Identification of the Target of the Retrograde Response that Mediates Replicative Lifespan Extension in *Saccharomyces cerevisiae*

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ABSTRACT The retrograde response signals mitochondrial status to the nucleus, compensating for accumulating mitochondrial dysfunction during *Saccharomyces cerevisiae* aging and extending replicative lifespan. The histone acetylase Gcn5 is required for activation of nuclear genes and lifespan extension in the retrograde response. It is part of the transcriptional coactivators SAGA and SLIK, but it is not known which of these complexes is involved. Genetic manipulation showed that these complexes perform interchangeably in the retrograde response. These results, along with the finding that the histone deacetylase Sir2 was required for a robust retrograde response informed a bioinformatics screen that reduced to four the candidate genes causal for longevity of the 410 retrograde response target genes. Of the four, only deletion of *PHO84* suppressed lifespan extension. Retrograde-response activation of *PHO84* displayed some preference for SAGA. Increased *PHO84* messenger RNA levels from a second copy of the gene in cells in which the retrograde response is not activated achieved >80% of the lifespan extension observed in the retrograde response. Our studies resolve questions involving the roles of SLIK and SAGA in the retrograde response, pointing to the cooperation of these complexes in gene activation. They also finally pinpoint the gene that is both necessary and sufficient to extend replicative lifespan in the retrograde response. The finding that this gene is *PHO84* opens up a new set of questions about the mechanisms involved, as this gene is known to have pleiotropic effects.

KEYWORDS mitochondria; transcriptional coactivation; SAGA/SLIK; Sir2; Pho84

THE retrograde response is an intracellular signaling pathway that communicates mitochondrial dysfunction to the nucleus, resulting in myriad changes in nuclear gene expression (Parikh *et al.* 1987; Liu and Butow 2006). These changes portend major metabolic adjustments and enhanced resistance to stress. Among those that stand out are the up-regulation of anaplerotic pathways, the generation of α-ketoglutarate for biosynthetic processes, and the ability to mobilize acetate both for energy and for net production of macromolecules in the absence of a fully functional, mitochondrial electron transport chain (Jazwinski 1999). The retrograde response was first identified in *Saccharomyces cerevisiae* (budding yeast), but it has since been demonstrated in *Caenorhabditis elegans*, *Drosophila melanogaster*, *Mus musculus*, and in human cells in tissue culture (Dell'agnello *et al.* 2007; Passos *et al.* 2007; Caldeira da Silva *et al.* 2008; Lapointe and Hekimi 2008; Copeland *et al.* 2009; Lee *et al.* 2010; Yang and Hekimi 2010; Durieux *et al.* 2011; Liu *et al.* 2011; Walter *et al.* 2011; Liu *et al.* 2012; Jazwinski 2015; Mishur *et al.* 2016). The retrograde response is a compensatory pathway (Jazwinski 2014) whose activation extends yeast replicative lifespan (RLS) (Kirchman *et al.* 1999). Similarly, it has a life extending effect in the other aging models listed above (Jazwinski 2015). Interestingly, the metabolic gene expression changes found under conditions of nutrient limitation that extend yeast RLS resemble those shown in the retrograde response (Jiang *et al.* 2000, 2002; Wang *et al.* 2010). The primary signal that triggers the yeast retrograde

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response is the drop in mitochondrial membrane potential (Miceli *et al.* 2011), although reduced ATP levels may play a secondary role (Zhang *et al.* 2013). Reactive oxygen species (ROS) signaling is not involved; however, mitochondrial ROS are a signal that is relevant for chronological lifespan extension in yeast (Pan *et al.* 2011).

One of the multitude of changes in nuclear gene expression is an increase in *CIT2* messenger RNA (mRNA) levels, which is most frequently used as a diagnostic for activation of the retrograde response (Liao *et al.* 1991). However, induction of this gene is not necessary for RLS extension upon retrograde-response activation (Kirchman *et al.* 1999). Given the large number of genes activated (Epstein *et al.* 2001; Traven *et al.* 2001), the task of identifying the one gene or the many genes that act together to affect longevity is a daunting task that has not been completed up until now.

Our interest in the role of chromatin-dependent gene regulation in yeast longevity (Jazwinski 1999, 2005) led us to examine the impact of transcriptional coactivator and corepressor complexes on RLS. We determined that the histone deacetylases Rpd3 and Sir2 both have large effects on RLS but in opposite directions (Kim *et al.* 1999). Deletion of *RPD3* extends, while deletion of *SIR2* curtails it. In fact, Sir2 was shown to function in yeast longevity by two mechanisms (Kaeberlein *et al.* 1999). Rpd3 is part of the Rpd3L transcriptional corepressor complex with Sin3 that typically possesses the subunits Ume1, Ume6, Sds3, Sap30, and Pho23 (Yang and Seto 2008; Lardenois *et al.* 2015) and opposes the Gcn5-containing coactivator complex at many sites throughout the genome (Huisinga and Pugh 2004; Lardenois *et al.* 2015). This implies that activation of transcription of certain genes is important for longevity. This conclusion was supported by our observation made concurrently with our work on Rpd3 that deletion of *GCN5* shortens RLS. This analysis was expanded to examine the potential function of Gcn5 in the retrograde response, and we determined that deletion of *GCN5* prevents activation of *CIT2* and the extension of RLS caused by retrograde-response activation (Kim *et al.* 2004).

The histone acetyltransferase Gcn5 is part of the large transcriptional coactivator complex SAGA, which is conserved throughout phylogeny (Sterner *et al.* 1999; Srivastava *et al.* 2015). SAGA dominates over another transcriptional coactivator TFIID at ~10% of yeast genes, which contain TATA boxes and are primarily involved in stress responses (Huisinga and Pugh 2004). However, SAGA can be found throughout the yeast genome, as shown recently (Bonnet *et al.* 2014). SAGA is also responsible for the retention of DNA circles in mother cells during the yeast RLS (Denoth-Lippuner *et al.* 2014).

More recently, a transcriptional coactivator complex called SLIK/SALSA, closely related to SAGA, was identified (Pray-Grant *et al.* 2002; Sterner *et al.* 2002). It is distinguished from SAGA by the absence of Spt8 and the presence of Rtg2 protein in its place. The conversion of SAGA to SLIK requires the specific, proteolytic truncation of another SAGA component, Spt7 (Wu and Winston 2002; Mischerikow *et al.* 2009; Spedale *et al.* 2010). SAGA with full-length Spt7 contains Spt8, while in SLIK with the truncated Spt7, the Spt8 is substituted by Rtg2 (Pray-Grant *et al.* 2002). SLIK was claimed to be the coactivator involved in the retrograde response by virtue of the detection of Rtg2 at the promoter of *CIT2* and by an increase in *CIT2* mRNA levels (Pray-Grant *et al.* 2002). However, these events were induced by growth on acetate in this study, which requires a fully active mitochondrial electron transport chain, unlike the retrograde response described earlier. Induction of *CIT2* expression in this retrograde response is activated by mitochondrial dysfunction and requires *RTG2* (Liao and Butow 1993). Although Rtg2 appears to function only in the cytoplasm to support the translocation of the transcription factor Rtg1–Rtg3 into the nucleus, which is required for induction of retrograde-response target genes (Sekito *et al.* 2000), we documented a separate role for Rtg2 in the nucleus in promoting genome stability (Borghouts *et al.* 2004). Thus, the function of the SLIK complex in retrograde signaling is not entirely clear.

Clarification of the role of SLIK compared to SAGA in the retrograde response triggered by mitochondrial dysfunction from the perspective of both gene activation and RLS extension remains to be accomplished. Here, we have separated the activities of these closely related complexes by gene manipulation, and we have determined the phenotypic effects of each complex. This analysis shows that they function largely interchangeably. We have used this information in conjunction with other gene expression studies to narrow the focus to 4 genes of the 410 retrograde-response target genes. Testing these 4 genes individually has allowed us for the first time to identify the gene whose activation is both necessary and sufficient to result in the RLS extension afforded by the retrograde response. As part of these analyses, we have also uncovered a new role of Sir2 in retrograde signaling and RLS extension.

Materials and Methods

Yeast strains and growth conditions

All strains used originated from *S. cerevisiae* strain YPK9 (*MAT***a**, *ade2-101*^{ochre} *his3-Δ200 leu2-Δ1 lys2-801*^{amber} *trp1-Δ63 ura3-52* [*rho*$^{+}$]) and its rho^{0} derivative YJR2 (Kirchman *et al.* 1999; Miceli *et al.* 2011).

SPT7 mutants were created as follows: *SPT7* was amplified from yeast genomic DNA by PCR and cloned into pRS406 (Sikorski and Hieter 1989) between the *Eco*RI and *Xho*I sites. The *SPT7* sequence was verified by DNA sequencing. Site-directed mutagenesis was performed to create the L1141V, L1142V, and S200 (Spt7 without amino acids 1125–1150) mutants. Site-directed mutagenesis was performed using the Quick Change Site-Directed Mutagenesis protocol (Stratagene, La Jolla, CA) and further modified (Wang and Malcolm 2002). The primers used for mutagenesis were: 5′-ATC TGT TCC ATT ACA G G*TA* CTG ACT ACT CAG TTT C-3′ (*SPT7* L1141V forward), 5′-G AAA CTG AGT AGT CAG *TAC* C TGT AAT GGA ACA GAT-3′ (*SPT7* L1141V reverse), 5′-CT

GTT CCA TTA CAG TTA G*TG* ACT ACT CAG TTT CAA AC-3′ (*SPT7* L1142V forward), 5′-GT TTG AAA CTG AGT AGT *CAC* TAA CTG TAA TGG AAC AG-3′ (*SPT7* L1142V reverse), 5′-TTT GGT TTT AGA GAG CTT GGG GAA ACC AAA GTG CAG G-3′ (*SPT7* S200 forward), 5′-C CTG CAC TTT GGT TTC CCC AAG CTC TCT AAA ACC AAA-3′ (*SPT7* S200 reverse). (Sequence changes for single amino acid substitutions are italicized.) Mutants were confirmed by DNA sequencing. The pRS406 plasmids with the mutants were digested with *Nru*I and individually transformed into the yeast strain by selecting for uracil prototrophy. Positive strains were grown and counterselected using fluorouracil, to evict the copy of *URA3*. Mutant strains were confirmed by sequencing of genomic DNA.

