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Regulation of Yeast Chronological Life Span by TORC1 via Adaptive Mitochondrial ROS Signaling

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SUMMARY

Here we show that yeast strains with reduced target of rapamycin (TOR) signaling have greater overall mitochondrial electron transport chain activity during growth that is efficiently coupled to ATP production. This metabolic alteration increases mitochondrial membrane potential and reactive oxygen species (ROS) production, which we propose supplies an adaptive signal during growth that extends chronological life span (CLS). In strong support of this concept, uncoupling respiration during growth or increasing expression of mitochondrial manganese superoxide dismutase significantly curtails CLS extension in *tor1Δ* strains, and treatment of wild-type strains with either rapamycin (to inhibit TORC1) or menadione (to generate mitochondrial ROS) during growth is sufficient to extend CLS. Finally, extension of CLS by reduced TORC1/Sch9p-mitochondrial signaling occurs independently of Rim15p and is not a function of changes in media acidification/composition. Considering the conservation of TOR-pathway effects on life span, mitochondrial ROS signaling may be an important mechanism of longevity regulation in higher organisms.

INTRODUCTION

Active signaling through the conserved TOR pathway limits life span in budding yeast (Bonawitz et al., 2007; Kaeberlein et al., 2005; Lavoie and Whiteway, 2008; Pan and Shadel, 2009; Powers et al., 2006), nematodes (Vellai et al., 2003), fruit flies (Kapahi et al., 2004), and mice (Selman et al., 2009). In fact, treatment of mice with the TOR inhibitor rapamycin, even starting relatively late in life, extends life span, representing the first pharmacological antiaging regimen in mammals (Harrison et al., 2009). The TOR pathway controls many aspects of cell physiology, including ribosome biogenesis, translation, autophagy, cell growth, and proliferation (Wullschleger et al., 2006), and hence it remains unclear precisely which aspects of TOR signaling contribute most significantly to aging and life span regulation (Blagosklonny and Hall, 2009).

In budding yeast, TOR signaling requires two PI3-kinase-like, serine-threonine kinases encoded by the *TOR1* and *TOR2* genes, which assemble into two signaling complexes (TORC1 and TORC2) with unique subunit compositions (Loewith et al., 2002). TORC1 can contain either Tor1p or Tor2p, while TORC2 only contains Tor2p, rendering *tor2Δ* lethal and *tor1Δ* a viable genetic background with reduced TORC1 signaling. Signaling through rapamycin-sensitive TORC1 regulates ribosome biogenesis and translation in part via activation of the protein kinase Sch9p, which is an ortholog of mammalian ribosomal S6 kinase 1 (S6K1) (Urban et al., 2007). Yeast TORC2 appears to control separate pathways involved in cell-cycle regulation and cytoskeleton dynamics and is much less sensitive to rapamycin (Cybulski and Hall, 2009).

Mitochondria are involved in aging and age-related pathology (Balaban et al., 2005; Bonawitz and Shadel, 2007; Shadel, 2008) and are the cornerstones of the mitochondrial and free radical theories of aging (Balaban et al., 2005; Wallace, 2005). Mitochondrial respiration is a major source of superoxide, which directly causes oxidative damage to mitochondrial and cellular molecules or is converted to other ROS that induce aging-associated damage (Balaban et al., 2005). We (Bonawitz et al., 2007; Bonawitz et al., 2006; Pan and Shadel, 2009) and others (Barros et al., 2004; Fabrizio et al., 2003; Lavoie and Whiteway, 2008; Longo et al., 1999; Piper et al., 2006) have documented complex relationships between TOR signaling, mitochondrial function, ROS, and yeast chronological life span (CLS), defined by the length of time yeast cells can survive in a nondividing population in postdiauxic and stationary-phase cultures (Fabrizio and Longo, 2007; Parrella and Longo, 2008). We showed that CLS extension in *tor1Δ* yeast requires mitochondrial respiration and is associated with an increase in translation of mtDNA-encoded subunits of the oxidative phosphorylation (OXPHOS) system (Bonawitz et al., 2007) and OXPHOS complex density, which are mediated through downregulation of Sch9p (Bonawitz et al., 2007; Pan and Shadel, 2009). Importantly, the observed increases in mitochondrial translation and mitochondrial oxygen consumption are only manifest during logarithmic and early postdiauxic growth stages (Pan and Shadel, 2009). Although

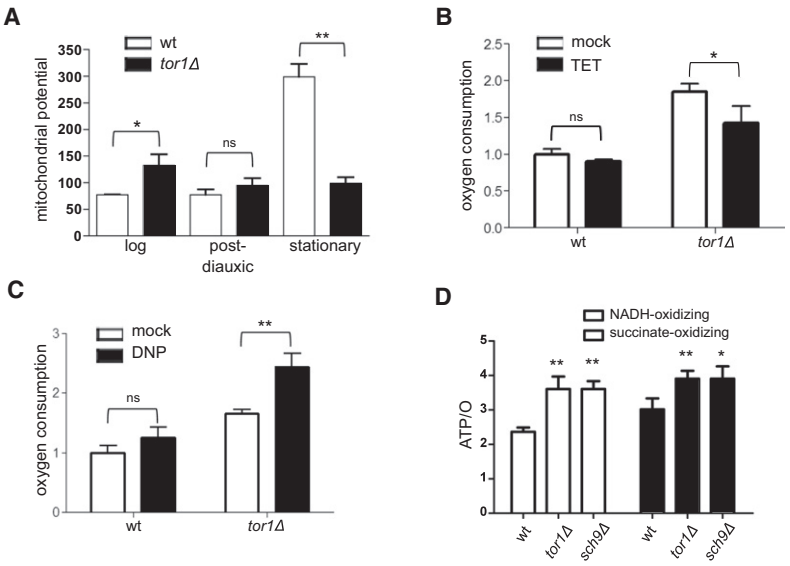

Figure 1. Analysis of Mitochondrial Respiration Parameters In Vivo and In Vitro in *tor1Δ* and *sch9Δ* Strains

(A) Mitochondrial membrane potential of DBY2006 (wt) and *tor1Δ* strains during mid-log (12 hr after inoculation), late-log/postdiauxic (20 hr after inoculation), and early stationary phase (36 hr after inoculation) measured by DiOC6 staining.

(B) Oxygen consumption of DBY2006 (wt) and *tor1Δ* in the presence (TET) or absence (mock) of 100 μM TET.

(C) Oxygen consumption of wild-type (wt) and *tor1Δ* in the presence or absence of 10 μM DNP.

