Mitochondrial Retrograde Signaling

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Annu. Rev. Genet. 2006. 40:159–85

First published online as a Review in Advance on June 13, 2006

The *Annual Review of Genetics* is online at http://genet.annualreviews.org

This article's doi: 10.1146/annurev.genet.40.110405.090613

0066-4197/06/1215-0159$20.00

Key Words

mitochondria, signaling, retrograde response, nutrient sensing, RTG pathway

Abstract

Mitochondrial retrograde signaling is a pathway of communication from mitochondria to the nucleus under normal and pathophysiological conditions. The best understood of such pathways is retrograde signaling in the budding yeast *Saccharomyces cerevisiae*. It involves multiple factors that sense and transmit mitochondrial signals to effect changes in nuclear gene expression; these changes lead to a reconfiguration of metabolism to accommodate cells to defects in mitochondria. Analysis of regulatory factors has provided us with a mechanistic view of regulation of retrograde signaling. Here we review advances in the yeast retrograde signaling pathway and highlight its regulatory factors and regulatory mechanisms, its physiological functions, and its connection to nutrient sensing, TOR signaling, and aging.

Contents

INTRODUCTION

The maintenance of mitochondrial function during cell growth and development depends on contributions from the nuclear genome, which encodes the vast majority of mitochondrial proteins, and the mitochondrial genome, which encodes a limited but essential number of mitochondrial proteins, most of which are components of the oxidative phosphorylation apparatus (40). Because of the quantitatively predominant role of the nuclear genome in the biogenesis of the organelle, much attention over the past several decades has been directed to the analysis of anterograde regulation, which is the control of the flow of material and information from the nucleus and cytoplasm to the mitochondria. More recent studies have revealed that mitochondria are also engaged in retrograde regulation, in which cells respond to changes in the functional state of the organelle via changes in nuclear gene expression. Retrograde regulation encompasses a wide assortment of cellular activities, including nutrient sensing, growth control, aging, and other signaling processes that function in metabolic and organelle homeostasis. Many of these processes occur by novel mechanisms that utilize a rich repertoire of regulatory factors, some of which are unique to retrograde regulation pathways. A central issue in retrograde regulation is determination of the molecules that initiate mitochondrial signaling and how those signals are transduced to effect the relevant changes in nuclear gene expression.

The general process of mitochondrial signaling is conserved from simple eukaryotes such as yeast to humans. However, the

molecular mechanisms by which signaling and signal transduction occur do not appear to be conserved across species. These mechanistic differences are likely to reflect fundamental differences both in metabolic activities and their regulation between animal cells and yeast and their intrinsic responses to different environmental cues not shared between metazoan cells and those of a unicellular fungus such as yeast, whose primary activities are geared toward nutrient sensing. In this review, we focus primarily on the retrograde pathways in yeast. [For examples of retrograde pathways in animal cells see (7, 8, 88), summarized in (12).]

REGULATORS OF THE YEAST RETROGRADE PATHWAY

An early attempt to study the effects of mitochondrial DNA (mtDNA) mutations on nuclear gene expression was reported in 1987 (106). Using the complementary DNA subtraction method, nuclear gene transcripts that respond to mtDNA defects were identified. Two types of nuclear-encoded transcripts were identified: class I transcripts that respond to the general mitochondrial respiratory capacity and class II transcripts that respond to petite-specific mtDNA mutations. Those results, in principle, established the existence of a signal transduction pathway from mitochondria to the nucleus.

The prototypical target gene of the retrograde pathway is *CIT2*, which encodes a peroxisomal isoform of citrate synthase (80). In cells with compromised mitochondrial functions, for example, those without mtDNA (ρ^0 petites), *CIT2* expression is increased by as much as 30-fold (80). Analysis of the 5′-flanking sequence of *CIT2* revealed a UAS (upstream activation site) element necessary and sufficient to drive retrograde *CIT2* expression (79). Identification of the *cis*-acting element in the *CIT2* promoter region enabled the identification and isolation of *trans*-acting factors and other regulators of the regulatory pathway. Using *CIT2* promoter-driven reporter genes, multiple positive and negative regulators in the RTG pathway have been identified (62, 79, 84–86, 121) (**Figure 1**). Analysis of these factors has greatly increased our understanding of the regulation of this pathway.

POSITIVE REGULATORS OF THE RTG PATHWAY

Rtg1p and Rtg3p

RTG1 and *RTG3* encode basic helix-loop-helix leucine zipper-(bHLH/Zip) type transcription factors. They form a heterodimer and bind to the promoter region of target genes (**Figure 1**). The transcriptional activation domain of the Rtg1/3p complex is contained within Rtg3p (120, 122). Unlike most bHLH-type transcription factors, which bind to the consensus sequence, CANNTG, called the E box (92), Rtg1/3p binds to an unusual site, GTCAC, termed the R box (62, 79). Rtg3p is a phosphoprotein, whereas Rtg1p is not (122). Activation of Rtg3p correlates with its partial dephosphorylation and its nuclear translocation with Rtg1p (122).

Rtg2p

Rtg2p is a cytoplasmic protein with an N-terminal ATP (adenosine triphosphate) binding domain belonging to the actin/Hsp70/sugar kinase superfamily (10, 76). The ATP binding domain of Rtg2p is most similar to that of bacterial exopolyphosphatase/guanosine pentaphosphate phosphohydrolase (76), whose structure has been solved recently (77). Despite the functional diversity of these ATP binding domain proteins and negligible overall sequence similarity, three-dimensional structures of these ATP binding domains are surprisingly similar (58). Structures of these ATP binding domains reveal two subdomains with a pseudodyad symmetry, with the active site predicted to be in the cleft formed between the two subdomains. Rtg2p is required for Rtg3p dephosphorylation and

Mitochondrial DNA (mtDNA): the hereditary element existing in mitochondria, encoding a few essential components in the electron transport chain and the ATP synthase complex

ρ^0: cells without mitochondrial DNA

bHLH/Zip: a DNA binding domain existing in a group of eukaryotic proteins that mediates specific dimerization and DNA binding