The *ADA2*, *SPT7*, *SPT8*, *MDH1*, and *PHO84* deletion strains were all constructed similarly. Deletion strains were purchased from Open Biosystems. The respective deletion cassettes containing the kanMX marker were amplified from genomic DNA using PCR primers containing sequences flanking the deleted genes. These were purified and used in a one-step gene replacement, by selecting for G418 resistance. All strains were verified by PCR using primers flanking the gene and an internal primer specific for kanMX. The *BAP2* deletion strain was constructed differently. First, the natMX4 module in plasmid pAG25 (Goldstein and McCusker 1999) was amplified by PCR using primers containing *BAP2* upstream and downstream sequences. The purified DNA was then transformed into the relevant yeast strain by selection for resistance to nourseothricin. Strains were confirmed by PCR using primers flanking the gene and an internal primer specific for natMX4.

The 2XPHO84 strain was created by first replacing the *ura3-52* mutant in YPK9 by the *URA3* gene PCR amplified from plasmid pRS406 and selection for uracil prototrophy. Then, *PHO84* was PCR amplified from genomic DNA with its promoter and 3′-UTR, and this DNA was cloned into pRS406. *PHO84*, flanked by *URA3* sequences, was released from the plasmid by restriction enzyme digestion and gel purified. This fragment was then transformed into the YPK9 *URA3* strain. Growth on 5-fluorouracil was used to select for YPK9 *ura3-52*::*PHO84*. This strain was confirmed by DNA sequencing.

Yeast strains were grown in YPD (2% peptone, 1% yeast extract, 2% glucose) or for selection of strains for prototrophy in SC medium lacking the relevant nutrient (Sherman 1991). For extrachromosomal ribosomal DNA circle (ERC) determinations, adenine sulfate was added to YPD medium to a final concentration of 50 μg/ml (YPAD). Solid medium contained 2% agar. Incubations were at 30°. YPK9 and its derivatives were cultured overnight in YPG (2% peptone, 1% yeast extract, 3% glycerol) prior to spotting on YPD plates for initiation of RLS determinations.

RNA isolation and reverse transcription, real-time quantitative PCR

One to 5 ml of yeast culture was harvested in midexponential growth phase. Cells were washed, resuspended in 400 μl TES buffer (10 mM Tris-HCl, 10 mM EDTA, 0.5% SDS, pH 7.5), and incubated for 1 hr at 65° with 400 μl of acidic phenol (Sigma, St. Louis, MO; pH 4.3) with mixing every 15 min. The samples were placed on ice for 5 min and centrifuged. The aqueous phase was removed and extracted with chloroform. RNA was precipitated at −20° by adding 1/10 volume of 3.0 M sodium acetate (pH 5.3) and 2.5 volume of 100% ethanol. Precipitates were centrifuged, washed with 70% ethanol, and dissolved in 80 μl of sterile, deionized water. RNA solutions were frozen and stored at −80°. Up to 15 μg of RNA was mixed with buffer RDD and DNase I (30 Kunitz units, QIAGEN RNase-Free DNase Kit) and incubated at 25° for 30 min. Subsequently, the RNA was cleaned using the QIAGEN RNeasy Mini Kit.

Up to 2 μg of RNA was mixed with TaqMan Reverse Transcription buffer, 25 μM $MgCl_2$, 2.5 mM (each) dNTP mix, 50 μM random hexamers, 40 units RNase inhibitor and 125 units reverse transcriptase (Applied Biosystems Reverse Transcription kit). Incubation was for 10 min at 25°, followed by 45 min at 48°, and finally 5 min at 95°. The resulting complementary DNA (cDNA) was directly used for quantitative PCR (qPCR). For qPCR, 5 μl of this cDNA was mixed with 20 μl BioRad iTaq Universal SYBR Green mix and the respective primers. The PCR program consisted of one incubation of 5 min at 50° and 40 cycles of 1 min at 95° and 1 min at 60°, using an Applied Biosystems 7300 Real-Time PCR system. The primers were: 5′-CGA AAT CTA CCC CAT CCA TGC-3′ (*CIT2* forward) and 5′-TCC CAT ACG CTC CCT GGA ATA-3′ (*CIT2* reverse), 5′-ACG GTT TGG AAA GAG CTT CT-3′ (*PHO84* forward) and 5′-TCA GAT TAC CGA CAG CAG TAT CA-3′ (*PHO84* reverse), and 5′-TTC CAT CCA AGC CGT TTT GT-3′ (*ACT1* forward) and 5′-CAG CGT AAA TTG GAA CGA CGT-3′ (*ACT1* reverse).

A standard curve was generated using serial dilutions for *ACT1*. The expression of *CIT2* and *PHO84* was normalized to that of *ACT1*, as a constitutive control, in each sample, and the relative levels of the *CIT2* and *PHO84* mRNA were calculated from the standard curve. The resident Applied Biosystems 7300 system software was used. Results are expressed as mRNA levels relative to the *ACT1* mRNA levels in the sample. Assays were in at least three replicates, and error bars are the SE.

RLS determination

A 1-μl drop of an overnight yeast culture was spotted on an agar plate. Budding cells were micromanipulated to an isolated spot on the plate. The buds were retained to initiate the experiment. Plates were incubated at 30°. Each time the retained cell budded, the resulting bud was removed, and the cell (now a mother cell) was counted one generation older. This was continued until the mother cell stopped dividing and lost refractility. The total number of buds produced by the cell is the age in generations. There were 40 cells for each strain in each determination, except that there were 80 cells each for YPK9 and YPK9 *ura3*::*PHO84* in Figure 7B. RLS of each strain within an experiment was compared using the Mann–Whitney test. Two-tailed *P*-values