(D) ATP/O ratio of mitochondria isolated from wild-type (wt), *tor1Δ*, and *sch9Δ* strains under NADH- or succinate-oxidizing conditions. Error bars represent the mean ± SD with p values from a Student's unpaired t test denoted as follows: * = $p \leq 0.05$, ** = $p \leq 0.01$, and "ns" denoting no significance ($p > 0.05$) between the indicated comparisons. See also Figures S1 and S2.

wild-type yeast exhibit minimal TOR activity in stationary phase, *TOR1* or *SCH9* deletion results in fewer ROS in stationary phase, accompanied by upregulation of Sod2p, the yeast mitochondrial manganese superoxide dismutase (Bonawitz et al., 2007). Based on these results, we proposed that relieving TOR inhibition of respiration during growth stages preconditions yeast to better survive the stressful conditions of stationary phase (Bonawitz and Shadel, 2007; Pan and Shadel, 2009).

Active TOR signaling represses stress responses and entry into stationary phase, in part, by inhibiting the transcription factors Msn2p/4p and Gis1p (Cameroni et al., 2004). These transcription factors are activated by the kinase Rim15p, lack of which curtails the CLS extension of strains with genetically or pharmacologically repressed TOR activity (Fabrizio et al., 2001; Wei et al., 2008). Thus, there is substantial evidence that reduced TOR signaling derepresses Rim15p, which activates downstream transcription factors to augment CLS extension (Agarwal et al., 2005; Fabrizio et al., 2001; Wei et al., 2008).

It has recently been proposed that the accumulation of acetic acid in the culture medium creates a toxic environment that limits CLS (Burtner et al., 2009; Kaeberlein, 2010) and that deletion of *SCH9* extends CLS by increasing resistance to acid stress (Burtner et al., 2009). Similarly, TORC1 regulates metabolic reconfiguration in stationary phase, resulting in altered levels of ethanol and glycerol that promote survival (Fabrizio et al., 2005; Wei et al., 2009). The connections between TOR-regulated mitochondrial respiration, metabolite accumulation, stress-resistance pathways, and other aspects of cell physiology implicated in yeast CLS remain largely unknown (Fontana et al., 2010).

In this study, we have revealed that reduced TORC1 signaling mediates CLS extension, to a significant degree, by cell-intrinsic regulation of mitochondrial respiratory coupling that elevates mitochondrial membrane potential and ROS during growth. Furthermore, we provide evidence that elevated ROS (e.g., superoxide) during growth is an adaptive mitochondrial signal that programs the downregulation of mitochondrial potential and ROS in stationary phase to promote longevity.

RESULTS

Reduced TORC1 Signaling Enhances Mitochondrial Respiratory Capacity and Coupling

To date, the relative contributions of TORC1 and TORC2 signaling with regard to CLS and age-associated mitochondrial phenotypes have not been established. To this end, we analyzed a yeast strain lacking the *TCO89* gene, which encodes a protein subunit unique to TORC1 (Reinke et al., 2004). Compared to an isogenic wild-type control strain, this strain has a significantly longer CLS (Figure S1A) and, as we reported previously in *tor1Δ* strains (Bonawitz et al., 2007), had increased mitochondrial oxygen consumption (Figure S1B), but decreased mitochondrial and cellular ROS in early stationary phase (Figures S1C and S1D). We next analyzed strains that were deleted for either *AVO2* or *BIT61*, nonessential genes that encode protein subunits unique to TORC2 (Wullschleger et al., 2005), and found that they also exhibited increased CLS (Figure S1E), however to a lesser degree than deletion of *TCO89* (Figure S1A). Similarly, mitochondrial oxygen consumption was increased moderately in these strains (Figure S2F). The reductions in mitochondrial and cellular ROS were similar in *bit61Δ*, *avo2Δ* and *tco89Δ* strains (Figures S1C, S1D, S1G, and S1H). These data solidify the hypothesis that reduced TORC1 activity specifically mediates the majority of the previously ascribed effects of reduced TOR signaling on mitochondrial function, ROS and CLS, but also demonstrate that TORC2 can similarly, although less dramatically, affect these parameters through a mechanism that remains to be determined.

Mitochondrial membrane potential is determined by a balance between electron transport chain-driven pumping of protons outward across the inner mitochondrial membrane and dissipation of the proton gradient, either by ATP synthase-driven proton translocation or unproductive proton leak. We measured mitochondrial membrane potential during various growth and stationary phases. At 36 hr postinoculation, when cultures are in early stationary phase, *tor1Δ* strains had lower membrane potential than wild-type strains (Figure 1A), similarly to what we

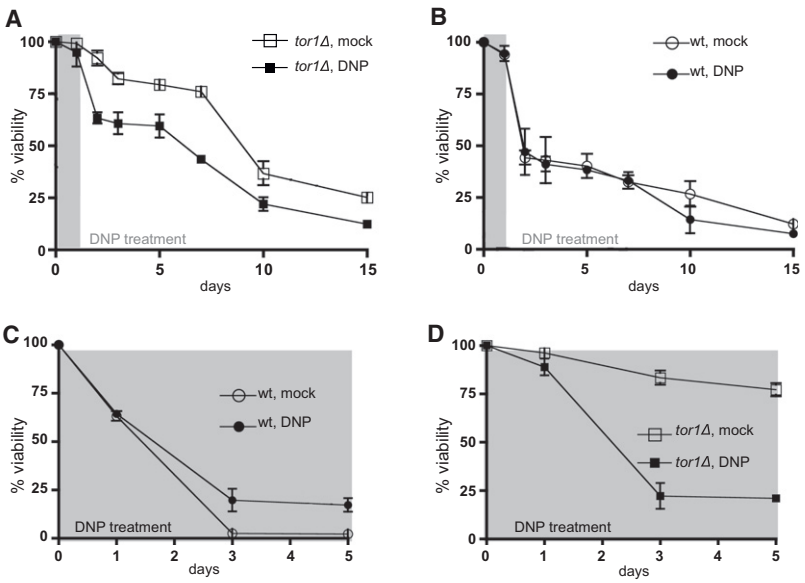

Figure 2. Effects of Uncoupling Respiration on CLS of Wild-Type and *tor1Δ* Strains

(A and B) CLS assays (viability as a function of time after inoculation) of *tor1Δ* (A) or wild-type (B) strains treated with 10 μM DNP (*tor1Δ*, DNP) or vehicle (*tor1Δ*, mock) for the first 24 hr after inoculation (gray box).