ATP: adenosine triphosphate

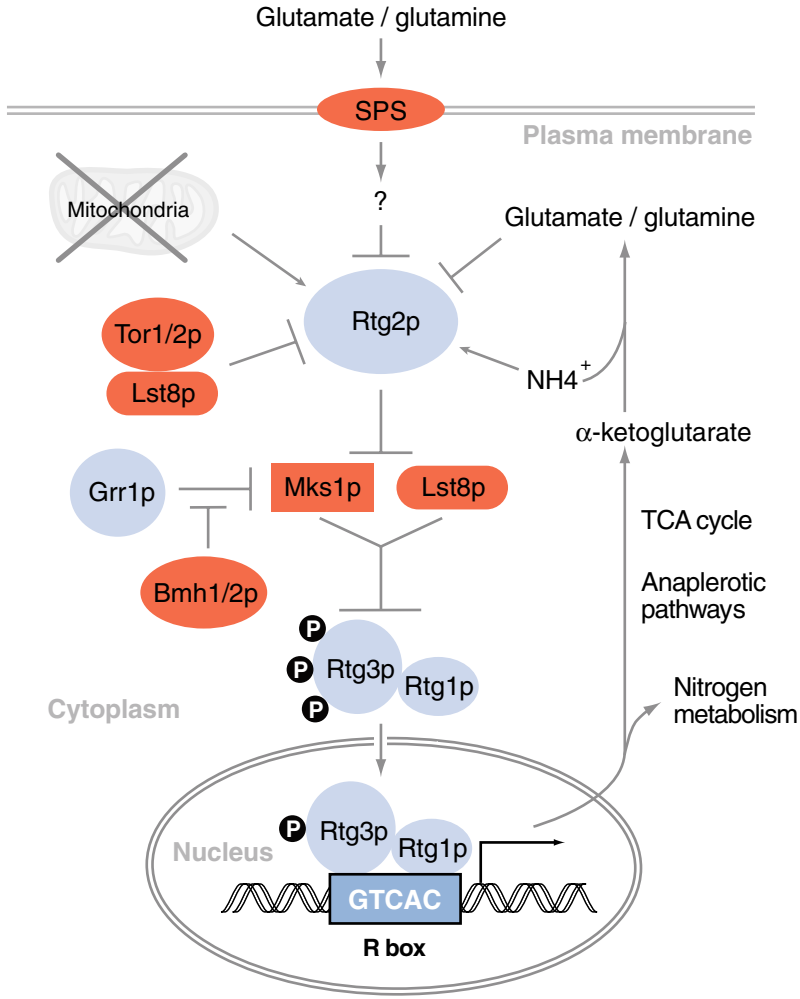

Figure 1

A model for the regulation of the RTG pathway. Positive and negative regulators of the RTG pathway are shown in blue and orange, respectively. Lst8p functions at two sites, with one upstream and the other downstream of Rtg2p. Activation of the RTG pathway leads to a higher level of glutamate and glutamine, which in turn inhibit the RTG pathway. This constitutes a feedback control for retrograde signaling. External glutamate and glutamine are sensed through the SPS amino acid sensing pathway to inhibit Rtg2p.

nuclear translocation of Rtg1/3p. Integrity of the ATP binding domain of Rtg2p is essential for its function (85).

Grr1p

Grr1p functions as a positive regulator of the retrograde pathway by mediating ubiquitination of a negative regulator, Mks1p (see below), resulting in Mks1p's ubiquitination and degradation (86). The conjugation of ubiquitin, a 76-amino acid protein, to a protein either tags it for degradation or modulates its function (56, 94, 138). The protein ubiquitination process is catalyzed through a cascade of enzymatic reactions mediated by

enzymes known as E1, E2, and E3. Grr1p is the F box component of the SCF^{Grr1} (Skp1-Cdc53/Cullin-F box protein) E3 ubiquitin ligase, which serves as an adapter complex to bring its substrate, through Grr1p's leucine-rich repeat (LRR) domain, to the vicinity of the E2 ubiquitin-conjugating enzyme Cdc34 (24, 142). The SCF^{Grr1} E3 ubiquitin ligase targets proteins for degradation in multiple pathways, including the G1 cyclins, Cln1 and Cln2 (24), and Mth1p and Std1p, two negative regulators in the glucose induction pathway (37, 70, 127).

NEGATIVE REGULATORS OF THE RTG PATHWAY

Mks1p

Mks1p negatively regulates the RTG pathway by promoting the phosphorylation of Rtg3p and inhibiting the nuclear translocation of Rtg1/3p (27, 121). Mks1p, a cytoplasmic protein, has been implicated in several pathways. It was originally identified as a negative regulator in the RAS-cyclic AMP pathway (93). It was also known as *LYS80*, a negative regulator in the lysine biosynthesis pathway (36). Mks1p has been proposed as a positive regulator of [URE3] prion formation (30). Most of the phenotypes exhibited by *mks1* mutant cells were later attributed to Mks1p's role as a negative regulator in the RTG pathway (28, 121, 131). Mks1p functions downstream of Rtg2p but upstream of Rtg1/3p (28, 121, 131). Mks1p is regulated through reversible binding with Rtg2p and through SCF^{Grr1}-dependent ubiquitination and subsequent degradation (85, 86).

Lst8p

Lst8p is an essential protein consisting of seven WD-repeats (tryptophan-aspartate repeats) (84, 114). A WD-repeat is a sequence motif consisting of ~40 amino acids, multiples of which are predicted to form a β-propeller structure as exemplified in the G protein β subunit (99, 126). *LST8* was originally identified via a mutation synthetically lethal with the *sec13-1* mutation (114). In *lst8-1* mutant cells, targeting of the general amino acid permease Gap1p to the plasma membrane is defective. Lst8p is an integral component of two TOR (target of rapamycin) kinase complexes, TORC1 and TORC2 (87, 113, 141). Genetic data suggest that Lst8p negatively regulates the RTG pathway, with one site upstream and the other site downstream of Rtg2p (17, 84).

14-3-3 Proteins

One mechanism for 14-3-3 proteins to function as negative regulators in the RTG pathway is by preventing Mks1p from SCF^{Grr1}-dependent ubiquitination and degradation (85, 86). 14-3-3 proteins are conserved in all eukaryotic organisms examined and often exist as multiple isoforms (29, 133). They are involved in a diverse array of cellular functions by interacting with different partner proteins, which are often phosphorylated. There are two functionally redundant 14-3-3 proteins in yeast, encoded by *BMH1* and *BMH2* (43, 134). Bmh1p is more abundant than Bmh2p (43, 116). Deletion of both genes is lethal in most strain backgrounds. Another possible mechanism for Bmh1/2p to regulate the RTG pathway is to bind to Rtg3p and keep it in an inactive state (135).

TRANSCRIPTIONAL ANALYSIS OF THE RETROGRADE RESPONSE

RTG-Target Genes

There are two groups of *RTG*-target genes (15, 79, 80, 83). The first group includes *CIT2* and *DLD3*, which show a robust retrograde response, and the second group includes several tricarboxylic acid (TCA) cycle genes, which do not show an obvious retrograde response. Analysis of expression of these *RTG*-target genes not only reveals the mode of their regulation by Rtg1/3p, but also provides

SCF: Skp1-Cdc53/Cul1-F box protein

Leucine-rich repeats (LRR): a protein domain that generally folds into an arc or horseshoe shape, providing a structural framework for protein and protein interactions

WD-repeats: short motifs of ~40 amino acids in length that often terminate in a tryptophan-aspartate (W-D) dipeptide, generally believed to form a circularized β-propeller structure that serves as a rigid scaffold for protein interactions

TOR: target of rapamycin

NAD: nicotinamide adenine dinucleotide

TCA cycle: tricarboxylic acid cycle

information on physiological functions of the RTG pathway.