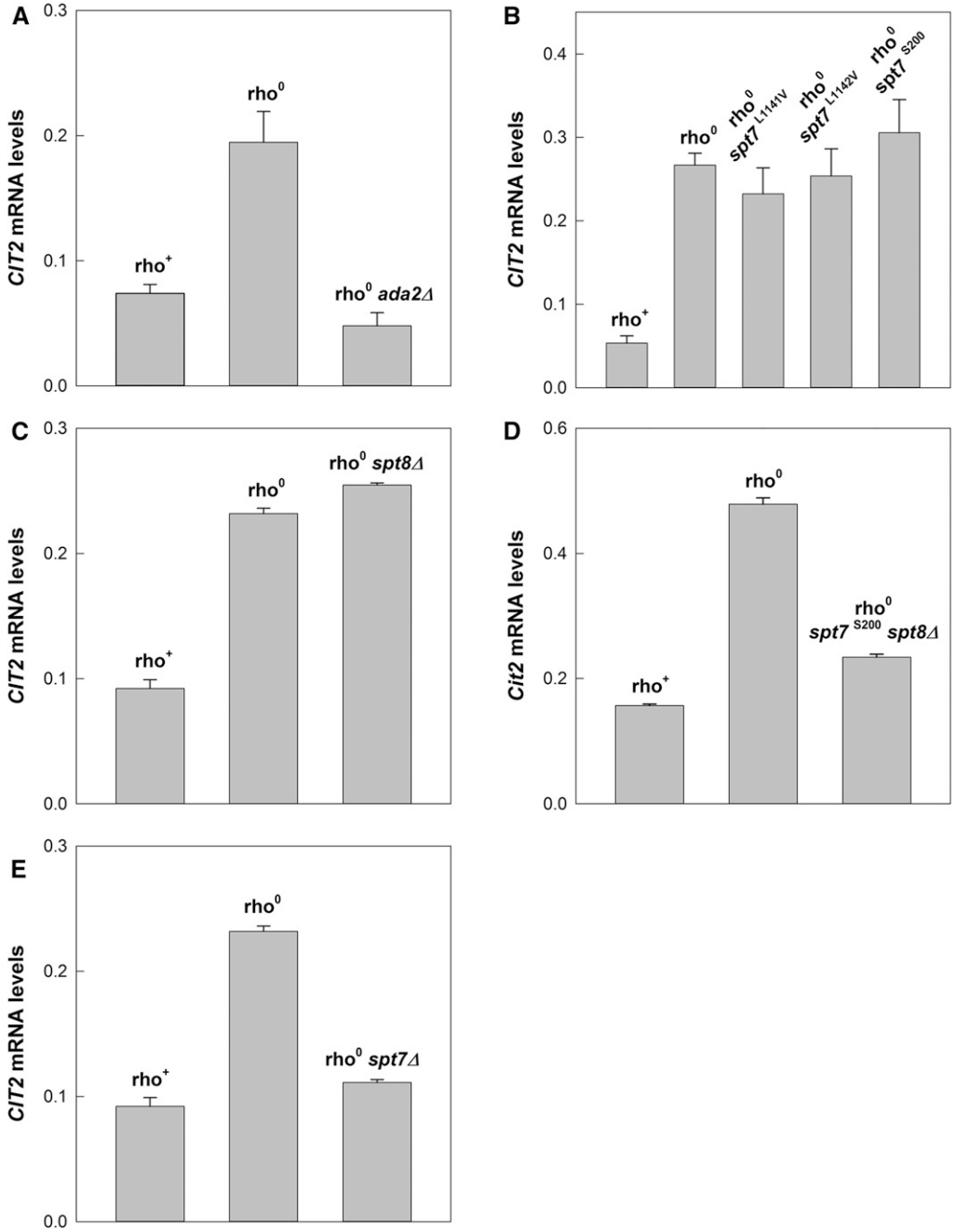

Figure 1 Activation of the retrograde response in rho^0 strains lacking SAGA or SLIK. Activation of the retrograde response was assessed by comparing the *CIT2* mRNA levels (relative to *ACT1*) by RT-qPCR in the rho$^+$ strain YPK9 and its rho^0 derivative YJR2. (A) Deletion of *ADA2*, a core component of SAGA/SLIK in rho^0 cells. (B) Elimination of SLIK in rho^0 cells by the S200 mutation in *SPT7*, which prevents the processing of this protein to allow SLIK formation and comparison to point mutations in the specific Pep4 cleavage site encompassed by S200. (C) Elimination of SAGA in rho^0 cells by deletion of *SPT8*, which is a component of SAGA but not SLIK. (D) Elimination of both SLIK and SAGA in rho^0 cells by the S200 mutation in *SPT7* and the deletion of *SPT8*, respectively. (E) Elimination of both SLIK and SAGA in rho^0 cells by removal of the common component Spt7.

are shown. Lifespans of at least two separate clones of each mutant strain were tested to ascertain reproducibility.

ERC determination

The protocol used has been described (Borghouts *et al.* 2004), and the modifications are presented here. Cells were collected by centrifugation from 5-ml overnight cultures in YPAD medium, to a concentration of 0.8–1.0 $\times$ 10^8/ml. The cells were washed with sterile, deionized water, and the pellets were resuspended in 240 μl of YD Digestion buffer (Zymo Research). To the suspension, 10 μl of R-Zymolyase (Zymo Research) were added, and the suspension was incubated at 37° for 1 hr. After addition of 240 μl of YD Lysis Buffer (Zymo Research, Irvine, CA) the suspension was mixed well by gently rolling the tube. Chloroform (500 μl) was added. The lysate was mixed thoroughly for 1 min and centrifuged for 2 min at >10,000 rpm. The upper phase was aspirated to a clean tube and two volumes of ethanol were added to precipitate yeast DNA (Kim *et al.* 2004). The DNA pellet was washed with 70% ethanol and resuspended in sterile, deionized water. DNA (10 μg) was digested with *Spe*I (New England BioLabs, Ipswich, MA) for at least 6 hr at 37°, which releases a 4.1-kb DNA fragment containing the *ACT1* gene. Digests were loaded into lanes of a 1% agarose gel containing TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) and electrophoresed for 24 hr at 1.8 V/cm. A parallel lane of

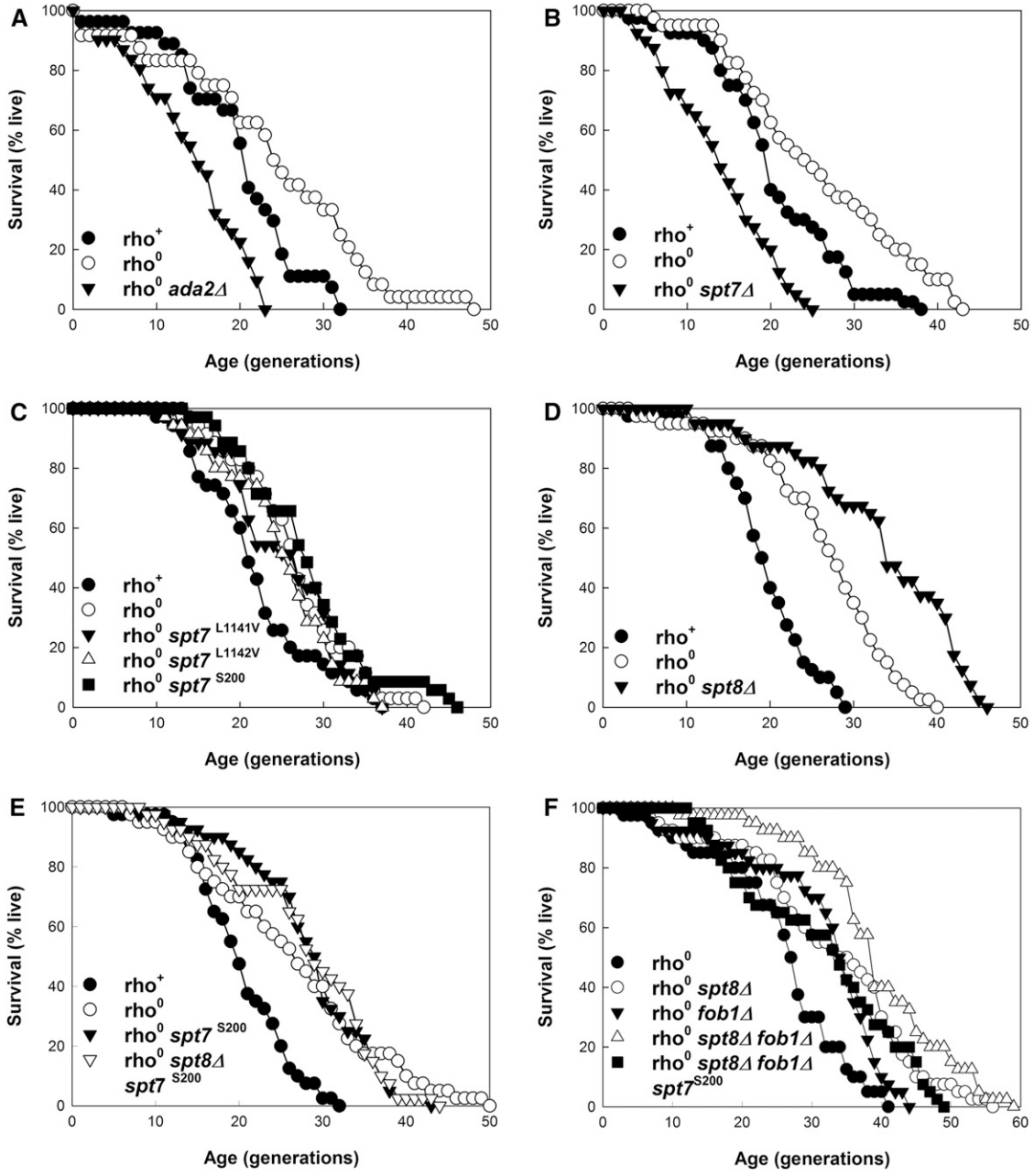

Figure 2 RLS extension in rho^0 strains lacking SAGA or SLIK. (A) Deletion of *ADA2*, a core component of SAGA/SLIK. Mean RLS are 19.9, 25.6, and 13.8, for rho$^+$, rho^0, and rho^0 *ada2Δ* strains, respectively. Lifespans for the first and second strain and for the second and third strain are significantly different ($P = 0.009$ and $P < 0.001$, respectively). (B) Elimination of both SLIK and SAGA in rho^0 cells by removal of the common component Spt7. Mean RLS are 19.5, 24.7, and 12.7, for rho$^+$, rho^0, and rho^0 *spt7Δ* strains, respectively. Lifespans for the first and second strain and for the second and third strain are significantly different ($P = 0.022$ and $P < 0.001$, respectively). (C) Elimination of SLIK in rho^0 cells by the S200 mutation in *SPT7*, which prevents the processing of this protein to allow SLIK formation and comparison to point mutations in the specific Pep4 cleavage site encompassed by S200. Mean RLS are 20.9, 25.8, 24.3, 24.1, and 27.2, for rho$^+$, rho^0, rho^0 *spt7* ^{L1141V}, rho^0 *spt7* ^{L1142V}, and rho^0 *spt7* ^{S200} strains, respectively. Lifespans for the first and second strain are significantly different ($P = 0.003$). There is no significant difference between the lifespans of the second and the third, fourth, and fifth strains (*P*-values of 0.502, 0.43, and 0.466, respectively). (D) Elimination of SAGA by deletion of *SPT8*, which is a component of SAGA but not SLIK. Mean RLS are 18.5, 25.8, and 32.6, for rho$^+$, rho^0, and rho^0 *spt8Δ* strains, respectively. Lifespans for the first and second strain and for the second and third strain are significantly different (each $P < 0.001$). (E) Combined elimination of SLIK and SAGA by the S200 mutation in *SPT7* and the deletion of *SPT8*. The mean RLS for the rho$^+$, rho^0, rho^0 *spt7* ^{S200}, and rho^0 *spt8Δ spt7* ^{S200} strains were 19.4, 25.5, 27.4, and 27.0, respectively. Lifespans for the first and second strains are significantly different ($P = 0.006$), but those for the second and third and second and fourth are not ($P = 0.345$ and $P = 0.373$, respectively). (F) Combined prevention of ERC accumulation by *FOB1* deletion and elimination of SAGA and SLIK by *SPT8* deletion and the *spt7* ^{S200} mutation, respectively. The mean RLS for the rho^0, rho^0 *spt8Δ*, rho^0 *fob1Δ*, rho^0 *spt8Δ fob1Δ*, and rho^0 *spt8Δ fob1Δ spt7* ^{S200} strains are 25.0, 32.1, 30.6, 38.5, and 30.7, respectively. The differences in lifespan between the rho^0 strain and all of the others were significant. Except for the triple mutant, the *P*-values are 0.005 or lower, while for the triple mutant, $P = 0.026$. There are no significant differences between the rho^0 *spt8Δ* strain and the rho^0

the gel was loaded with DNA-size markers. Gels were blotted onto Hybond-N$^+$ nylon membranes (Amersham, Piscataway, NJ) according to the protocol provided by the manufacturer. 35S ribosomal DNA (rDNA) and *ACT1* coding (401–1233 bp of the ORF) (Borghouts *et al.* 2004) fragments were employed as templates to produce DIG-labeled probes to detect ERC and the housekeeping gene *ACT1*. After probing for ERC, the blots were stripped and reprobed for *ACT1*. Detailed procedures are described in the DIG High Primer DNA Labeling and Detection Starter Kit II (Roche, Indianapolis, IN). Chemiluminescence detection and quantitation was carried out on the BioRAD ChemiDocTM XRs+ Imaging System, and the resident Quantity One software was used for quantitation. ERC levels from each strain were normalized to the housekeeping gene *ACT1*.

