enhances heterochromatin formation at subtelomeres. These Rad53p-dependent epigenetic changes occur in the absence of canonical DDR signaling. Rad53p therefore transduces both beneficial (mtROS) and detrimental (lack of mtDNA) mitochondrial signals to elicit either lifespan extension or cell cycle arrest, but how multiple signals might be integrated remains unknown. Additionally, given that complete loss of mtDNA represents a physiologically extreme circumstance that also induces extensive metabolic and transcriptional reprograming (Butow and Avadhani, 2004; Traven et al., 2001), it is not known how less severe mtDNA instability influences longevity. In this study, we used strains lacking the mitochondrial BER enzyme Ntg1p to examine the involvement of mtDNA copy number and stability in aging of budding yeast and potential intersections of different mitochondrial and nuclear signaling modes to Rad53p.

2. Materials and methods

2.1. Yeast growth and chronological lifespan measurement

All yeast strains used in this study are derivatives of DBY2006 (MATa *his3-Δ200 leu2-3,-112 ura3-52 trp1-Δ1 ade2-1*) or BY4742 (MATα *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) and are listed in Table S1. Deletion strains were generated via gene replacement with *URA3* or *KanMX6* cassettes and transformed using the lithium acetate method. Ntg1p was tagged at the C-terminus with either GFP-KanMX6 or HA-KanMX6. Strains overexpressing Rnr1p were transformed with the plasmid pBAD71-RNR1 (2 μ, *URA3*) (Lebedeva and Shadel, 2007) or pRS316 (*CEN, URA3)* as a control empty vector. Chronological lifespan was determined as described (Schroeder et al., 2013). For adaptive menadione treatment, saturated cultures from single colonies were diluted to an OD_{600} of 0.01 in 50 mL fresh minimal media containing only essential amino acids and grown at 30 °C, 200 RPM until the OD_{600} reached 0.5. Either menadione (50 μM final concentration for DBY2006 background, 80 μM final concentration for BY4742 background) or an equivalent volume of ethanol (no treatment control) was added, and cultures were grown until the OD_{600} reached 2.0, approximately 24 h after inoculation. At this point, cells were pelleted and resuspended in media from an untreated parallel culture. For lifespan measurement under caloric restriction (CR), cells were grown in minimal media supplemented with essential amino acids and 0.5% glucose. CLS measurements were initiated 24 h (day 1 of CLS) or 48 h (day 2 of CLS) following adaptive menadione treatment and were analyzed in triplicate for all strains.

2.2. ROS measurements and oxygen consumption

Measurements of cellular superoxide were performed as described (Bonawitz et al., 2006, 2007; Pan et al., 2011). Oxygen consumption was measured using a Clark electrode (SI130) paired with an oxygen meter (model 782, Stathkelvin Instruments). Respiration of 1 mL of cells at either OD_{600} = 0.5 or collected at day one of CLS (48 h after inoculation) was measured as %oxygen/minute/OD_{600} for biological triplicate samples and normalized to the wild-type rate, which was set to one.

2.3. Microscopy

Yeast expressing *NTG1*-GFP at OD_{600} = 0.5 were visualized with an Olympus IX-71 inverted microscope. Prior to image acquisition, 1 mL of cells were stained with MitoTracker Red and DAPI for 15 min at 30 °C at final concentrations of 100 nM and 5 ng/mL, respectively. Three microliters of 0.2% low melting point agarose in SD medium warmed to 50 °C was pipetted onto glass slides and allowed to cool briefly before 3 μL of stained cells were added, mixed by pipetting, and covered with a glass coverslip. All images were acquired within 30 min of slide preparation using Metamorph software. Merged images were generated in Adobe Photoshop.

2.4. Subcellular fractionation

Mitochondria and nuclei were isolated from exponentially growing cells (OD_{600} = 0.5) as described (Daum et al., 1982). The crude nuclear pellet, isolated after the second round of dounce homogenization, was purified further by resuspension in Ficoll buffer and centrifugation as described (Mosley et al., 2009).

2.5. Western blotting

Yeast whole-cell extracts were prepared from cells at OD_{600} = 0.5 treated with 0.01% MMS or water (vehicle control) for 30 min