The prototypical target gene of the RTG pathway is *CIT2* (79, 80). The promoter region of *CIT2* contains an inverted repeat of R box sequences that provides the binding site for the Rtg1/3p heterodimer. Mutations in the R box sequences abolish the binding of Rtg1/3p to the *CIT2* promoter, resulting in failure of both basal and retrograde expression of *CIT2*. The bHLH family transcription factors form homo- or heterodimers to bind to the target DNA sequence (92). Rtg1p and Rtg3p function as a heterodimer because neither alone is able to bind to an R box site (62). *CIT2* encodes the peroxisomal isoform of citrate synthase, an enzyme in the glyoxylate cycle that enables yeast cells to utilize two carbon compounds, such as acetate and ethanol, as sole carbon source. The rest of the glyoxylate cycle genes, *ICL1* (encoding isocitrate lyase), *MDH3* (encoding malate dehydrogenase), and *MLS1* (encoding malate synthase), are not subject to retrograde regulation (14).

DLD3, encoding a cytoplasmic isoform of D-lactate dehydrogenase, is also subject to retrograde regulation (15). Using a promoter-trapping method developed by Burns et al. (11), *DLD3* was isolated in a genetic screen for genes whose expression requires Rtg2p. Similar to *CIT2*, expression of *DLD3* is elevated in ρ^0 cells, and its basal and retrograde-induced expression requires functional *RTG1/2/3*. An inverted repeat of R box sequences also exists in the promoter region of *DLD3* and is required for its optimal expression. A physiological function for the retrograde expression of *DLD3* is unknown, but it is likely to be involved in regenerating NAD^+ due to the potential buildup of NADH in respiration-deficient cells.

Expression of the second group of *RTG*-target genes is not elevated in ρ^0 cells (83). This group includes genes encoding the first three enzymes in the TCA cycle, namely *CIT1* (encoding a mitochondrial isoform of citrate synthase), *ACO1* (encoding aconitase), and *IDH1/2* (encoding NAD^+-dependent isocitrate dehydrogenase). Expression of these four genes switches from Hap2-5p control to Rtg1/3p in cells with compromised mitochondrial function. This switch reflects two important functions of the TCA cycle: supplying ATP through catabolic oxidative metabolism and providing metabolic intermediates for anabolic biosynthesis. In cells grown under respiratory conditions, expression of the TCA cycle genes, and many genes involved in oxidative metabolism, is under the global control of heme-responsive transcription factor Hap1p and the heme-independent Hap2-5p transcription factor complex (39, 101, 118, 147). As mitochondrial respiratory function is compromised, for example, in cells without mtDNA, the TCA cycle is blocked at the step of succinate dehydrogenase (SDH), but part of the TCA cycle upstream of SDH still operates to provide metabolic intermediates for biosynthesis (**Figure 2**). The switch to Rtg1/3p control of these early TCA cycle genes ensures continuance of essential anabolic processes, such as glutamate and lysine biosynthesis.

Among the four early TCA cycle genes, regulation of *CIT1* expression is the best studied. Analysis of the *CIT1* promoter region revealed two *cis*-acting elements controlling glucose-repressed and -nonrepressed expression of *CIT1*, respectively (118, 119). The *cis*-acting element responsible for *CIT1* expression under respiratory conditions is the Hap2-5p binding site, whereas the *cis*-acting element responsible for *CIT1* expression under glucose-repressed condition is an R box site (83). As the mitochondrial respiratory function decreases, the expression of *CIT1* is increasingly dependent on Rtg1/3p and less dependent on Hap2-5p.

This dual *HAP-RTG* control of gene expression extends to *ACO1*, *IDH1*, and *IDH2* (42, 53, 83, 139). In clear contrast, expression of the other TCA cycle genes, which encode proteins catalyzing steps of the TCA cycle downstream of isocitrate dehydrogenase, is independent of the *RTG* genes in

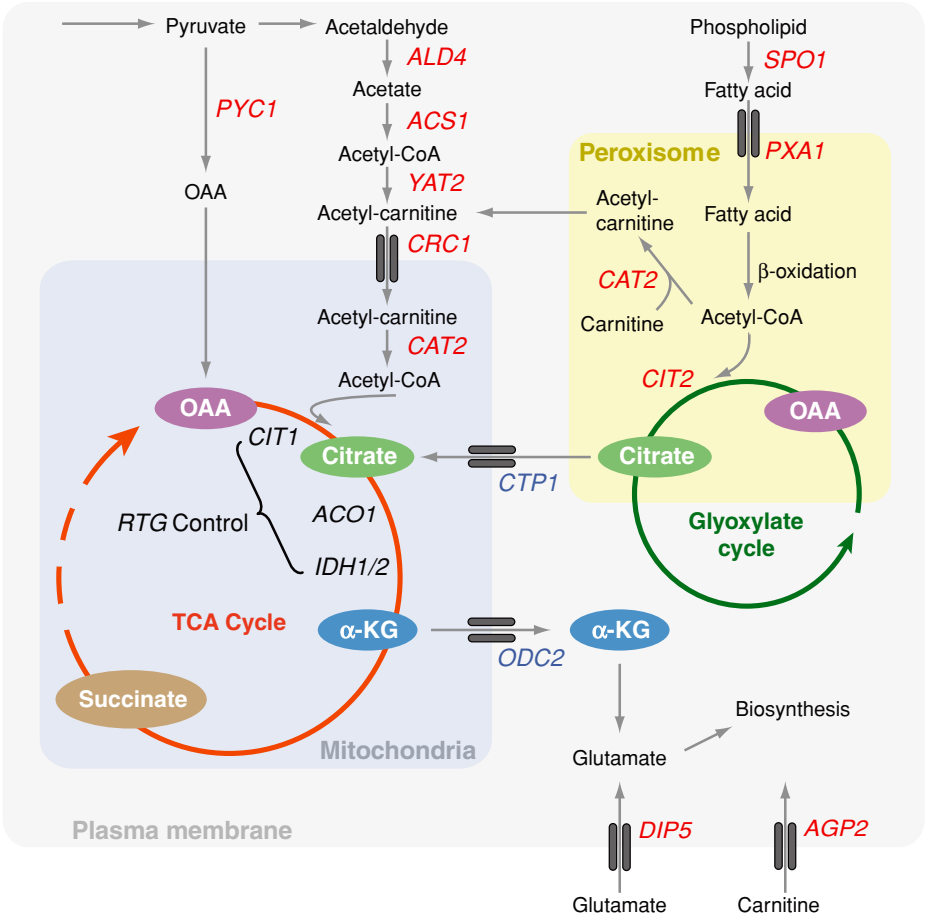

Figure 2

Metabolic reconfiguration in respiration-deficient cells. Only genes involved in glutamate biosynthesis and anaplerotic pathways are shown. The broken line in the TCA cycle indicates the block from succinate to oxaloacetic acid (OAA) in respiration-deficient cells. Genes whose expression is elevated in ρ^0 cells are indicated in red. Two genes, *CTP1* and *ODC2*, indicated in blue and known to be up-regulated in *mks1*Δ cells, are included as likely target genes of the RTG pathway. A metabolic reconfiguration of the retrograde pathway ensures a sufficient level of α-ketoglutarate for glutamate synthesis to meet the demand of nitrogen supply for biosynthetic reactions.