Data availability

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article. Strains are available upon request.

Results

Either SLIK or SAGA can support activation of CIT2 in the retrograde response

Previously, we showed that deletion of *GCN5* abrogated the increase in *CIT2* mRNA levels in rho^0 cells compared to the rho$^+$ cells from which they were derived (Kim *et al.* 2004). Gcn5 can function in a complex smaller than SAGA that contains Ada2 (Grant *et al.* 1997). To ascertain whether this complex is responsible for the role of Gcn5 in *CIT2* expression, we examined the effect of deletion of *ADA2* on *CIT2* mRNA levels. As shown in Figure 1A, deletion of the gene prevented the increase in *CIT2* mRNA in rho^0 cells. Thus, Gcn5 does not function alone in the activation of the retrograde response. However, Gcn5, along with Ada2 is also part of the large SAGA and SLIK complexes (Pray-Grant *et al.* 2002).

To distinguish the potential function of SAGA and SLIK in activation of the retrograde response, it was necessary to eliminate them in turn. Rtg2 replaces Spt8 in SLIK. This requires truncation of Spt7 at a specific site (Pray-Grant *et al.* 2002; Wu and Winston 2002; Mischerikow *et al.* 2009; Spedale *et al.* 2010). Therefore, we subjected *SPT7* to site-directed mutagenesis to eliminate this specific Pep4 protease cleavage site. Three separate mutants were generated: L1141V, L1142V, and S200. The first two are point mutants in the Pep4 cleavage site. The last one is a short deletion that eliminates 25 amino acids spanning it, and it is known to leave the uncleaved Spt7 available for participation in SAGA complex activity while preventing SLIK formation (Wu and Winston 2002; Mischerikow *et al.* 2009; Spedale *et al.* 2010). None of these mutations eliminated induction of *CIT2* mRNA in rho^0 cells (Figure 1B). This result indicates that SLIK is not necessary for activation of the retrograde response.

Elimination of SAGA was readily accomplished by deletion of *SPT8*, which is unique to this complex (Pray-Grant *et al.* 2002). In the absence of Spt8, Spt7 undergoes truncation to allow Rtg2 to become part of the complex, resulting in SLIK formation. As shown in Figure 1C, deletion of *SPT8* did not prevent the induction of *CIT2* mRNA in rho^0 cells. Thus, SAGA is not necessary for activation of the retrograde response.

SLIK and SAGA can readily be eliminated in tandem by combining the S200 mutation in *SPT7*, which eliminates the proteolytic cleavage site in the protein with the deletion of *SPT8*. As shown in Figure 1D, this genetic manipulation virtually abolished *CIT2* induction, suggesting that either SLIK or SAGA is necessary for transcriptional coactivation of this gene. This was confirmed by deletion of *SPT7*, as Spt7 is an essential component of both SLIK and SAGA (Pray-Grant *et al.* 2002). Deletion of *SPT7* prevented the increase in *CIT2* mRNA levels in rho^0 compared to rho$^+$ cells (Figure 1E). We conclude that either SLIK or SAGA can facilitate the activation of the retrograde response, acting interchangeably. The results also confirm that the *SPT7* mutants that prevent truncation of the protein do not disturb its function in the SAGA complex (Wu and Winston 2002). In addition, they indicate that smaller Gcn5-containing complexes cannot replace SLIK or SAGA in the retrograde response.

Either SLIK or SAGA can support RLS extension in the retrograde response

Because the retrograde-response target gene or genes that are responsible for extension of yeast longevity are not known, induction of *CIT2* is not sufficient as a readout for lifespan extension. The RLS of rho^0 cells is significantly longer than that of rho$^+$ cells (Kirchman *et al.* 1999), and this extension is prevented by deletion of *GCN5* (Kim *et al.* 2004). This shows that the Gcn5 histone acetyltransferase is essential for RLS extension in the retrograde response; however, it does not indicate whether it acts alone or in a complex. To assess the possibility that the Gcn5–Ada2 complex is involved, we deleted *ADA2*. This deletion suppressed the RLS extension observed in rho^0 cells (Figure 2A), indicating that Gcn5 does not act alone.

Gcn5 and Ada2 not only act as a complex, but they also are components of larger protein complexes. Therefore, it was necessary to determine the effects of SAGA and SLIK on RLS, again notwithstanding the effects on *CIT2* induction. Elimination of Spt7 by deleting the gene suppressed the

fob1Δ or the triple mutant (*P*-values of ≥0.419). However, the differences between the rho^0 *spt8Δ fob1Δ* and the rho^0 *spt8Δ* ($P = 0.034$) and the rho^0 *fob1Δ* ($P < 0.001$) strains are significant, while those for the rho^0 *fob1Δ* and the triple mutant are not ($P = 0.862$). YPK9 is the rho$^+$ strain and YJR2 is its rho^0 derivative.

RLS extension in rho^0 cells (Figure 2B), indicating that either or both SAGA and SLIK are required.

To distinguish between the possibilities, the three *SPT7* mutants in the Pep4 cleavage site were tested. None of the mutations prevented RLS extension (Figure 2C), indicating that SLIK was not essential and leaving SAGA as the player. To verify that SAGA is the responsible transcriptional coactivator complex that can facilitate RLS extension in rho^0 cells, *SPT8* was deleted because its product is unique to SAGA. However, this did not prevent the increase in lifespan (Figure 2D), showing that either SLIK or SAGA can perform as the transcriptional coactivator in the retrograde response. Surprisingly, deletion of *SPT8* resulted in a further increase in RLS in rho^0 cells (Figure 2D). Thus, elimination of Spt8 likely affects additional cellular processes, potentially masking effects of the gene deletion. Mutation of *SPT7*, blocking the proteolytic processing of the protein that allows SLIK formation suppressed the additional RLS extension seen in the rho^0 *spt8Δ* strain (compare Figure 2D with Figure 2E), apparently leaving this additional cellular process intact while eliminating *CIT2* induction (Figure 1D).

Activation of the retrograde response results in a progressive and extensive accumulation of ERCs during the RLS in both rho$^+$ and rho^0 cells, although much more extensive in the latter (Borghouts *et al.* 2004), as a result of recombination within the 100–200 tandem repeats of rDNA on chromosome 12 (Szostak and Wu 1979; Clark-Walker and Azad 1980; Larionov *et al.* 1980; Park *et al.* 1999; Johzuka and Horiuchi 2002). Such a burden of ERC normally curtails yeast RLS (Sinclair and Guarente 1997). However, rho^0 cells can tolerate as much as five times the number of ERCs about the midpoint of their RLS as rho$^+$ cells at a point when the latter have nearly exhausted their life expectancy, and this appears due to the partial compensation of the negative effects of the ERC by an active retrograde response (Borghouts *et al.* 2004). ERC production is inhibited by deletion of *FOB1*, and this increases RLS in rho$^+$ cells (Defossez *et al.* 1999; Johzuka and Horiuchi 2002). Interestingly, elimination of ERC by deletion of this gene results in a synergistic increase in RLS when combined with the rho^0 genotype (Borghouts *et al.* 2004) (Figure 2F), reflecting the deleterious effects on RLS of ERC in rho^0 just as in rho$^+$ cells. It has been suggested that ERC accumulation curtails yeast RLS by titration of replication proteins by these episomes (Sinclair and Guarente 1997).