(C and D) CLS assays of wild-type (C) or *tor1Δ* (D) strains treated with 10 μM DNP from inoculation until termination of the aging experiment. Error bars represent the mean ± SD.

reported previously at 24 hr postinoculation (Pan and Shadel, 2009). Yet during mid-logarithmic growth (12 hr postinoculation), *tor1Δ* strains showed elevated membrane potential compared to wild-type strains, while no difference was observed between these strains at 20 hr postinoculation, as cultures approach the postdiauxic shift (Figure 1A). These differences are likely due to the wild-type strain building and maintaining membrane potential in early stationary phase (following increased respiration during diauxic and postdiauxic growth), while the *tor1Δ* null strain has greater respiration early in logarithmic phase that steadily decreases as a function of time in culture (Bonawitz et al., 2007). When electron transport chain activity is coupled tightly to ATP synthesis, inhibition of the ATP synthase prevents dissipation of the proton gradient to result in decreased electron transport and mitochondrial oxygen consumption. To identify the source of elevated membrane potential in *tor1Δ* yeast during growth and determine if reduced TORC1 signaling affects mitochondrial coupling, we evaluated relative responses to respiration inhibitors in vivo and respiration in isolated mitochondria from logarithmically growing yeast. When treated with the ATP synthase inhibitor triethyl-tin bromide (TET), cultures of the *tor1Δ* strain, but not the wild-type strain, responded by decreasing oxygen consumption (Figure 1B), indicating that ATP production is better coupled to electron transport in the *tor1Δ* strain. When cultures of these strains were treated with dinitrophenol (DNP), which causes proton leak and complete uncoupling of electron transport from ATP synthesis, the *tor1Δ* strain robustly increased oxygen consumption, while wild-type strains were relatively unaffected (Figure 1C). Consistent with in vivo results, we observed higher ATP/O ratios, NADH- and succinate-driven oxygen consumption, and increased complex II and IV activities in mitochondria isolated from *tor1Δ* and *sch9Δ* strains compared to wild-type (Figures 1D, S2A, and S2B). From these results, we conclude that during early logarithmic growth, reduced TOR signaling increases electron transport chain capacity and coupled ATP synthesis.

Coupled Respiration Contributes to Reduced TORC1-Mediated CLS Extension

TORC1 is most active during logarithmic growth, downregulated as cells progress through postdiauxic growth (late logarithmic), and largely inactive in stationary phase (Wullschleger et al., 2006), yet reduced TORC1 signaling results in extended stationary phase survival and CLS. To test the hypothesis that increased coupling and membrane potential during growth contributes to extended CLS in yeast with reduced TORC1 signaling, we treated *tor1Δ* and wild-type yeast cultures with DNP for the first 24 hr of growth (Figures 2A and 2B, gray box) and then shifted yeast into conditioned media lacking the drug. This growth-phase DNP treatment virtually eliminated the extended CLS of the *tor1Δ* strain relative to vehicle-only controls, but had little effect on CLS in identically treated wild-type strains (Figures 2A and 2B). In contrast, adding DNP to wild-type cultures during growth and stationary phase slightly increased CLS (Figure 2C), as reported previously (Barros et al., 2004). However, similar to DNP treatment during growth only, DNP treatment of *tor1Δ* cultures during growth and stationary phase severely attenuated CLS extension (Figure 2D). Together, these results indicate that mild uncoupling during stationary phase can slightly extend CLS of wild-type strains, but that increased coupled respiration during some phase of active growth is important for extension of CLS imparted by reduced TORC1 signaling.

Elevated Mitochondrial ROS during Growth Are Needed for Full CLS Extension

The results presented so far suggested that reduced TORC1 signaling enhances respiratory coupling during growth to somehow elicit an adaptive, CLS-extending response in stationary phase. Although mitochondrial membrane potential could be sensed directly, we pursued a hypothesis in which elevated membrane potential during growth generates mitochondrial ROS, which have known functions in cellular signaling (Hamanaka and Chandel, 2009; Linnane et al., 2007; Starkov, 2008).

As we documented previously (Bonawitz et al., 2007), *tor1Δ* strains have reduced ROS based on DHE staining relative to wild-type strains in early stationary phase (Figure 3A). However, during logarithmic growth, where we already have documented increased mitochondrial membrane potential (Figure 1A), *tor1Δ* strains have elevated DHE staining (Figure 3A). To determine if

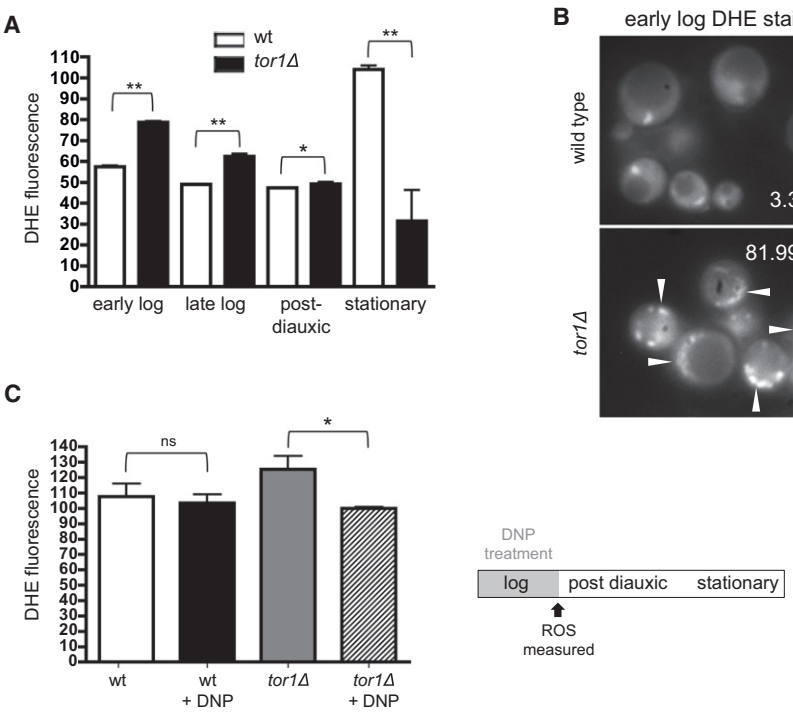

Figure 3. Coupled Respiration Drives Mitochondria ROS Production in *tor1Δ* Yeast

(A) FACS analysis of DHE-stained wild-type and *tor1Δ* strains during early log (6 hr after inoculation), late log (16 hr after inoculation), postdiauxic (20 hr after inoculation), and stationary phase (36 hr after inoculation).

(B) Microscopy of DHE fluorescence in wild-type (top) and *tor1Δ* (bottom) in early log (6 hr after inoculation). Arrows indicate punctate and tubular, mitochondria-like structures. Numbers represent the average percentage of cells displaying more than two foci of DHE fluorescence per cell plus the standard deviation of three biological replicates. Over 200 cells were counted for each replicate.