respiration-deficient cells (83). Taken together, expression of genes encoding the first three steps of the TCA cycle enzymes comes under increasing control of the *RTG* genes as mitochondrial respiratory function is reduced. This combinatorial control of early TCA cycle gene expression by Hap2-5p and Rtg1/3p represents a novel strategy for cells to adapt to changes in carbon source and mitochondrial functional state.

Genome-Wide Transcriptional Profiling of Cellular Responses to Mitochondrial Dysfunction

Genome-wide transcriptional profiling of respiration-competent versus respiration-deficient cells lacking mtDNA has provided information on the scope of the retrograde response (33, 132). Two such studies yielded somewhat different results, which are likely

OAA: oxaloacetic acid or oxaloacetate

ρ^+: cells with mitochondrial DNA

due to the strains used and the medium selected for culturing cells. Traven et al. used a W303 strain, which has a weak or no retrograde response of *CIT2* (73, 132), whereas Epstein et al. used a strain known to have a robust retrograde response (33, 80). The choice of carbon source was also different; glucose, a repressing carbon source, was used in one case (132), and raffinose, a nonrepressing but fermentable carbon source, in the other (33). That disparity seemingly explains elevated expression of a large number of genes involved in mitochondrial biogenesis in ρ^0 cells in Traven et al.'s study, which is likely due to earlier onset of glucose derepression in such cells. It is unclear what accounts for these strain differences, but it is possible that multiple parallel retrograde pathways operate to affect different sets of target gene expression.

One prominent feature from genome-wide transcriptional analysis in cells with dysfunctional mitochondria is the elevated expression of genes involved in (anaplerotic) pathways that would supply acetyl-CoA and citrate to mitochondria (33) (**Figure 2**). For example, there is a dramatic proliferation of peroxisomes with concomitant high expression of genes involved in fatty acid β-oxidation that would lead to increased acetyl-CoA production. Elevated expression of *PYC1*, encoding cytosolic pyruvate carboxylase, would lead to increased production of oxaloacetate (OAA) from pyruvate. Increased expression of *CIT2* would increase production of citrate from acetyl-CoA and oxaloacetate. The citrate transporter encoded by *CTP1*, whose expression is up-regulated in an *mks1*Δ strain (28), is expected to be up-regulated in ρ^0 cells to transport citrate into mitochondria. There is also evidence to indicate increased transport of acetyl-CoA into mitochondria. There exist two pathways for acetyl-CoA transport into mitochondria (32, 129, 136, 137). One requires Cit2p, which functions with other enzymes in the glyoxylate cycle to convert acetyl-CoA into glyoxylate cycle intermediates, such as succinate and citrate. Those intermediates can then be transported into mitochondria via carrier proteins. The second pathway converts acetyl-CoA and carnitine into acetylcarnitine through three carnitine acetyl-CoA transferases: Cat2p, Yat1p, and Yat2p. Acetylcarnitine is then transported into mitochondria in exchange for carnitine via the carnitine:acylcarnitine antiporter, Crc1p. Once in the mitochondria, the mitochondria-localized Cat2p catalyzes the reverse reaction to release acetyl units and regenerate carnitine. Carnitine is transported into yeast cells through the plasma membrane carnitine transporter Agp2p (137). A *cit2*Δ mutation is synthetically lethal with mutation in either *CRC1*, *AGP2*, *CAT2*, *YAT1*, or *YAT2* on medium with acetate, ethanol, or oleic acid as the sole carbon source (129, 137). Increased expression of *CIT2*, *CRC1*, *AGP2*, and to a lesser extent, *CAT2* and *YAT2*, suggests a concerted effort to utilize the acetyl group in cells with dysfunctional mitochondria, consistent with the observed proliferation of peroxisomes and increased expression of genes involved in β-oxidation of fatty acids in ρ^0 cells.

Another feature of genome-wide transcriptional profiling is the elicitation of different genome responses with different perturbations of mitochondrial respiratory function (33). Genome responses to treatment of ρ^+ cells with three different inhibitors of mitochondrial function, antimycin (inhibitor of mitochondrial electron transport by blocking the reoxidation of reduced cytochrome b), CCCP (uncoupler of ATP synthesis by dissipating the proton gradient across the inner mitochondrial membrane), and oligomycin (a specific inhibitor of the F0 component of the F1-F0 ATP synthase complex) were examined and compared to that elicited in ρ^0 cells. In terms of changes in global gene expression, antimycin is most similar to ρ^0 cells, whereas there is little overlap of up-regulated genes between CCCP or oligomycin treatment and ρ^0 cells. There is also a significant overlap of up-regulated genes between CCCP and antimycin or oligomycin treatment. These

results suggest that different retrograde responses are activated according to specific mitochondrial defects.

The retrograde response can also be activated by defects in genes encoding TCA cycle enzymes (14). Genome-wide responses to mutations in 15 genes encoding each of the 8 TCA cycle enzyme components was examined using DNA microarrays (95). There are common sets of genes whose expression is up- or down-regulated in all these mutants, reflecting a shift away from oxidative metabolism. There is a group of genes whose expression alternates between up-regulation and down-regulation in TCA cycle gene mutants as one moves pairwise down the TCA cycle, with mutations in the Mdh1p-Cit1p (malate dehydrogenase-citrate synthase) and Kgd1/2p-Lsc1/2p (α-ketoglutarate dehydrogenase-succinyl-CoA ligase) pair of enzymes leading to down-regulation, and with mutations in the intervening pair of Aco1p-Idh1/2p (aconitase-isocitrate dehydrogenase) and Sdh1-4p-Fum1p (succinate dehydrogenase-fumarase) of enzymes leading to upregulation. This alternating pattern of gene expression correlates well with a growth-suppression phenotype (41, 108), in which an *idh1* mutant, which is defective in growth on nonfermentable carbon sources, can be rescued by mutations in Mdh1p-Cit1p and Kgd1/2p-Lsc1/2p (108). In the *CIT1* case, the *idh1* mutant suppression phenotype by a *cit1* mutation is likely due to neutralization of changes in the aforementioned set of genes, which show an alternating pattern of gene expression. These results suggest that a novel signaling pathway senses a specific TCA cycle enzyme defect by detecting the level of a TCA cycle intermediate that would be accumulated in the mutant. The mechanism for this alternating pattern of gene expression is unknown.