Previously, we found that *GCN5* deletion markedly reduced ERC levels in rho^0 cells, in which Rtg2 protein plays a part (Kim *et al.* 2004). We wondered whether the increase in RLS by elimination of SAGA resulting from *SPT8* deletion in rho^0 cells could be due to an effect on ERC, as well. As expected (Borghouts *et al.* 2004), the deletion of *FOB1* reduced ERC levels in rho^0 cells (Figure 3). Deletion of *SPT8* had a similar effect, although to a lesser extent (Figure 3). When both genes were deleted, ERCs were reduced to the level seen in the *FOB1* deletion. Next, we examined the effects of these gene deletions on RLS. As seen in Figure 2F,

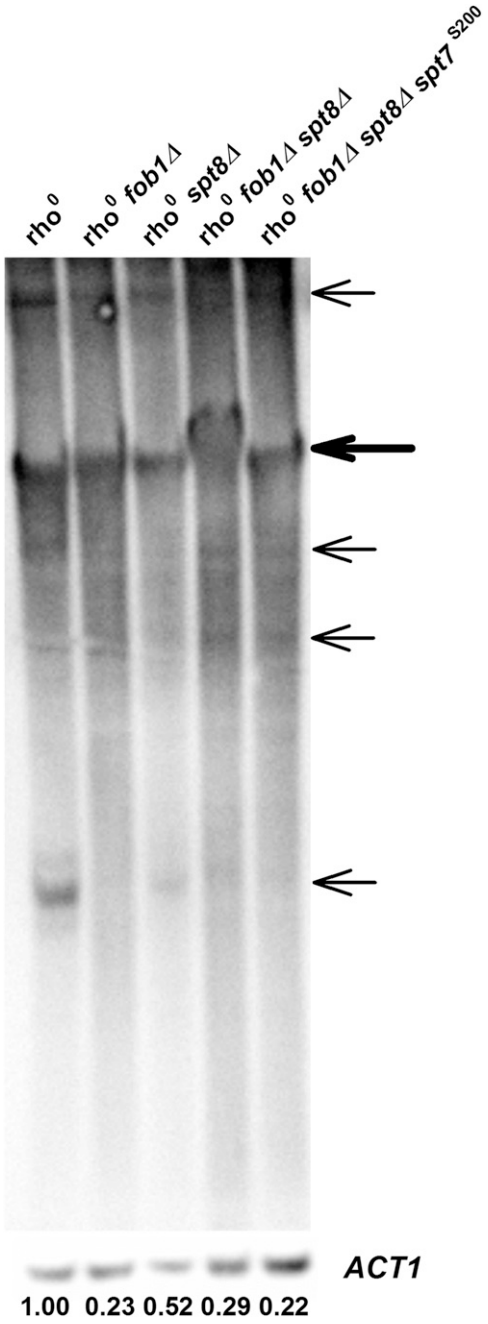

Figure 3 Analysis of ERCs in rho^0 strains. DNA prepared from the indicated rho^0 strains was electrophoresed and Southern blots were probed for rDNA and *ACT1* (see *Materials and Methods*). Bands representing the hybridization signal for genomic rDNA are indicated by the thick arrow, while the ERC bands are indicated by the thin ones. For quantification of ERCs, the sums of the signal intensities for the ERC bands in each lane were normalized against the hybridization signals of *ACT1* in those lanes, and the results are shown at the bottom of each lane with the intensity of the parental rho^0 strain set to 1.00. Similar results were obtained in three separate experiments.

both *FOB1* and *SPT8* deletion extended RLS in rho^0 cells, and combining them yielded a further increase. When the *SPT7* S200 mutation that prevents SLIK formation was combined with the deletions in *FOB1* and *SPT8*, this further increase in

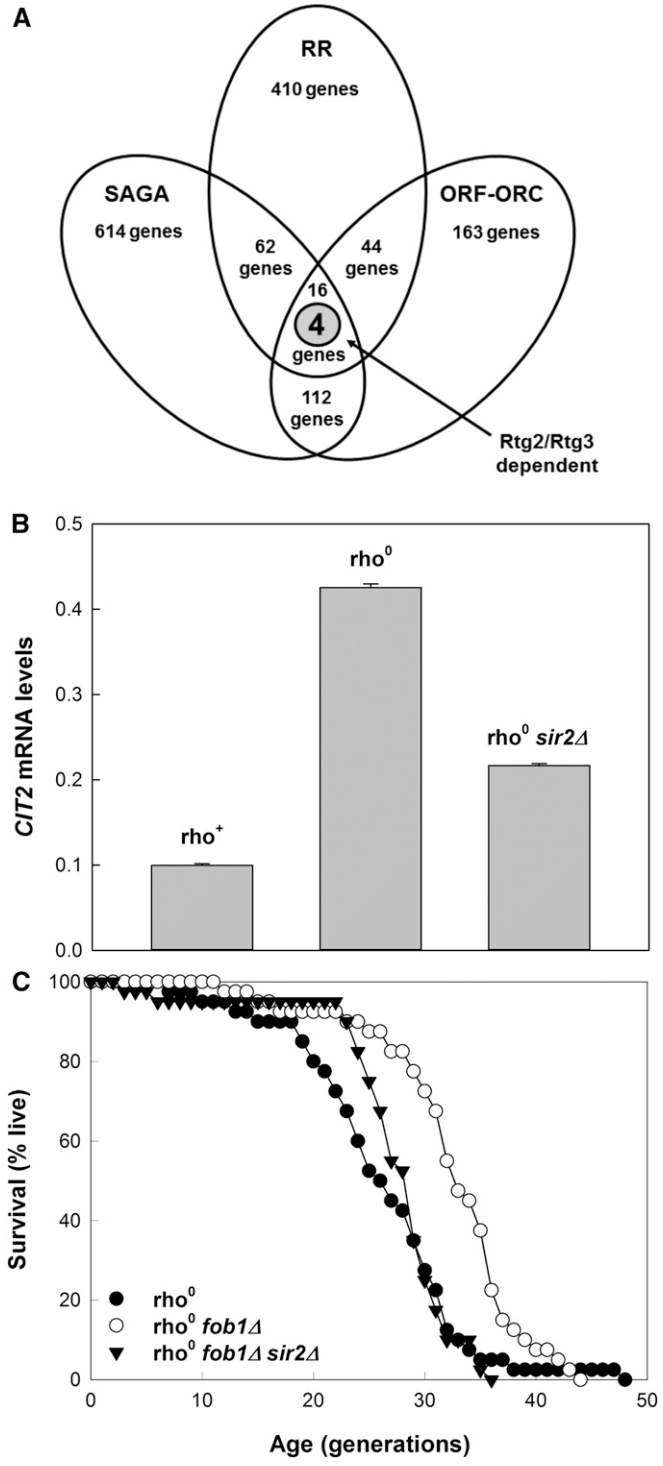

Figure 4 Identification of the genes that are dependent on the retrograde response, SAGA, and ORF-ORC. (A) The dependent genes in each of these three cases were identified in published sources (Epstein *et al.* 2001; Huisinga and Pugh 2004; Shor *et al.* 2009). The three databases were merged to find shared sets of genes. This identified four genes common to all three that are also Rtg2 and Rtg3 dependent in the retrograde response. (B) Effect of *SIR2* deletion on the induction of *CIT2* expression in the retrograde response. (C) *SIR2* deletion suppresses RLS in rho^0 cells independently of effects on ERCs, which are eliminated by *FOB1* deletion. Mean RLS are 25.1, 31.4, and 26.4, for rho^0, rho^0 *fob1Δ*, and rho^0 *fob1Δ sir2Δ* strains, respectively. Lifespans for the first and second strain and for the second and third strain are significantly different (each $P < 0.001$). As discussed in the text, the results in B and C provide additional rationale for inclusion of ORF-ORC genes in the analysis shown in A. RR, retrograde response.

RLS was suppressed to the level seen in the presence of either deletion alone (Figure 2F). ERC levels remained low in the triple mutant (Figure 3). We interpret these complex results as follows: Elimination of SAGA and SLIK by deletion of *SPT8* and the S200 mutation in *SPT7* prevents the induction of *CIT2* and extension of RLS in the retrograde response. However, the *SPT8* deletion affects another cellular process, which may be involved in ERC maintenance. The difference between Gcn5 and Spt8 is likely due to the fact that deletion of *GCN5* eliminates both SLIK and SAGA, while deletion of *SPT8* only prevents SAGA formation. This is addressed further in the *Discussion*.

Search for retrograde-response target genes that potentially affect RLS extension

There are 410 gene expression changes that are found in rho^0 cells compared to rho$^+$ cells (Epstein *et al.* 2001; Traven *et al.* 2001). One or more of these are the likely effector of the increase in RLS in the retrograde response. The involvement of SAGA/SLIK in induction of the retrograde-response diagnostic gene *CIT2* and especially in the RLS extension afforded by activation of the response indicates that the effector(s) of longevity should be found at the intersection of the 614 yeast genes at which SAGA dominates and these 410 genes (Figure 4A).

We also considered another set of genomic sites, called ORF-origin recognition complexes (ORCs), in this bioinformatics analysis. The ORC that is important in DNA replication (Bell 2002) also binds to silencers in the yeast genome where it recruits silent information regulator (Sir) proteins (Bose *et al.* 2004; Fox and McConnell 2005). These silent genomic sites bind ORCs tightly, as compared to many active replication origins (Palacios DeBeer *et al.* 2003). A subset of the ORC-binding sites outside silenced genomic regions can also bind ORCs tightly, but they do not function as origins of replication. They are generally found in close proximity to ORFs of highly transcribed, metabolic genes (Shor *et al.* 2009). The 163 sites with these characteristics have been named ORF-ORCs. The consideration of ORF-ORC genes was motivated by the fact that the retrograde response involves a major metabolic adaptation (Epstein *et al.* 2001; Traven *et al.* 2001). Based on the known interactions of ORC and Sir complexes in silent and transcribed regions of the yeast genome (Shor *et al.* 2009), we reasoned that these ORF-ORCs are potential sites for Sir2 recruitment in the retrograde response. Indeed, Sir2 has been shown to associate with 24% of the most frequently transcribed genes (Tsankov *et al.* 2006). We therefore examined the effect of *SIR2* deletion on expression of *CIT2*. As shown in Figure 4B, deletion of *SIR2* decreased induction of *CIT2* by ~50% in rho^0 cells in which the retrograde response is activated.