(C) DHE fluorescence of wild-type and *tor1Δ* strains treated with 10 μM DNP (+DNP) or vehicle for the first 20 hr of growth. FACS analysis was performed in late logarithmic/postdiauxic growth (as indicated to the right). Error bars represent the mean ± SD with p values indicated as described in the legend to Figure 1. See also Figure S3.

reduced TORC1 signaling specifically elevates mitochondrial ROS, we analyzed the pattern of DHE staining in logarithmically growing cells. Upon oxidation, DHE intercalates into proximal nucleic acids (usually DNA) to generate a fluorescent signal (Vanden Hoek et al., 1997; Benov et al., 1998). In wild-type cells, low levels of ROS throughout the cytoplasm induce DHE intercalation into nuclear DNA, resulting in a single focus of fluorescence (Figure 3B, top). In contrast, in *tor1Δ* strains, the presence of multiple foci of DHE fluorescence and tubular structures (Figure 3B, bottom, arrows) in over 80% of cells indicates DHE intercalation with mtDNA following oxidation by mitochondrial ROS. Finally, to determine if coupled respiration drives ROS production during growth in *tor1Δ* yeast, we treated cells with DNP for the first 24 hr of growth and measured DHE fluorescence at the conclusion of DNP treatment. Consistent with more uncoupled respiration in wild-type yeast during growth, DNP did not affect wild-type cellular ROS (Figure 3C). However, DNP treatment reduced DHE staining of *tor1Δ* to wild-type levels, supporting that reduced TORC1 signaling couples respiration, increases membrane potential, and drives mitochondrial ROS production during growth. Since DHE is primarily used to detect superoxide, but can also react with other ROS, we analyzed wild-type and *tor1Δ* strains with DHR, which preferentially reacts with hydrogen peroxide (Henderson and Chappell, 1993). In contrast to our DHE results, *tor1Δ* strains exhibit consistently lower DHR staining than wild-type (Figure S3A). Finally, overexpression of *SOD2*, which encodes mitochondrial manganese superoxide dismutase, significantly curtails CLS extension in a *tor1Δ* strain, but has no effect in the isogenic wild-type strain (Figure S3B). Therefore, we conclude that mitochondrial superoxide produced during growth in a TORC1-inhibited strain can act as an adaptive signal to extend CLS.

Growth-Phase TORC1 Inhibition or Enhanced Mitochondrial ROS Extends CLS

If mitochondrial superoxide during growth determines a significant portion of extended CLS in strains with reduced TORC1 activity, elevating mitochondrial superoxide levels in wild-type yeast should extend CLS. We treated wild-type and *tor1Δ* yeast with menadione (MD), which participates in redox cycling to generate superoxide (Castro et al., 2008). FACS analysis of DHE-stained cells indicated that MD treatment causes a significant increase in cellular superoxide of wild-type cells, and the pattern of DHE fluorescence colocalizes with mitochondria-targeted GFP in >50% of MD-treated cells (Figures S4A and S4B), indicating that mitochondrial superoxide is being produced. As shown in Figure 4A, MD treatment for the first 24 hr of growth extends CLS of wild-type yeast relative to vehicle-treated controls. Similar to reduced TORC1 signaling, MD treatment during growth also led to an adaptive decrease in mitochondrial membrane potential and DHE staining in stationary phase (Figures 4C and 4D). Therefore, elevating mitochondrial superoxide during growth in wild-type strains phenocopies the reduced mitochondrial membrane potential and reduced superoxide levels observed in stationary phase of *tor1Δ* strains and extends CLS. Furthermore, the inability of the same treatment to further alter these stationary-phase parameters and extend CLS in the *tor1Δ* strain (Figures 4B–4D) strongly suggests that, during growth, reduced TORC1 signaling and elevated mitochondrial superoxide function in the same pathway to regulate CLS.

Growth phase appears to be a critical window during which reduced TORC1 signaling influences CLS. Consistent with this hypothesis, treating a wild-type strain with rapamycin just during growth increased CLS (Figure 4E), which was accompanied by

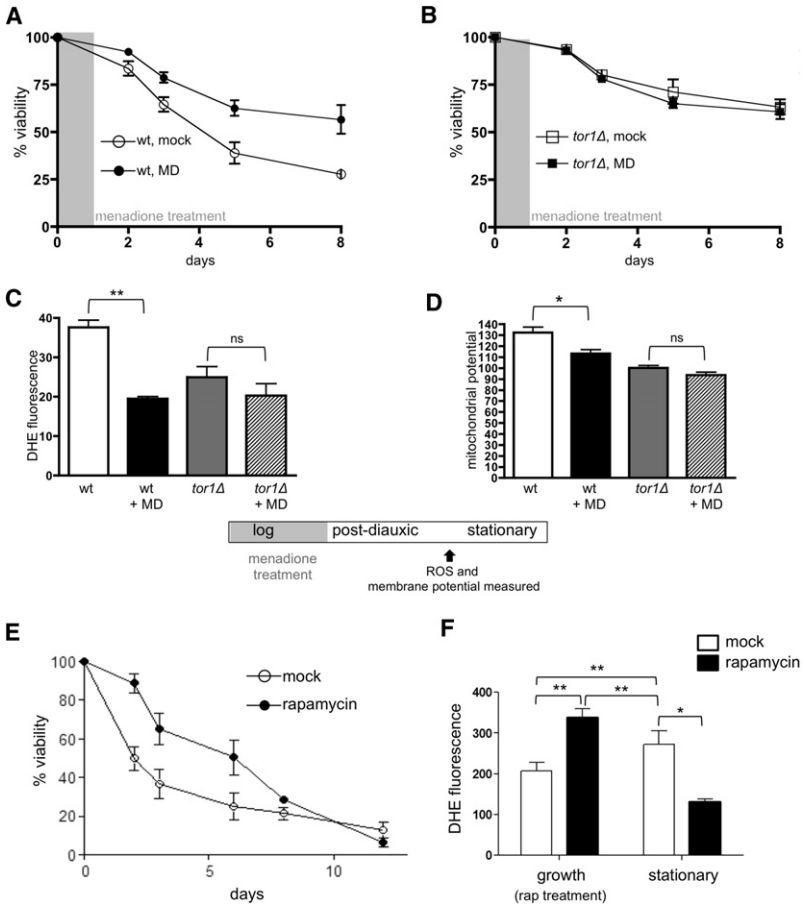

Figure 4. Elevating Superoxide via Menadione or Rapamycin Treatment Only during Growth Extends CLS of Wild-Type Yeast

(A and B) CLS assays of wild-type (A) or *tor1Δ* (B) strains treated with 1 μM menadione (MD) or vehicle (mock) for the first 24 hr of growth (gray box).

(C) FACS analysis of stationary-phase DHE staining of wild-type (wt) and *tor1Δ* strains that were treated with menadione (+MD) or vehicle for the first 24 hr of growth.

(D) Stationary-phase mitochondrial membrane potential measured by DiOC6 fluorescence of wild-type and *tor1Δ* strains treated as described in (C).

(E) CLS assays of a wild-type strain treated with 200 nM rapamycin or vehicle (mock) for the first 24 hr of growth (gray box).

(F) DHE fluorescence of a wild-type strain treated with rapamycin or vehicle (mock) for the first 24 hr of growth. Bars on the left indicate DHE staining during growth with and without rapamycin treatment; bars on the right indicate DHE staining in stationary phase following rapamycin treatment during growth. Error bars represent the mean ± SD with p values indicated as described in the legend to Figure 1. See also Figure S4.

significantly elevated DHE staining during growth and reduced DHE staining in stationary phase relative to untreated cells (Figure 4F). Importantly, neither MD nor rapamycin treatment in stationary phase extended CLS (Figures S4C and S4D), confirming that increased mitochondrial ROS during growth phase is a key determinant of CLS.