RTG-Independent Retrograde Responsive Genes

Genome-wide analysis of gene expression in cells with compromised mitochondrial function revealed a large number of genes whose expression is independent of *RTG* genes, indicating the existence of other retrograde signaling pathways (33, 132). One such *RTG*-independent retrograde response is elevated expression of the *PDR5* gene in cells devoid of mtDNA (25, 47, 149). *PDR5* encodes a plasma membrane-localized protein, which belongs to the ATP binding cassette family of membrane transporter proteins and mediates multidrug resistance (3, 6). Expression of *PDR5* requires Pdr1p and Pdr3p, two homologous zinc finger transcription factors (2, 3, 65). Elevated expression of *PDR5* in ρ^0 cells requires Pdr3p but not Pdr1p. One mechanism for this retrograde response is elevated expression of *PDR3* in ρ^0 cells, which is subject to auto-regulation by its translated product Pdr3p. An unknown posttranslational modification of Pdr3p also leads to its activation in ρ^0 cells. Elevated expression of *PDR3* and *PDR5* in ρ^0 cells requires Lge1p (148), a nuclear protein that plays a role in the ubiquitination of histone H2B, together with the ubiquitin ligase Bre1p and the ubiquitin-conjugating enzyme Rad6p (59). The mechanism by which Lge1p mediates retrograde response of *PDR3* and *PDR5* is not known, but this function is clearly separable from its role in the Rad6p-dependent ubiquitination of histone H2B (148). Taken together, these data lead to the following model: A signal from mtDNA-less mitochondria activates Pdr3p, which in turn auto-regulates its own expression; auto-amplification of Pdr3p drives expression of its target genes, including Pdr5p (97).

Mutations in various nuclear genes affecting mitochondrial function were examined to pinpoint mitochondrial lesions responsible for *PDR5* activation in ρ^0 cells (149). Those mutations specifically affecting the functional status of the F0 component of ATP synthase complex activate retrograde expression of *PDR5*. Although Rtg1p plays some role in affecting Pdr3p activity (47), the signals for *RTG*-dependent retrograde response and retrograde activation of *PDR5* are likely to be

different. Retrograde activation of *PDR5* can be observed in cells grown in glucose, whereas such a growth condition produces very little activation of *RTG*-dependent retrograde response because mitochondrial respiratory function is already low in ρ^+ cells.

Another *RTG*-independent pathway is increased expression of *ATO3* in ρ^0 cells (33, 46). *ATO3* encodes a putative ammonia/ammonium outward transporter localized on the plasma membrane, whose expression is also induced during transition from the acid to the alkali phase in yeast colonies grown on solid substrates (46, 103). Yeast colonies grown on solid medium alternately acidify and alkalinize the medium as a mechanism of intercolony communication (104). The alkaline compound ammonia is released during the alkali phase to communicate with other colonies and a growth inhibition zone is formed between two adjacent colonies. Mutations in *ATO3*, as well as in its two homologous genes, *ATO1* and *ATO2*, reduce ammonia production during the alkali phase (103). Ammonia production requires amino acids in the growth medium and the SPS amino acid sensing pathway, which is comprised of Ssy1p, an amino acid sensor on the plasma membrane, and two downstream factors, Ptr3p and Ssy5p (38, 104, 150). Consistent with the role of amino acids in ammonia production via Ato3p, expression of *ATO3* is dependent on Gcn4p, a transcriptional activator in the general amino acid control pathway, and Ssy1p, both of which are involved in amino acid metabolism (46). A physiological function for retrograde responsive expression of *ATO3* remains to be determined.

PHYSIOLOGICAL FUNCTIONS OF THE RTG PATHWAY

A Central Role of the RTG Pathway for Glutamate Biosynthesis

An indication of the RTG pathway involvement in glutamate metabolism is that all *rtg* mutants are glutamate auxotrophs (62, 79), reflecting *RTG*-dependent expression of genes involved in glutamate biosynthesis, namely *CIT1*, *CIT2*, *ACO1*, *IDH1*, and *IDH2* (**Figure 2**). Activities of these gene products lead to the production of α-ketoglutarate, the direct precursor for glutamate. *aco1* single and *cit1 cit2* double mutant cells are also glutamate auxotrophs (42, 71). Failure to detect a glutamate auxotroph phenotype in *idh1* or *idh2* mutant cells is due to the production of α-ketoglutarate via Idp1p, a mitochondrial isoform of NADP-dependent isocitrate dehydrogenase (52). Consistent with the transcriptional factor switch of early TCA cycle gene expression from Rtg1/3p to Hap2-5p in cells with increasing mitochondrial respiratory activity, *rtg* mutants are not glutamate auxotrophs under glucose nonrepressed conditions, for example, in ρ^+ cells grown on raffinose medium (83). Glutamate has been shown to inhibit expression of these early TCA cycle genes as well as *CIT2* (52, 71, 119, 139), which can now be viewed as a feedback control of glutamate biosynthesis. Although the exact target protein acted upon by glutamate or its derivative remains to be determined, it is certain that glutamate repression of expression of these genes is via inhibition of the RTG pathway.

Why do cells need to specifically ensure the production of glutamate in respiration-deficient cells? Glutamate, together with its downstream metabolite glutamine, provides all the nitrogen used in biosynthetic reactions (89). Thus, glutamate needs to be maintained at a certain level to meet biosynthetic needs. Consistently, limiting glutamate/glutamine production via a mutation in *GDH1*, encoding the main anabolic glutamate dehydrogenase, leads to a 39% reduction in total cellular amino acids, whereas increasing glutamate/glutamine production via a mutation in *MKS1*, which leads to activation of the RTG pathway, results in a 43% increase of total cellular amino acids (16). Therefore, one main function of the RTG pathway is to ensure that a sufficient level of α-ketoglutarate for glutamate synthesis is made to meet the demand

of nitrogen supply for biosynthetic reactions, especially in respiration-deficient cells.