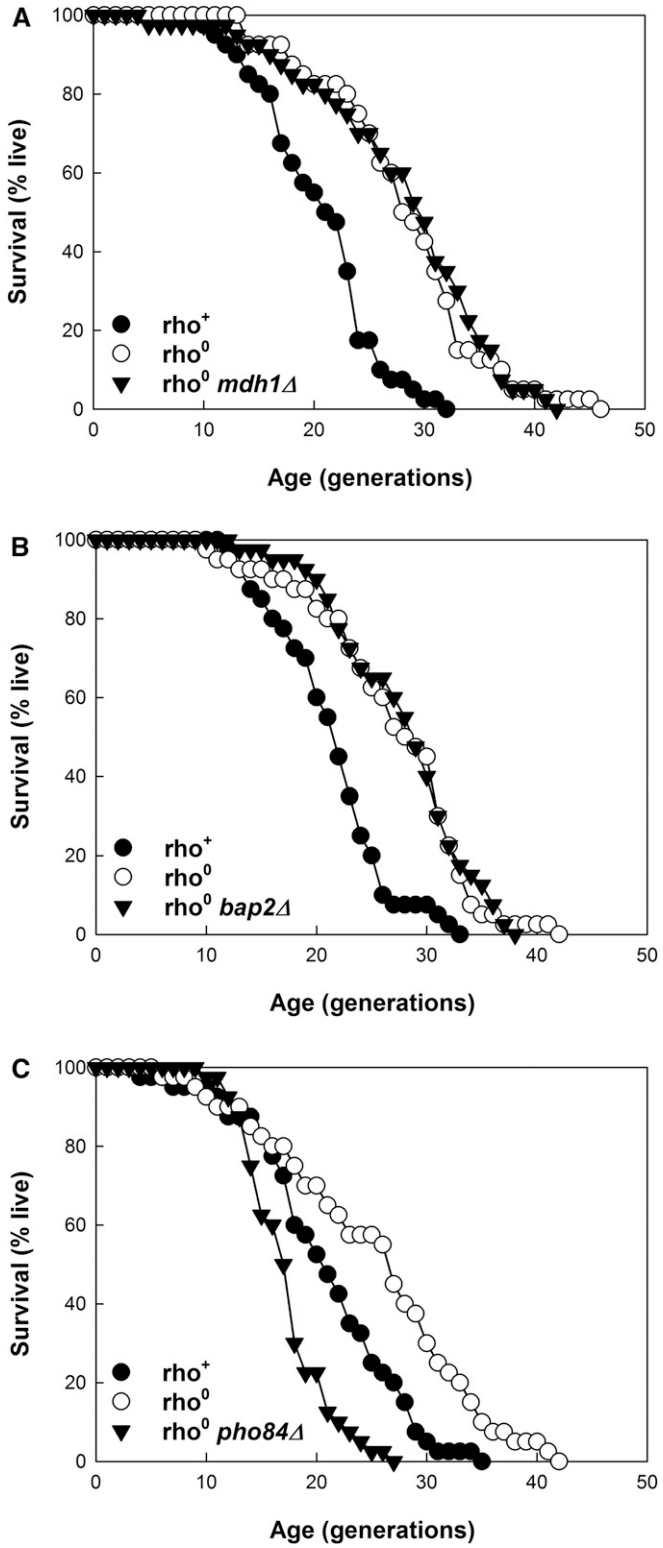

Figure 5 Effect of deletion of the genes at the intersection of the retrograde response, SAGA, and ORF-ORC on RLS extension in rho^0 strains. (A) Deletion of *MDH1*. Mean RLS are 19.7, 27.3, and 27.3, for rho$^+$, rho^0, and rho^0 *mdh1Δ* strains, respectively. Lifespans for the first and second strain are significantly different ($P < 0.001$). They are not significantly different for the second and third strain ($P = 0.736$). (B) Deletion of *BAP2*. Mean RLS are 20.5, 26.2, and 27.0, for rho$^+$, rho^0, and rho^0 *bap2Δ* strains, respectively. Lifespans for the first and second strain are significantly different ($P < 0.001$). They are not significantly different for the second and third strain ($P = 0.765$). (C) Deletion of *PHO84*. Mean RLS are 19.9, 24.3, and 16.4 for rho$^+$, rho^0, and rho^0 *pho84Δ* strains, respectively. Lifespans for the first and second strain and for the second and third strain are significantly different ($P = 0.019$ and $P < 0.001$, respectively).

This raises the question of whether *SIR2* deletion compromises the RLS extension afforded by activation of the retrograde response. However, deletion of *SIR2* enhances ERC production and this suppresses RLS (Kaeberlein *et al.* 1999), confounding interpretation of its effect in rho^0 cells. Deletion of *FOB1* eliminates this confounder by suppressing ERC formation (Kaeberlein *et al.* 1999). Figure 4C demonstrates that deletion of *SIR2* in rho^0 cells lacking Fob1 resulted in suppression of RLS. This supports the role of Sir2 in the retrograde response, independent of its effects on ERC production. It also provides additional rationale for inclusion of ORF-ORC genes in our bioinformatics analysis.

Not all genes induced in rho^0 cells require the retrograde regulators Rtg2 and Rtg3 (Epstein *et al.* 2001). Both regulators are required for RLS extension in rho^0 cells (Kirchman *et al.* 1999; Borghouts *et al.* 2004). Of the 16 genes at the intersection of rho^0 dependence, SAGA dominance, and ORF-ORC association, only 4 genes require Rtg2 and Rtg3 for activation in the retrograde response (Figure 4A). These genes are *MDH1*, *BAP2*, *PHO84*, and *CIT2*.

PHO84 is necessary for RLS extension in the retrograde response

If our bioinformatics approach was successful, the effector(s) of RLS extension would be among the four genes identified above. The effector could be a single gene or combination of the four genes, which involves a total of 15 potential combinations. We decided to first delete each of the genes individually in rho^0 cells to determine the effect on RLS. We already had the answer for *CIT2*. Deletion of this gene does not curtail the RLS of the cells (Kirchman *et al.* 1999). Deletion of *MDH1* and *BAP2*, individually, also did not affect the RLS extension in rho^0 cells (Figure 5, A and B, respectively). However, deletion of *PHO84* completely abrogated RLS extension (Figure 5C). This indicates that *PHO84* is necessary for the RLS extension caused by activation of the retrograde response. Thus, this is at least one of the retrograde-response effectors of longevity.

It has been shown previously that *CIT2*, *MDH1*, *BAP2*, and *PHO84* are among the retrograde-response target genes (Epstein *et al.* 2001). As discussed above, activation of *CIT2* requires either SAGA or SLIK (Figure 1). We wanted to verify that this is also the case for *PHO84*, the gene whose activation is necessary for the RLS extension in the retrograde response. Previously, it was shown that expression of *PHO84* is decreased in *spt7* null mutants (Nishimura *et al.* 1999), implicating SLIK and/or SAGA. Therefore, we examined the effects of the various mutants that affect SLIK and SAGA

differentially (Figure 6A). The S200 mutant of *SPT7*, which prevents SLIK assembly, caused only a small (6%) reduction in the activation of *PHO84* in rho^0 cells. On the other hand, deletion of *SPT8*, which prevents SAGA formation, lowered *PHO84* mRNA levels by ~28%. This suggests that SAGA plays a greater role in expression of this gene than does SLIK, although the transcription of *PHO84* supported by SLIK appears sufficient for effective RLS extension (Figure 2D). However, combination of the S200 mutation in *SPT7* with the *SPT8* deletion virtually abolished *PHO84* expression in rho^0 cells (Figure 6A). This was also the case when *SPT7* was deleted. These results demonstrate that either SLIK or SAGA function as transcriptional coactivators of *PHO84* in the retrograde response. Thus, retrograde-response activation of *PHO84* can utilize either SLIK or SAGA interchangeably, with a preference for the latter. Nevertheless, one or the other must be available for expression of this gene and extension of RLS.

The requirement for Sir2 for RLS extension in the retrograde response implies that deletion of *SIR2* would suppress induction of *PHO84*. This was indeed the case (Figure 6B), providing further support for the essential role of *SIR2* in gene induction and RLS extension in rho^0 cells.

PHO84 is sufficient for RLS extension in the retrograde response

The results above raise the question of whether *PHO84* is sufficient for RLS extension in the retrograde response. To answer this, we inserted a second copy of *PHO84* into the genome of our rho$^+$ strain at the *ura3-52* locus. As shown in Figure 7A, the extra copy of *PHO84* resulted in a 27% increase in *PHO84* mRNA levels. This compares to the 83% increase observed in the rho^0 derivative of the parental strain. Thus, the extra copy of the gene provides an increase in the expression of the gene that is 33% of that observed when the retrograde response is induced in rho^0 cells. We then determined the RLS of the rho$^+$ strain with and without the extra copy of *PHO84* (Figure 7B). The mean RLS of the strain with the extra copy was 14% greater. This compares to the 37% increase observed in the rho^0 strain (Figure 7B), which corresponds to ~86% of the increase in RLS that would be expected if RLS was a linear function of *PHO84* mRNA levels. These results indicate that *PHO84* is sufficient for RLS extension in the retrograde response, in addition to being necessary. However, it is possible that another gene or genes can augment the effect of *PHO84* even further, as there is still a small increment of longevity that appears to remain to reach the RLS of the rho^0 strain.