Reduced TORC1-Mediated CLS Extension Is Cell-Intrinsic and Rim15p-Independent

It was recently reported that extracellular accumulation of metabolic organic acid by-products is the major parameter that limits yeast CLS (Burtner et al., 2009). As reported by others (Burtner et al., 2009), we found that neutralizing the culture medium extended CLS of wild-type and *tor1Δ* yeast strains (Figure 5A), although the latter to a lesser extent. Because our results implicate mitochondrial function as a key determinant of CLS, we investigated mitochondrial parameters in neutralized and untreated cultures and found that, in wild-type strains, oxygen consumption is gradually modulated in response to neutralization, resulting in a reduced rate of oxygen consumption 24 and 48 hr (Figures 5B and S5A) after neutralizing the media. Media neutralization also reduced DHE staining and mitochondrial membrane potential in wild-type stationary-phase cultures (Figures 5C and 5D), supporting the correlation between these parameters and CLS. Together, these results support a mitochondrial response to media neutralization in wild-type yeast that is important for CLS extension. Interestingly, although media neutralization extended CLS of a *tor1Δ* strain to some extent, oxygen consumption, DHE staining, and mitochondrial membrane potential in stationary phase were not additionally decreased in this strain (Figures 5C, 5D, and S5B). These results suggest that reducing TORC1 activity does not extend CLS exclusively by reducing media acidification and that media neutralization partially determines CLS by mechanisms that are independent of mitochondrial function.

We next examined whether any cell-extrinsic factor was responsible for the ability of reduced TOR signaling to extend CLS. We grew wild-type, *tor1Δ*, and *sch9Δ* strains to the beginning of stationary phase (when the largest changes in pH manifest) and exchanged the growth medium for the remainder of the aging experiment. This media exchange between either wild-type and *tor1Δ* (Figure 5E) or wild-type and *sch9Δ* (Figure 5F) cultures had no effect on CLS of any of the strains. This demonstrates that the effects of reduced TORC1 activity on CLS are cell-intrinsic and not a function of acidification or increases in ethanol in stationary-phase media that we observed in *tor1Δ* and *sch9Δ* cultures (Figure S6).

The Rim15p kinase relays input from several signaling pathways, including TORC1-Sch9p, to transcription factors that mediate cellular stress responses and is an important determinant of yeast CLS. Hence, we examined if Rim15p functions downstream of the TORC1/Schp9 mitochondrial pathway to regulate CLS. In agreement with the findings of others (Wei et al., 2008), we observe that deletion of *RIM15* curtails CLS (Figure 6A). However, deletion of *SCH9* extended CLS of the *rim15Δ* strain to a similar degree as it does its isogenic wild-type control strain, demonstrating that *SCH9* deletion can extend CLS independently of Rim15p. Furthermore, CLS of the

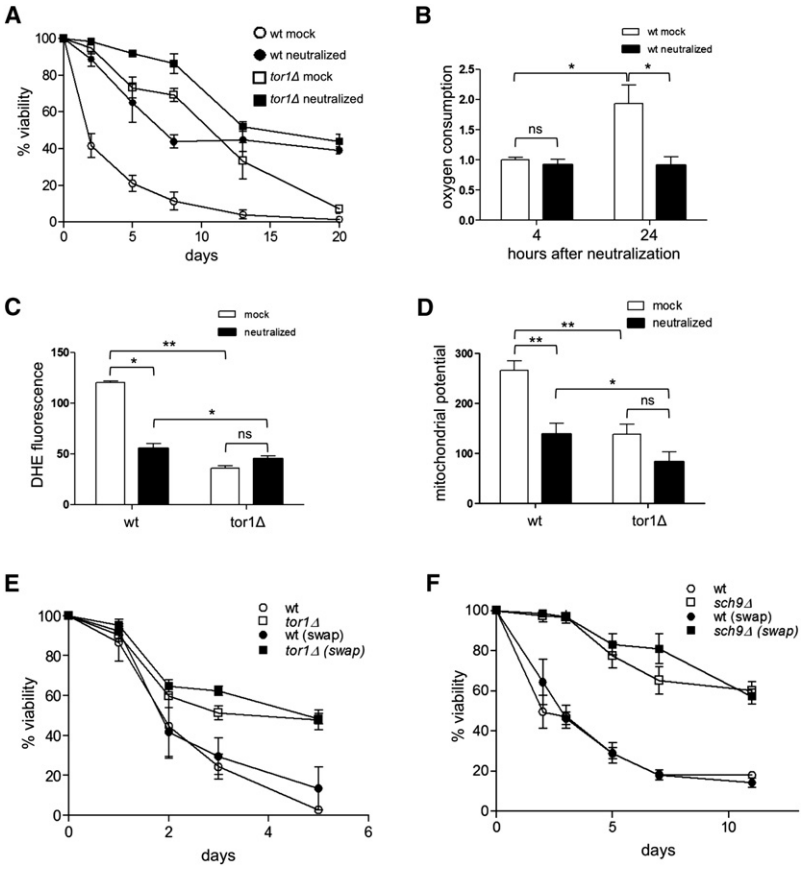

Figure 5. Media Acidification Limits Chronological Life Span in Synthetic Dextrose Medium, but Extended CLS in *tor1Δ* and *sch9Δ* Strains is Due to Cell-Intrinsic Effects

(A) CLS curves of wild-type (wt) and *tor1Δ* in unneutralized (mock) and NaOH-neutralized (neutralized) SD media. Media was neutralized at the beginning of stationary phase.

(B) Oxygen consumption of neutralized (wt neutralized) or not (wt mock treated) wild-type cells 4 and 24 hr after neutralization.

(C) Stationary phase DHE fluorescence of wild-type (wt) and *tor1Δ* cells neutralized or untreated (mock).

(D) Mitochondrial membrane potential of cells treated as described in (C).

(E) CLS of wild-type and *tor1Δ* in original media (open symbols) or subjected to media swap (swap) in stationary phase.

(F) Same as (E), except wild-type and *sch9Δ* strains were analyzed. Error bars represent the mean ± SD with p values indicated as described in the legend to Figure 1. See also Figures S5 and S6.

rim15Δ/sch9Δ strain was intermediate to wild-type and *sch9Δ* (Figure 6A), showing that much, but not all, of the CLS-extending effects of *SCH9* deletion in a wild-type strain can be attributed to Rim15p activation.