Expression of genes involved in the lysine biosynthesis pathway is highly induced in *mks1* mutants (36, 111). Increased activities of citrate synthase, aconitase, and isocitrate dehydrogenase and an increased level of α-ketoglutarate were observed in *mks1* mutants (36). A high level of α-ketoglutarate is expected to increase metabolic flux into the lysine biosynthesis pathway and raises the level of α-aminoadipate semialdehyde, a lysine biosynthesis pathway intermediate and an inducer of Lys14p, a transcriptional activator for genes involved in lysine biosynthesis (35, 110). These data suggest that the effect of *mks1* mutations on activation of gene expression in the lysine biosynthesis pathway is likely to be indirect. In a genome-wide transcriptional analysis of wild type versus an *mks1* mutant, Dilova et al. found that up-regulated genes in *mks1* mutant cells include target genes of the RTG pathway, consistent with Mks1p's role as a negative regulator in the RTG pathway, and those involved in lysine biosynthesis (28). Introduction of an *rtg3* mutation abolishes induced expression of genes involved in lysine biosynthesis in *mks1* mutant cells, indicating that the effect of *mks1* mutation on the lysine biosynthesis pathway is secondary to the RTG pathway activation. Only a very small number of genes displayed significant differences, and most were up-regulated in *mks1* mutant cells. Those few genes include *DIP5*, which encodes a plasma membrane glutamate/aspartate transporter (112); *ADH3*, which encodes a mitochondrial isoform of alcohol dehydrogenase (1); and two encoding mitochondrial carrier proteins; *CTP1*, which encodes a citrate transporter (64); and *ODC2*, which encodes an oxodicarboxylate transporter (105). All four genes are related to glutamate metabolism: Ctp1p transports citrate formed in the cytoplasm via Cit2p into mitochondria to provide material for α-ketoglutarate formation; Odc2p transports α-ketoglutarate and α-ketoadipate out of mitochondria for glutamate and lysine biosynthesis, respectively; Dip5p transports glutamate into cells from the extracellular environment; Adh3p shuttles out NADH generated in the mitochondrial matrix, mainly due to glutamate biosynthesis, in cells incapable of respiration (100) (**Figure 2**). The small number of genes affected by an *mks1* mutation and their overwhelming involvement in glutamate metabolism suggest that the RTG pathway is a dedicated system to maintain glutamate homeostasis.

Mitochondrial DNA Maintenance

The RTG pathway affects mtDNA maintenance through regulation of the *RTG*$^{-}$ target gene, *ACO1*, thus linking retrograde signaling to mitochondrial biogenesis (19). An *mks1*Δ mutation, by activating *ACO1* expression, can complement mtDNA maintenance defects in cells lacking the mtDNA packaging protein Abf2p (18). Rtg1/3p and Hap2-5p play a redundant role in *ACO1* expression to maintain mtDNA because *rtg hap* double mutant cells cannot maintain mtDNA (19). A transcriptional factor switch between Rtg1/3p and Hap2-5p for *ACO1* expression ensures that cells are able to maintain mtDNA under various growth conditions (53, 83, 139). Early indications linking Aco1p to mtDNA inheritance came from identification of Aco1p among proteins cross-linked to mtDNA and the observation of unstable mtDNA in *aco1* mutant cells (66, 95). The mtDNA loss phenotype is much more severe in *aco1* mutants than mutations in any other TCA cycle gene, suggesting that involvement of Aco1p in mtDNA maintenance is not due to a defect in the TCA cycle function (95). Bifunctionality for aconitase is not unprecedented. The mammalian cytosolic aconitase can function as an iron regulatory protein (IRP) through disassembly of its iron-sulfur cluster (55). IRPs bind to the iron regulatory elements in the untranslated region of target mRNAs that encode proteins involved in iron homeostasis, including ferritin (involved in iron storage) and the transferrin receptor, by affecting

translation efficiency or mRNA stability (31). Bifunctionality of yeast Aco1p becomes evident after examining the effects of mutations in three cysteine residues, Cys382, Cys445, and Cys448, which are involved in iron-sulfur cluster coordination. All three mutants are glutamate auxotrophs and unable to use nonfermentable carbon source, which indicate a lack of aconitase activity. However, expression of the Cys448 mutant under the control of its own promoter and overexpression of the Cys382 and Cys445 mutants under control of the *ADH1* promoter allows maintenance of mtDNA. These results clearly separate two functions of aconitase, an enzymatic activity and an mtDNA maintenance activity. A role for Aco1p in mtDNA maintenance provides a mechanism to coordinate expression of genes involved in oxidative metabolism from the nuclear genome and those from mtDNA, which encodes components in the electron transport chain and ATP synthase.

NLS: nuclear localization signal

REGULATORY MECHANISMS AND SIGNALS

Nuclear Translocation and Activation of Rtg1/3p

One key regulatory step in the regulation of the RTG pathway is nuclear translocation of Rtg1/3p (122). When the RTG pathway is inactive, Rtg3p is hyperphosphorylated and localized in the cytoplasm together with Rtg1p (**Figure 3**). Upon activation of the RTG pathway, Rtg3p becomes partially dephosphorylated and translocates into the nucleus. Once in the nucleus, Rtg1p and Rtg3p form a heterodimer and bind to the R box sequence in the promoter region of target genes. The basic region in the bHLH domain of Rtg3p also functions as a bipartite nuclear localization signal (NLS). Nuclear translocation of Rtg1p and Rtg3p requires Rtg2p. Deletion of Rtg1p results in constitutive nuclear localization of Rtg3p even in *rtg2*Δ cells, which

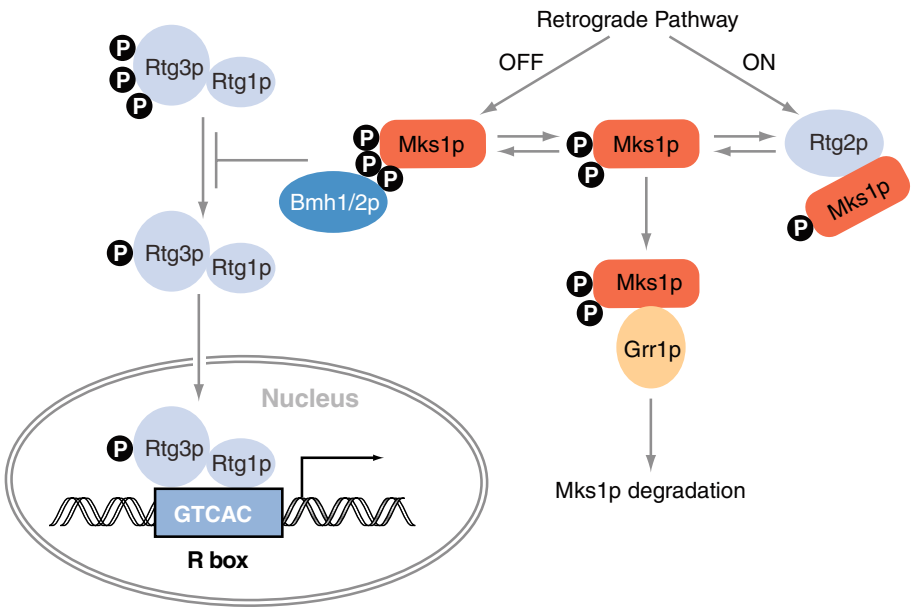

Figure 3

Regulation of *RTG*-dependent gene expression by a dynamic interaction between Rtg2p and Mks1p. When the RTG pathway is active, Rtg2p binds to and inactivates Mks1p. When the RTG pathway is inactive, Mks1p dissociates from Rtg2p and interacts with 14-3-3 proteins. The Bmh1/2p bound form of Mks1p is the active form to inhibit Rtg1/3p nuclear translocation. Grr1p-dependent degradation of free Mks1p ensures an efficient switch between the Rtg2p-Mks1p complex and Bmh1/2p-Mks1p complex.

suggests a negative regulatory role of Rtg1p in the cytoplasm. Thus, Rtg1p plays an unusual dual role for RTG pathway regulation: a positive role in the nucleus to dimerize with Rtg3p to activate target gene expression and a negative role in the cytoplasm to prevent nuclear translocation of Rtg3p.