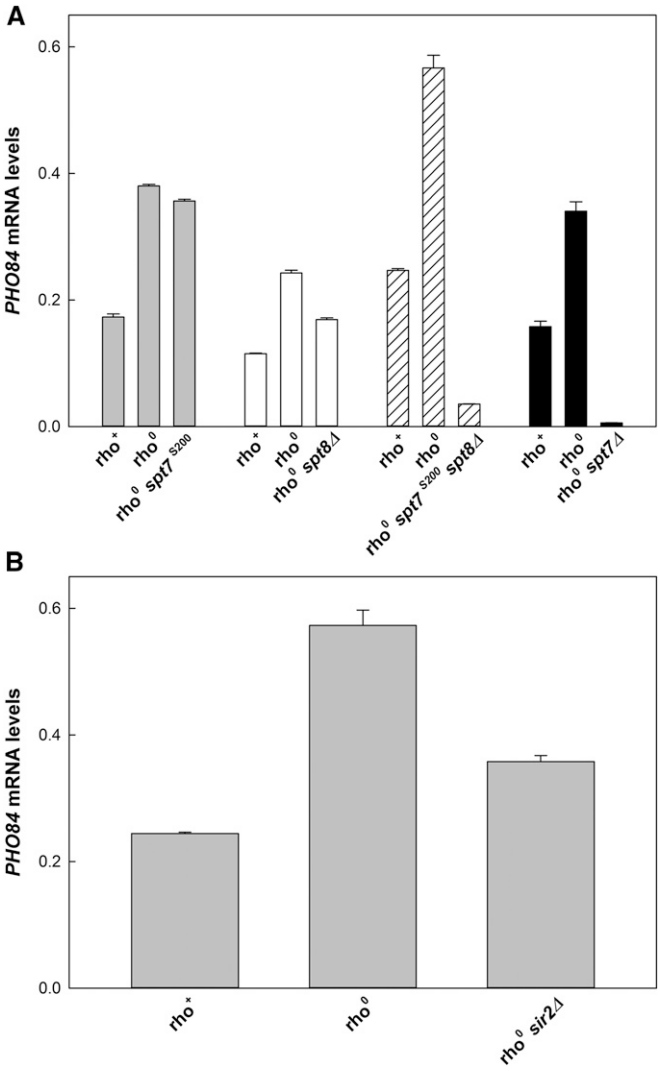

Figure 6 Effects of elimination of SLIK and SAGA and *SIR2* on activation of *PHO84* in rho^0 cells. (A) SLIK was eliminated by the S200 mutation in *SPT7*. SAGA was eliminated by deletion of *SPT8*. SLIK and SAGA were eliminated together by combining the S200 mutant with the *SPT8* deletion or by the deletion of *SPT7*. (B) Effect of *SIR2* deletion on expression of PHO84 in rho^0 cells. YPK9 is the rho$^+$ strain and YJR2 is its rho^0 derivative.

Discussion

Our initial goal was to unravel the contributions of SAGA and SLIK in retrograde signaling of gene expression changes and RLS extension. We have found that these two, large transcriptional coactivator complexes act interchangeably in the induction of the retrograde-response diagnostic gene *CIT2* (Figure 1). This behavior of these complexes was paralleled in their effects on RLS extension in the retrograde response (Figure 2). The implication of SAGA and SLIK in the retrograde response provided an opportunity to winnow the potential retrograde-response target genes responsible for longevity extension (Figure 4A). This effort was aided by considering ORF-ORC genes in tandem. The rationale behind their inclusion stems from their association with metabolic gene expression. It is also supported by the effect of *SIR2* deletion on *CIT2* induction and by the ERC-independent effect of *SIR2* deletion on RLS extension in the retrograde response (Figure 4, B and C).

The juxtaposition of retrograde-response target genes, SAGA dominated genes, and ORF-ORC genes resulted in only

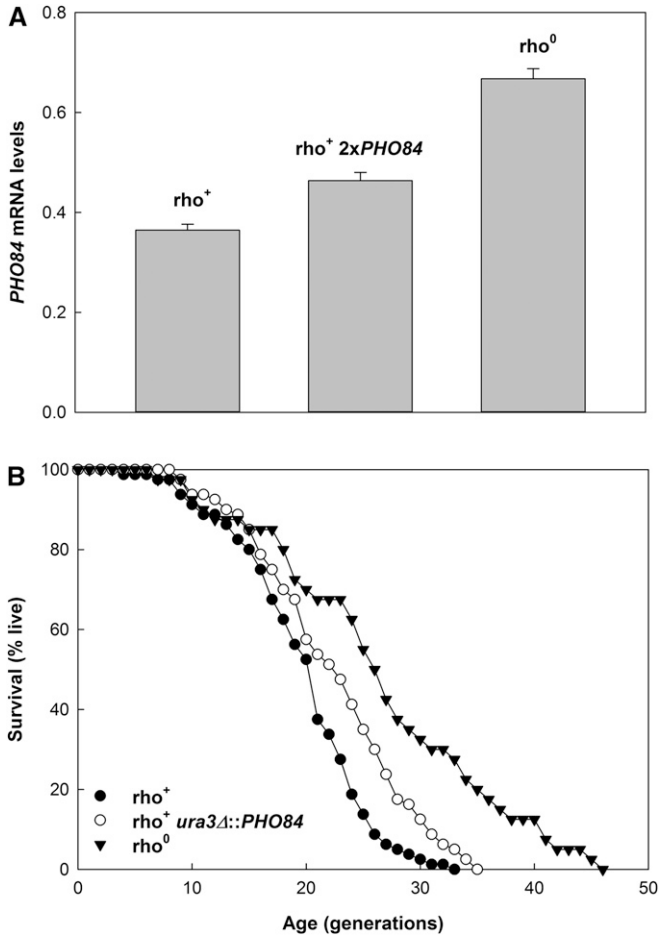

Figure 7 Effect of *PHO84* overexpression on the RLS of the rho⁺ strain. (A) The effect of an additional copy of the gene on *PHO84* mRNA levels (relative to *ACT1* mRNA) determined by RT-qPCR. The rho⁺ 2x*PHO84* strain possessed a second copy of the gene at the *ura3* locus (rho⁺ *ura3Δ*::*PHO84*). (B) The effect of an additional copy of *PHO84* in the rho⁺ strain YPK9 (rho⁺ *ura3Δ*::*PHO84*) compared to both the rho⁺ (YPK9) and its rho⁰ derivative strain (YJR2). Mean RLS are 18.8, 21.4, and 25.6 for rho⁺, rho⁺ *ura3Δ*::*PHO84*, and rho⁰ strains, respectively. Lifespans for the first and second strain and for the first and third strain are significantly different ($P = 0.014$ and $P < 0.001$, respectively). They also differ significantly for the second and third strain ($P = 0.021$).

four genes to test for a role in RLS extension. Only deletion of one, *PHO84*, suppressed RLS extension (Figure 5). Thus, this gene is necessary for RLS extension in the retrograde response. *PHO84* expression is regulated by SAGA and SLIK interchangeably, just like *CIT2* (Figure 6A). In addition, *SIR2* was required for robust expression of *PHO84* in rho⁰ cells (Figure 6B). Activation of *PHO84* expression is also sufficient for RLS extension. An extra copy of the gene in the rho⁺ cells extended RLS to at least 86% of that expected, based on the increase in *PHO84* mRNA levels (Figure 7). This potentially leaves some space for an additional retrograde-response target gene or genes to affect RLS. These additional genes may reside at the intersection of the Rtg2/Rtg3-dependent retrograde response and SAGA (Figure 4A), as our justification to include ORF-ORC genes was to some extent theoretical compared to the experimental data forming the basis for inclusion of the other sets of genes.

Our interest in SLIK was engendered by the discovery that it contains Rtg2 protein as an essential component in place of Spt8 and that Rtg2 can be found at the *CIT2* promoter where SLIK appears to regulate transcription (Pray-Grant *et al.* 2002). These properties of SLIK were described in terms of the retrograde response. However, growth on acetate as a carbon source was used in that study. The retrograde response was originally defined as a phenomenon that occurs when mitochondria are dysfunctional, a condition under which yeast cells cannot grow on acetate. However, our current study demonstrates that SLIK does function in the *bona fide* retrograde response along with SAGA.

Deletion of *SPT8* did not suppress RLS extension in rho⁰ cells (Figure 2D). In fact, it resulted in a further increase. One possibility is that elimination of SAGA leaves SLIK as the coactivator and a more efficient one at that. This is not likely because there is a preference for SAGA in *PHO84* activation (Figure 6A). It is also possible that Spt8 and SAGA play additional roles in RLS. Support for one such alternative role is provided by the decrease in ERC levels on *GCN5* deletion in rho⁰ cells (Kim *et al.* 2004). Deletion of *SPT8* had a similar effect on ERC levels (Figure 3). Although this effect was not as great as that of a *FOB1* deletion, it yielded the same effect on RLS (Figure 2F), which may reflect the high threshold for ERC tolerance in rho⁰ cells as they progress through their RLS (Borghouts *et al.* 2004). The fact that the *SPT7* S200 mutation suppressed the excess RLS extension in the presence of the *SPT8* deletion, whether or not *FOB1* is present (Figure 2, D–F), while the *SPT7* S200 mutation on its own did not affect the RLS of rho⁰ cells (Figure 2C) suggests that elimination of both SLIK and SAGA is required to prevent RLS extension in the retrograde response.