We next investigated whether changes in mitochondrial function and/or stress response pathways could explain the ability of *SCH9* deletion to extend CLS in the absence of Rim15p. First, *rim15Δ* strains had greater mitochondrial oxygen consumption during growth and greater mitochondrial membrane potential and ROS in stationary phase, the latter of which likely contribute to shortened CLS. As it does in a wild-type background, deletion of *SCH9* in the *rim15Δ* background further increased mitochondrial oxygen consumption (Figure 6B), yet stationary-phase mitochondrial membrane potential and ROS were reduced to below wild-type levels (Figures 6C–6E). Next, we determined if the ability of *SCH9* deletion to rescue the decreased CLS of a *rim15Δ* strain could also be explained by Rim15p-independent activation of Gis1p and Msn4p, transcription factors downstream of Rim15p. We measured abundance of two mRNAs, *SSA3* and *SHC1*, whose transcription is induced by Gis1p (Zhang and Oliver, 2010; Zhang et al., 2009). As expected, expression of both target mRNAs was increased in strains lacking *SCH9* (Figures S6A and S6B), and deletion of *RIM15* did not decrease Gis1p target gene expression, consistent with parallel pathways of Gis1p regulation (Zhang and Oliver, 2010; Zhang et al., 2009). However, deletion of both *RIM15* and *SCH9* substantially increased expression, suggesting that Gis1p might be hyperactive when both Rim15p and Sch9p regulation are removed. Additionally, nuclear localization of Msn4p, which is induced by stress and as cells enter stationary phase (Beck and Hall, 1999), is decreased in *sch9Δ/rim15Δ* relative to *sch9Δ* in early, but not late stationary phase (Figure S6C), suggesting that deleting *SCH9* can induce Msn4-GFP nuclear localization independently of Rim15p. Altogether, these results suggest that, in addition to altering mitochondrial respiration and ROS production, deleting *SCH9* can also enhance stress responses in a Rim15p-independent manner, both of which likely contribute significantly to the rescue of the curtailed CLS of a *rim15Δ* strain.

DISCUSSION

While the involvement of mitochondria in aging and longevity is well accepted, the precise mechanisms involved remain unresolved. Cellular oxidative damage and stress mediated by enhanced mitochondrial ROS production are commonly invoked as mediators of the mitochondrial effects on aging; however, the emergence of mitochondria and ROS as signaling entities has invited speculation on more intricate mitochondrial theories of aging (Fleury et al., 2002; Hamanaka and Chandel, 2009; Schieke et al., 2006; Starkov, 2008). In this study, we examined how TOR signaling influences mitochondrial function, ROS, and yeast CLS. From our results, we conclude that reduced TORC1 signaling reconfigures mitochondrial respiration toward a more coupled state that increases mitochondrial membrane potential and ROS production during active growth phases, providing an adaptive ROS signal that enhances survival in stationary phase and extends CLS (Figures 1A–2A and 7). The main results that support this conclusion are (1) mitochondria in *tor1Δ* strains have higher membrane potential and produce more mitochondrial ROS during growth due to enhanced coupling of electron transport to ATP synthesis (Figures 1 and 3), (2) uncoupling

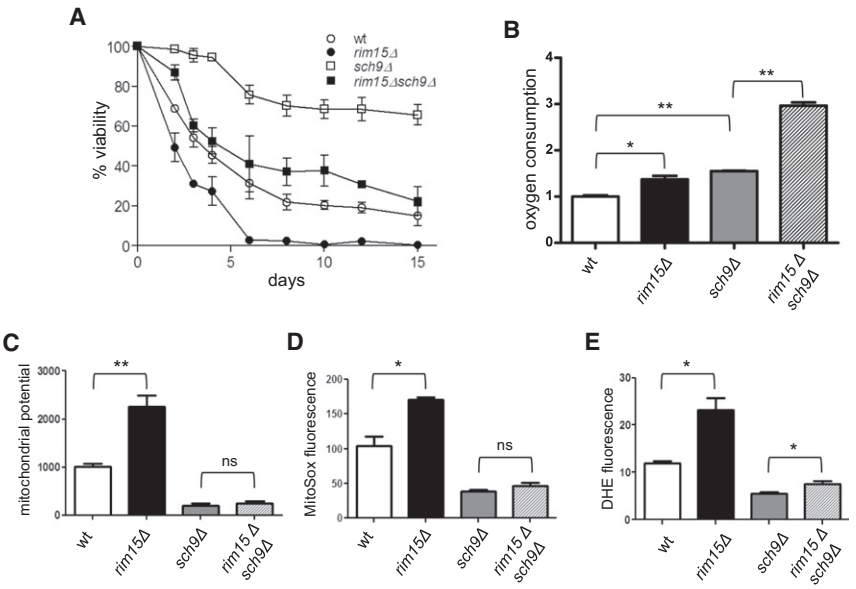

Figure 6. The Reduced Chronological Life Span of a *rim15Δ* Strain is Rescued by Deletion of *SCH9* via Effects on Mitochondrial Respiration and ROS

(A–E) Chronological life span (A), mitochondrial oxygen consumption (B), mitochondrial membrane potential (C), MitoSOX fluorescence (D), and DHE fluorescence (E) of DBY2006 (wt) and isogenic *rim15Δ*, *sch9Δ*, and *rim15Δ/sch9Δ* strains. Mitochondrial parameters were measured in early stationary phase as described in Experimental Procedures. Error bars represent the mean ± SD with p values indicated as described in the legend to Figure 1. See also Figure S7.

respiration during growth alone or overexpressing *SOD2* prevents CLS extension in *tor1Δ* strains (Figures 2, 3, and S3), and (3) inhibiting TORC1 with rapamycin or generating an artificial mitochondrial ROS signal with MD in a wild-type strain just during growth is sufficient to extend CLS (Figure 4).

As we showed previously for mitochondrial oxygen consumption and translation of mtDNA-encoded OXPHOS subunits (Pan and Shadel, 2009), mitochondrial membrane potential in *tor1Δ* strains is higher than in wild-type strains during active growth, but gradually declines to levels below wild-type in stationary phase (Figure 1A). Since yeast have no known uncoupling proteins, which in mammals can be induced to uncouple respiration and reduce membrane potential (Azzu et al., 2010; Kadenbach, 2003), we propose that mitochondrial membrane potential is likely greater during growth in *tor1Δ* strains due to (1) more electron transport chain complexes available to pump protons out of the matrix and (2) protons being shuttled back into the matrix in a more controlled fashion through ATP synthase, versus leaking across the inner membrane. Thus, in yeast, we conclude TORC1 dynamically controls the degree of respiration coupling, which in turn regulates mitochondrial membrane potential.