Phosphorylation of Rtg3p inversely correlates with activity of the RTG pathway. Rtg3p nuclear translocation, indicative of activation of the RTG pathway, correlates with its partial dephosphorylation, which requires Rtg2p (122). In *rtg2*Δ cells, Rtg3p is hyperphosphorylated and located in the cytoplasm. Partial dephosphorylation of Rtg3p is observed under many conditions that activate the RTG pathway, including loss of mtDNA (122), glutamate starvation, and rapamycin treatment (121). Hyperphosphorylation of Rtg3p and its inability to translocate into the nucleus in *rtg2*Δ cells can be reversed by an *rtg1*Δ or an *mks1*Δ mutation (121, 122). Most of the potential phosphorylation sites of Rtg3p lie in the N-terminal region comprising residues 2–279, deletion of which also leads to Rtg3p dephosphorylation and nuclear translocation. Rtg3p phosphorylation is subject to a feedback control (122). The phosphorylation state of a mutant Rtg3pΔ280–298 construct, which lacks an NLS and thus constitutively localizes in the cytoplasm, can be affected by coexpressing different Rtg3p constructs. Coexpression of Rtg3pΔ 280–298 with a functional Rtg3p, compared to a nonfunctional one, leads to more phosphorylation of Rtg3pΔ280–298, suggesting that both phosphorylation and dephosphorylation of Rtg3p can take place in the cytoplasm and that phosphorylation of Rtg3p is subject to a feedback control. Together, these data suggest that phosphorylation of Rtg3p is likely to be a regulatory step for the RTG pathway, with its dephosphorylation and phosphorylation in the cytoplasm leading to pathway activation and inactivation, respectively.

Mks1p is a phosphoprotein whose phosphorylation status mirrors that of Rtg3p (27, 121). The correlation of phosphorylation of Rtg3p and Mks1p raises the possibility that they are subject to phosphorylation by the same kinase. In *mks1*Δ cells, Rtg3p is dephosphorylated and localized in the nucleus, resulting in activation of the RTG pathway. It is plausible that Mks1p is required for the putative kinase to phosphorylate Rtg3p.

Once in the nucleus, the transcriptional activation domain of the Rtg1/3p complex is localized to Rtg3p. Rtg3p contains both N-terminal and C-terminal transcriptional activation domains, with the N-terminal domain providing the main activation activity (120, 122). The N-terminal transcriptional activation domain of Rtg3p is related to a so-called activation domain 1 (AD1) (90), which is specific to type I HLH transcription factors (91, 109). AD1 has a highly conserved LDFS motif, which activates transcription by recruitment of the SAGA histone acetyltransferase complex (90). The C-terminal activation domain of Rtg3p also interacts with Cyc8p-Tup1p, a well-known transcriptional repressor complex, probably through recruiting histone deacetylase (20). Deletion of *CYC8* reduces *CIT2* expression, whereas a *tup1* mutation increases basal level expression of *CIT2*. Cyc8p has also been found to be a coactivator for the transcriptional activator Gcn4p (72). These data suggest that besides its well-established repressor activity, Cyc8p can also function as a novel coactivator. Taken together, Rtg3p has both N-terminal and C-terminal transcriptional activation domains, which function synergistically to activate transcription of RTG-target genes by recruiting corresponding coactivators.

Dynamic Interaction Between Rtg2p and Mks1p

Rtg2p is required for the activation of Rtg1/3p, but it does not directly act on the Rtg1/3p complex (75, 122). How does Rtg2p regulate Rtg1/3p? The role of Rtg2p became clear when it was found to interact with Mks1p, a negative regulator of the RTG pathway (121) (**Figure 3**). The integrity of the

ATP binding domain of Rtg2p is essential for its function because point mutations located in or close to conserved regions in that domain result in loss of function of Rtg2p, which is mainly attributed to a failure of interaction with Mks1p (85). The N-terminal ATP binding domain of Rtg2p does not interact with Mks1p directly. Rather, the C-terminal region of Rtg2p interacts with Mks1p (Z. Liu, J. Thornton & R. A. Butow, unpublished results). The ATP binding and hydrolysis at the N-terminal ATP binding domain of Rtg2p probably regulate its C-terminal region's interaction with Mks1p.

All *rtg2* mutant phenotypes can be reversed by an *mks1* mutation, indicating that the main, if not the only, function of Rtg2p in the RTG pathway is to bind to and inactivate Mks1p. Rtg2p and Mks1p are localized diffusely throughout the cytoplasm (85, 121, 122). For an intracellular signaling pathway such as the RTG pathway to operate, interaction between Rtg2p and Mks1p is likely to be dynamic. To directly visualize a dynamic interaction, an actin cortical patch-recruiting assay was developed (85). By fusing Rtg2p to Aip1p, an actin interacting protein (117), the resultant Rtg2p-Aip1p fusion protein localizes to the actin cortical patches. When the Rtg2p-Aip1p fusion protein is coexpressed with Mks1p tagged with the green fluorescent protein (Mks1p-GFP), Mks1p-GFP is recruited to the actin cortical patches by Rtg2p-Aip1p when the RTG pathway is activated. Following inactivation of the RTG pathway, actin cortical patch localization of Mks1p-GFP disappears and Mks1p-GFP localizes diffusely in the cytoplasm. Together, an actin cortical patch-recruiting assay revealed that an interaction between Rtg2p and Mks1p activates the RTG pathway while their dissociation leads to pathway inactivation (**Figure 3**).

What signals to Rtg2p and Mks1p to regulate their interaction? Glutamate and glutamine are potent repressors of the RTG pathway (75, 83, 84) (**Figure 1**). Tate & Cooper proposed that ammonia is an activator of Rtg2p (130). However, glutamate and glutamine fail to disrupt an interaction between Rtg2p and Mks1p in an in vitro assay (Z. Liu, J. Thornton & R. A. Butow, unpublished results). The ATP binding domain of Rtg2p is important for its function, which raises the possibility that Rtg2p may sense ATP. The RTG pathway is activated by mitochondrial dysfunctions, and a defect in mitochondrial respiratory function would reduce cellular ATP levels. Thus, a low level of ATP may favor an interaction between Rtg2p and Mks1p, leading to activation of the RTG pathway.