SLIK and SAGA can be distinguished chromatographically. The *SPT7* S200 mutation prevents the formation of SLIK by blocking specific cleavage of Spt7, which in turn prevents Rtg2 from replacing Spt8 in SAGA (Pray-Grant *et al.* 2002; Wu and Winston 2002). SAGA still forms and contains all its subunits (Wu and Winston 2002). SAGA partial complexes can form that are missing certain subunits. Deletion of *GCN5* or of *SPT8* eliminates SAGA activity, but all the remaining subunits associate in the respective SAGA partial complexes (Wu and Winston 2002). This includes the specifically truncated Spt7, at least in the case of the *SPT8* deletion (Wu and Winston 2002). Spt7 appears to perform a scaffolding role for several SAGA components. Its absence eliminates SAGA as a complex (Grant *et al.* 1997). These properties of the SAGA/SLIK complex likely contribute to the results obtained here. The effect of *SPT7* deletion (Figure 2B) or *GCN5* deletion (Kim *et al.* 2004) on RLS in rho⁰ cells was very similar. RLS was shorter than in rho⁺ cells. Thus, the histone acetylase activity of Gcn5 within an intact SAGA/SLIK appears to be essential for maintaining RLS even at rho⁺ cell levels. The effects on *CIT2* expression of *GCN5* or *SPT7* deletion were not that severe (Kim *et al.* 2004) (Figure 1D), suggesting that

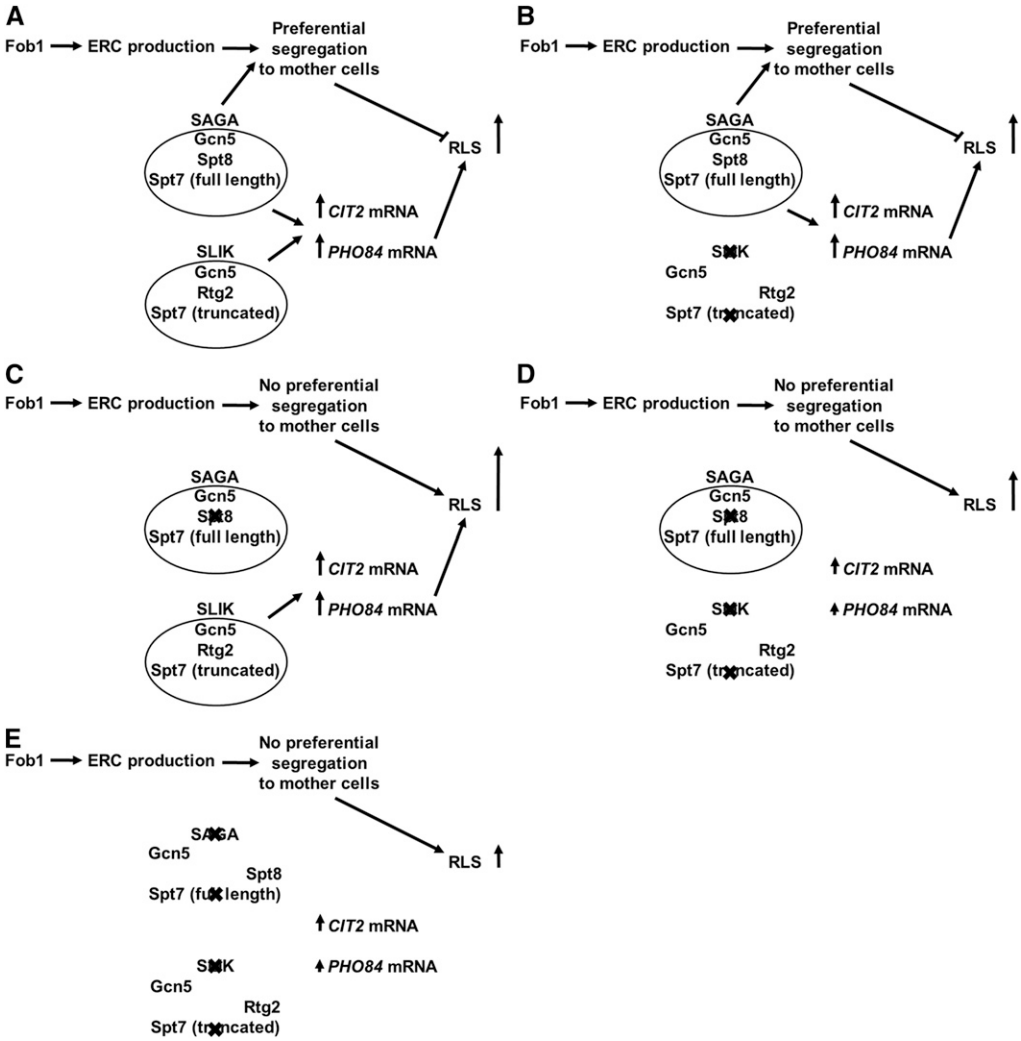

Figure 8 Schematic summarizing the discussion of the role of SAGA/SLIK in the RLS of rho^0 cells. (A) Depiction of the situation in wild-type cells. (B–E) The effects of elimination of SAGA/SLIK components on expression of retrograde-response target genes, segregation of ERCs to mother cells, and RLS. In B, there is no effect on RLS because preventing Spt7 cleavage leaves SAGA activity unaltered to support a wild-type retrograde transcriptional response, and eliminating the intact SLIK complex has no impact on ERCs. In C, RLS increases because eliminating Spt8 from SAGA reduces ERC accumulation in mother cells but leaves SLIK activity unaltered to support a wild-type retrograde response. In D, there is no effect on RLS, despite the loss of both an intact SLIK complex and SAGA complex activity and the attendant retrograde response, because the elimination of Spt8 from SAGA reduces ERC accumulation in mother cells. In E, RLS decreases by elimination of Spt7 and the resulting disruption of both SAGA and SLIK complexes even though the impairment of the retrograde response and reduction in ERC accumulation in mother cells is indistinguishable from that given by the situation in D. The different outcomes in D and E imply that SAGA has an additional unknown function(s) in promoting RLS that can be executed by the partial SAGA complex lacking only Spt8. Other possibilities include additional functions in the cell of SAGA components, such as Spt8, that have a negative effect on RLS when not assembled into intact SAGA complexes.

other mechanisms that maintain these rho$^+$ cell levels of RLS are in play. However, drastic effects of *SPT7* deletion on *PHO84* expression were seen (Figure 6A), reflecting its effect on RLS (Figure 2B). As shown in Figure 5C and Figure 7, *PHO84* is necessary and sufficient for RLS extension in the retrograde response.

The results presented here are consistent with the recent observation that SAGA plays a role in the asymmetric segregation of DNA circles, including ERCs, to mother cells, by their anchorage to nuclear pore complexes by SAGA (Denoth-Lippuner *et al.* 2014). These authors showed that this anchorage required SAGA components such as Gcn5, Spt3, and Sgf73. Deletion of these genes markedly reduced ERC content in aging mother cells. Denoth-Lippuner *et al.* (2014) also found that deletion of the SAGA component gene *SGF73* extended RLS, while the elimination of *GCN5* did not. They ascribed this difference to the multiple pathways on which SAGA impinges through its role as a transcriptional coactivator. Our present study identifies one such pathway. Disruption of SAGA by *SPT8* deletion here would relieve some of the ERC burden in mother cells to enhance their RLS. This effect would be particularly noticeable in rho^0 cells with their large accumulation of ERCs as they progress through their RLS (Borghouts *et al.* 2004). The additional increase in RLS of rho^0 cells possessing both *SPT8* and *FOB1* deletions (Figure 2F) is not directly reflected in their ERC content (Figure 3). However, ERC levels were determined in batch cultures that are primarily composed of young cells. The loss of asymmetric segregation of ERCs when *SPT8* is deleted would manifest itself to a greater extent as the yeasts progress through their RLS. Although deletion of *GCN5* abolishes asymmetric segregation of ERCs (Denoth-Lippuner *et al.* 2014), it also eliminates both SLIK and SAGA, masking any beneficial effect of ERC reduction on RLS of rho^0 cells, as observed earlier (Kim *et al.* 2004). It is noteworthy that elimination of SLIK by *SPT7* deletion or S200 mutation did not cause an increase in RLS (Figure 2, B and C), while deletion of *SPT8* did (Figure 2D). Thus, various components of SAGA and SLIK have

divergent effects on different aspects of these transcriptional coactivators' functions, probably including retention of ERCs in mother cells as well. Our model for the role of SAGA/SLIK in the RLS of rho^0 cells summarizing this discussion is shown in Figure 8. It is certainly possible that there are yet other cellular functions of SAGA. Recently, another study showed a role for SAGA in RLS (McCormick *et al.* 2014). This study implicated the deubiquitinase module of the transcriptional coactivator complex in rho$^+$ cells. Our parallel study in contrast, addresses the roles in RLS of both the SLIK and the SAGA core complexes in the retrograde response in rho^0 cells. Nevertheless, a connection between the two studies may exist, and this will be a subject for future study.

Our efforts to identify the effectors of the RLS extension in the retrograde response prompted examination of a potential role for *SIR2*. Deletion of this gene markedly suppressed induction of both *CIT2* (Figure 4B) and *PHO84* (Figure 6B) in rho^0 cells. It also abolished RLS extension in the retrograde response independently of the effects of *SIR2* on ERC production (Figure 4C). These results indicate a hitherto unappreciated role of *SIR2*.

PHO84 encodes a high-affinity inorganic phosphate transporter in the plasma membrane (Bun-Ya *et al.* 1991; Wykoff and O'Shea 2001). How does activation of this gene, which would result in an increase in phosphate import into the cell extend RLS? *PHO84* is pleiotropic, affecting several cellular processes. For example, overexpression of this gene triggers the endoplasmic reticulum unfolded protein response (ER-UPR) (Ofiteru *et al.* 2012). It has been shown that activation of this response in yeasts can extend RLS (Cui *et al.* 2015). Although the key ER-UPR genes *IRE1* and *HAC1* are not induced in rho^0 cells (Epstein *et al.* 2001), triggering of the ER-UPR at the level of Ire1 and/or Hac1 protein is an attractive possibility.

This study resolves the questions surrounding the roles of SAGA and SLIK in the retrograde response. In the process, we have bolstered the role of SAGA in ERC-mediated effects on yeast longevity, and we have uncovered a role for *SIR2* in the retrograde response. Importantly, we have succeeded in identifying the retrograde-response target gene responsible for RLS extension as *PHO84*. This finding opens up new avenues of research regarding the role of the retrograde response in yeast aging. Future efforts will be directed toward an elucidation of the proximal effector(s) of longevity downstream of *PHO84* triggered by mitochondrial dysfunction.

Acknowledgments

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