Since membrane potential is a major determinant of mitochondrial ROS production capacity (Kadenbach, 2003), we hypothesized that increased mitochondrial ROS may be an adaptive signal during growth involved in CLS extension imparted by reduced TORC1 signaling. Consistent with this concept, we observe more DHE staining in mitochondria-like patterns during growth in *tor1Δ* strains (Figures 3A and 3B). Furthermore, DHE readily reacts with superoxide, while DHR is more specific for hydrogen peroxide (Vanden Hoek et al., 1997; Benov et al., 1998; Henderson and Chappell, 1993). Because *tor1Δ* yeast have lower DHR fluorescence (Figure S3), we conclude that increased mitochondrial superoxide production during growth is an adaptive longevity signal in yeast. Our result that treating wild-type yeast with a sublethal concentration (1 μM) of MD during growth (as opposed to higher concentrations, see Fabrizio et al., 2005) generates a mitochondrial superoxide signal (Castro et al., 2008) to extend CLS strongly supports this conclusion (Figures 4A, S4A, and S4B). Additionally, similar treatment of a *tor1Δ* strain has no effect on CLS, and inhibition of TORC1 with rapamycin during the same growth window is sufficient to extend CLS of wild-type (Figures 4B and 4E). Finally, overexpression of *SOD2* specifically curtails the CLS of *tor1Δ* yeast (Figure S3B), consistent with a requirement for elevated mitochondrial superoxide in full life span extension of *tor1Δ* yeast. That *SOD2* overexpression alone significantly affects the CLS of *tor1Δ* is consistent with previous reports that combined *SOD1* and *SOD2* overexpression is required for CLS extension in wild-type yeast (Fabrizio et al., 2003) and that SOD overexpression has variable effects on CLS in strains with altered mitochondrial function and gene expression (Bonawitz et al., 2006). Our previous finding that hypoxia during growth extends CLS in wild-type, but not *tor1Δ,* strains may also support an adaptive signaling role for ROS, as hypoxia induces mitochondrial ROS production (Bonawitz et al., 2007; Dirmeier et al., 2002). We therefore propose that increasing mitochondrial superoxide production during growth elicits an adaptive response that reduces mitochondrial membrane potential and ROS in stationary phase and, in conjunction with enhanced stress responses, result in the robust CLS extension observed when TORC1 signaling is inhibited (Figure 7). Fully understanding the mechanism of superoxide-mediated mitochondrial adaptive signaling will require future identification of factors that sense elevated mitochondrial superoxide, which could include conserved ROS-sensing kinases, phosphatases, and transcription factors (Veal et al., 2007).

Rim15p activates a stress-responsive transcription program that contributes significantly to the extension of CLS in strains with reduced TOR signaling (Wei et al., 2008). Based on our results, we propose two pathways by which reduced TORC1 signaling ultimately regulates CLS: Rim15p-dependent stress resistance mechanisms that eliminate ROS (Wei et al., 2008; Fabrizio et al., 2001; Wanke et al., 2008; Agarwal et al., 2005) and Rim15-independent mitochondrial alterations (Figures 6B–6E) to produce fewer ROS (Figure 7). We also recognize the possibility for crosstalk between these branches (Burtner et al., 2009; Wanke et al., 2008). Determining precisely how Rim15p-dependent and Rim15p-independent TORC1 signaling cooperate to regulate yeast CLS will be an important focus of future work.

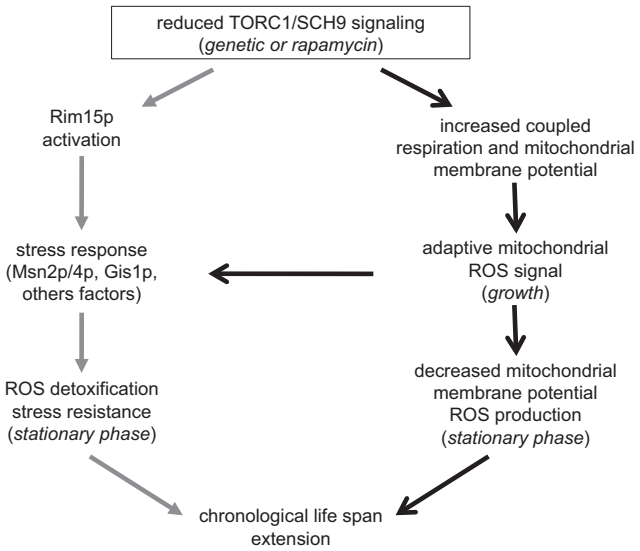

Figure 7. Adaptive Mitochondrial ROS Signaling and Activation of Rim15p-Dependent Stress Responses Collaborate to Mediate CLS Extension by Reduced TORC1 Signaling

A speculative model of how reduced TORC1 signaling extends CLS by activating both adaptive mitochondrial ROS signaling (right arm) and Rim15-dependent stress-resistance pathways (left arm). These responses would cooperate in CLS extension by enhancing ROS detoxification and stress resistance and by an adaptive response to elevated cellular superoxide and/or other ROS during growth that alters mitochondria function to decrease membrane potential and produce fewer ROS in stationary phase. This adaptive signal may activate some redox-sensitve factor that controls nuclear-encoded mitochondria and/or stress response genes (horizontal arrow), perhaps via epigenetic regulation, and results in altered respiration and enhanced stress responses in stationary phase. This model is meant to encapsulate those aspects of CLS extension by TOR inhibition involving ROS, mitochondria, and oxidative-stress resistance. We acknowledge that reduced TOR signaling has other effects on cell physiology that are also important for CLS, which are not pictured here.

Our results are consistent with a growing body of literature that mitochondrial ROS can act as mediators of adaptive/hormetic effects on yeast life span (Agarwal et al., 2005; Kharade et al., 2005; Piper et al., 2006), including a relevant recent study showing that caloric restriction elevates hydrogen peroxide in early stationary phase, which induces superoxide dismutase activity to help extend CLS (Mesquita et al., 2010; Weinberger et al., 2010). That we implicate superoxide as the adaptive/hormetic signaling molecule suggests that various ROS may promote adaptive signaling during different stages of growth (e.g., superoxide during logarithmic growth and H_2O_2 during stationary phase) or that there are mechanistic differences in life span extension induced by reduced TOR signaling and calorie restriction (CR). The observation that CR further extends the life span of *sch9Δ* strains supports the latter possibility (Wei et al., 2008; Weinberger et al., 2010). Furthermore, numerous studies in *C. elegans* provide strong evidence for mitochondria- and ROS-mediated adaptive/hormetic regulation of life span (Dillin et al., 2002; Gems and Partridge, 2008; Yang and Hekimi, 2010; Ristow and Zarse, 2010; Schulz et al., 2007). Finally, lower mitochondrial membrane potential, which we propose is a downstream consequence of adaptive mitochondrial signaling, correlates extremely well with longer life span in worms with genetic alterations in various longevity pathways (Lemire et al., 2009). In the context of these studies, our findings strongly suggest that pathways that conditionally regulate mitochondrial membrane potential and/or ROS production during specific growth or developmental windows, like TORC1, could be key regulators of longevity.

Media metabolites have also been shown to regulate yeast CLS (Aerts et al., 2009; Fabrizio et al., 2005; Wei et al., 2009). While our results (Figure 6A) are consistent with media acidification as one determinant of CLS (Burtner et al., 2009), we conclude from our media-swap experiments (Figures 6E and 6F) that reduced production or release of extracellular molecules does not underlie the extension of CLS in *tor1Δ* and *sch9Δ* strains, and hence the effects we describe in this study are cell-intrinsic. The additional finding that media neutralization reduces mitochondria membrane potential and cellular ROS (Figure 6) highlights the involvement of mitochondria in acid stress response and suggests that media acidification in yeast CLS experiments ultimately regulates general cell-intrinsic stress responses that are relevant to conserved mechanisms of aging. Finally, we observe a slight reduction in stationary-phase media ethanol levels in *tor1Δ* and wild-type cultures treated with rapamycin during growth (Figure S6). While our media-swap experiments indicate that differences in ethanol concentration at the time of swap do not determine CLS, we cannot exclude the possibility that enhanced extracellular ethanol depletion, via utilization as an alternate carbon source, in TOR-inhibited strains later in stationary phase contributes to CLS extension as proposed by others (Fabrizio et al., 2005). In fact, such metabolic reconfigurations may be part of the adaptive response in stationary phase to mitochondrial ROS signaling events during growth.

Reduced TORC1 signaling increases life span in organisms ranging from yeast to mammals and has other beneficial effects that are of potential therapeutic value for human disease (Fontana et al., 2010). Our results shed significant light on the involvement of mitochondrial adaptive/hormetic signaling in the TORC1 longevity pathway and therefore likely represent an important avenue that might be exploited in these regards.

EXPERIMENTAL PROCEDURES

Yeast Strains

All experiments were performed in the DBY2006 strain background (*MATα his3-Δ200 leu2-3,-112 ura3-52 trp1-Δ1 ade2-1*) or the BY4742 background (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) as indicated. The *tor1Δ* and *sch9Δ* strains have been described (Bonawitz et al., 2007; Pan and Shadel, 2009). The *RIM15* ORF in DBY2006 and in DBY2006 *sch9Δ* was deleted with a kanamycin cassette that was PCR amplified from the *rim15Δ* strain in the yeast DBY4742 knockout collection (Open Biosystems) with primers AATTA TCCCGGGTCCATATTGCCCTAGGTCTTG and AATTATCCCGGGGCCTCGA AATTGAGAAATGAA. Gel-purified amplicons were used in transformation and G-481-resistant colonies were selected. Successful integration was verified by PCR. The *sch9Δ* and *rim15Δ/sch9Δ* strains were transformed with an MSN4-GFP plasmid (Bonawitz et al., 2006). DBY2006 was transformed with the plasmid pYX142-SU9-GFP (Westermann and Neupert, 2000). All strains were grown in standard SD medium with appropriate nutrients (Sherman, 1991).

Oxygen Consumption and CLS Assays

Oxidative phosphorylation and coupling assays using purified mitochondria were performed as described (Ocampo et al., 2010). Cellular oxygen consumption was assayed as described (Bonawitz et al., 2007). Mean oxygen consumption as

percent oxygen/minute/OD_{600} ± standard deviation of three biological replicates normalized to wild-type is shown. Chronological life span was determined as in (Bonawitz et al., 2006, 2007). For strain comparisons and drug treatments in which all cultures demonstrated comparable growth rates, percent viability is plotted as a function of "days," with the day of inoculation indicated as day 0 (the cultures reach stationary phase at day 1). During rapamycin treatment or CLS assays of *sch9* and *sch9/rim15* strains, which show reduced logarithmic growth rate, culture inoculation was staggered such that all cultures reach stationary phase within several hours. Percent viability is plotted as a function of "days," with the day of wild-type inoculation indicated as day 0. Percent viability was determined by counting the number of cells stained with 0.4% trypan blue. Data points represent the mean of three replicates inoculated from single colonies of the same strain; error bars represent standard deviation.

Chemical Treatments

TET (Sigma) or DNP (MP Biomedicals) was added to SD medium to final concentrations of 100 μM and 10 μM, respectively. Cultures were then inoculated to OD_{600} of 0.1 and grown for 24 hr before oxygen consumption was monitored. An equal volume of drug vehicle (DMSO for TET or water for DNP) was added as a control. MD (Sigma) was added to SD medium to a final concentration of 1 μM from a 4 mg/ml stock; rapamycin (Sigma) was added to media to a final concentration of 200 nM. An equivalent volume of 100% ethanol was added to mock-treated cultures in MD and rapamycin experiments. For treatments during growth only, DNP or MD was added to 50 ml SD and cultures inoculated to an OD_{600} of 0.1 and grown for 24 hr. Cells were then pelleted, the medium containing drug or vehicle discarded, and cells were resuspended in filtered, equivalently conditioned medium from a parallel culture of the same strain. In the case of rapamycin (Figure 4E), drug treatment significantly slowed growth and was thus continued until the culture reached stationary phase, after which the media was swapped as described for MD and DNP. For drug treatments in stationary phase, drug or vehicle was added to cultures inoculated as above after 48 hr of growth. Due to light sensitivity, MD- and DNP-treated cultures were grown in the dark.

Fluorescence Microscopy

Fluorescence microscopy was conducted as described previously (Bonawitz et al., 2006). Briefly, 7 μl of culture expressing GFP was mounted without fixation and visualized with an Olympus IX-71 inverted fluorescence microscope using the GFP filter. For analysis of DHE fluorescence, cells were stained with DHE as described for Flow Cytometry and visualized using the rhodamine filter. Images were captured at 100× magnification with Olympus Metamorph software. Merged images were generated with Adobe Photoshop.

Flow Cytometry

All measurements were performed on a Becton-Dickinson FACSCalibur. Analysis of yeast cellular superoxide using DHE (FL3 channel) was conducted as described previously (Bonawitz et al., 2006). For analysis of mitochondrial superoxide, 500 μl of culture was pelleted, washed once with phosphate-buffered saline (PBS), and incubated with 5 μM MitoSOX (Molecular Probes, Inc.) in PBS for 45 min at 30°C. Cells were then washed twice with PBS, and fluorescence intensity in the FL3 channel was measured. Analysis of mitochondrial potential was performed with DiOC6 (FL1 channel) as described (Pan and Shadel, 2009).

Media-Neutralization and Media-Swap Experiments

Cultures were inoculated to OD_{600} of 0.1 in 50 ml SD and grown for 20 hr. Cells were then pelleted, and the medium was vacuum filtered. The pH of the medium was neutralized to between 6.5 and 7.0 with NaOH, and cells were resuspended in neutralized medium. In the media-swap experiments, cultures were grown for 48 hr and harvested by centrifugation. Cell pellets were subsequently resuspended in the filtered original medium (nonswap) or equivalently conditioned medium of the indicated strain (swap).

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at doi:10.1016/j.cmet.2011.03.018.

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