Degradation of Mks1p via the SCF^{Grr1} E3 Ubiquitin Ligase

Another level of control of the RTG pathway is that Mks1p is subject to SCF^{Grr1}-dependent ubiquitination and degradation (86). A *grr1*Δ mutation stabilizes Mks1p and blocks induced expression of *CIT2*. Grr1p has an LRR domain, which is predicted to form a horseshoe-shaped solenoid with a parallel β sheet on the concave side and mostly α-helix on the convex side (57). The concave surface and adjacent loops are the most common protein interaction interface on proteins carrying an LRR domain (50, 74). The SCF^{Grr1} E3 ubiquitin ligase usually targets phosphosubstrates, for example, phosphorylated Cln1 and Cln2 (4, 57). All *GRR1* dominant mutations, which result in a gain of one or two positive charges and lead to increased degradation of Mks1p, are mapped onto the concave surface of its LRR domain (86). A gain of positive charge on the concave surface would lead to a better interaction with phosphorylated Mks1p and hence more efficient degradation. Although Mks1p is hyperphosphorylated, the main degradation signal (degron) is a short conserved acidic domain region instead of phospho-degron.

How can an acidic domain replace a canonical phospho-degron? There are no structural data on how Grr1p recognizes its substrate. Due to the prevalent requirement of

phospho-degron for SCF E3 ubiquitin ligases, structural analysis of other SCF E3 ubiquitin ligases may provide clues to how Grr1p binds to its substrate (50, 102, 143). One common feature revealed by structural studies is the presence of positive electrostatic potential on the surface of an SCF E3 ubiquitin ligase that makes contact with its phosphosubstrate. There is also electrostatic interaction between an SCF E3 ubiquitin ligase and the phospho-residue of its substrate and in some cases, an adjacent acidic residue. Thus, it is conceivable that some short-lived proteins would employ acidic residues as their degrons. Some substrates have to be phosphorylated at multiple sites to be recognized by a cognate SCF E3 ubiquitin ligase (98, 140). An acidic domain containing multiple acidic residues would be an alternative for multiple phosphorylations.

Conservation of the acidic domain in the middle region of Mks1p among fungal homologs suggests its importance. It provides the docking site for Grr1p to bind (86). How is Mks1p degradation regulated if phosphorylation is not the major recognition motif for the SCF^{Grr1} E3 ubiquitin ligase? Mks1p is stable when it is complexed with Rtg2p (86). The acidic domain is required for binding to Rtg2p. It appears that Rtg2p binding to Mks1p precludes Grr1p binding, thus protecting Mks1p from degradation. Bmh1/2p protects Mks1p from Grr1p-dependent degradation in *rtg2*Δ mutant cells. Bmh1/2p binds to the N-terminal part of Mks1p, outside of the acidic domain, so the protective effect on Mks1p is not likely due to direct competition for binding with Grr1p. 14-3-3 proteins often bind to their substrates through phosphoserine or threonine at Rxx(x)pS/pT motifs (146). Mks1p has five such potential sites, mutation of which does not affect Bmh1/2p interaction or Mks1p functionality (86). Novel binding sites are likely used for interaction with Bmh1/2p. Taken together, these data indicate that the interaction of Mks1p with other factors mediates its recognition by Grr1p.

Mks1p switches between binding to Rtg2p and Bmh1/2p. If binding with either protects Mks1p from Grr1p-dependent degradation, how can Grr1p regulate the RTG pathway? The observation that Mks1p is subject to degradation by Grr1p to some extent suggests that there is a free pool of Mks1p (unbound to either Rtg2p or Bmh1/2p). A large pool of free Mks1p would make the switch of Mks1p between Rtg2p and Bmh1/2p less efficient. To ensure an efficient switch to dynamically regulate the RTG pathway, free Mks1p must be kept at a low level. Grr1p is likely to perform such a role by degrading free Mks1p (**Figure 3**).

THE RETROGRADE RESPONSE AND TOR SIGNALING

Identification of *LST8* as a negative regulator of RTG signaling implicates TOR signaling in regulation of the RTG pathway. Lst8p has been detected in TOR kinase complexes, which have been purified from yeast and metazoans (144). There are two distinctive TOR kinase complexes in yeast: a rapamycin-sensitive TORC1 complex (consisting of Tor1/2p, Lst8p, Kog1p, and Tco89p) and a rapamycin-insensitive TORC2 complex (consisting of Tor2p, Avo1p, Avo2p, Avo3p, and Bit61p) (87, 113, 141). Both complexes are also partly conserved in mammalian systems, with Raptor (mKog1p) and Rictor (mAvo3p) in the mTORC1 and mTORC2 complexes, respectively, suggesting evolutionarily conserved functions (51, 68, 69). GβL/mLst8p, the mammalian homolog of yeast Lst8p, exists in both mTORC1 and mTORC2 complexes. TORC1 function is shared by Tor1p and Tor2p and positively regulates anabolic processes, including ribosome biogenesis and protein translation, while inhibiting catabolic processes such as autophagy (144). TORC2, with a Tor2p specific function, is involved in actin cytoskeleton organization and cell wall integrity.

The original *lst8-1* mutant, which contains a mutation near the C terminus of the

NCR: nitrogen catabolite repression

protein, shows defects of targeting of general amino acid permease Gap1p and other amino acid permeases to the plasma membrane (114). Gap1p targeting to the plasma membrane was originally shown to be inhibited by glutamate (115). Chen & Kaiser have extended this effect to include all amino acids (16). They have found that failure of Gap1p targeting to the plasma membrane in an *lst8-1* mutant is due to activation of the RTG pathway, which leads to an increase in the levels of total intracellular amino acids. Unlike *lst8* mutants uncovered in *rtg2* bypass screens, the original *lst8-1* mutant does not bypass an *rtg2* mutation (84). Chen & Kaiser identified additional *lst8* mutants. Together, these *lst8* mutants can be grouped into two classes with respect to RTG pathway regulation (17). The class I *lst8* mutation, represented by the *lst8-1* mutant allele, does not bypass the Rtg2p requirement for the RTG pathway activation, unlike class II *lst8* mutations represented by the *lst8-2∼5* mutant alleles. However, in the presence of Rtg2p, the RTG pathway is constitutively activated and glutamate repression is compromised in both classes of *lst8* mutants. Structural modeling of Lst8p revealed that *lst8* mutations identified in *rtg2* bypass screens are all localized to the opposite side of the *lst8-1* mutation in the protein (84), providing a facile explanation for differential effects of two types of *lst8* mutations on the *rtg2* bypass phenotype. Mutations in the SPS amino acid sensing system produce a phenotype similar to that of the *lst8-1* mutation. Because targeting of several amino acid permeases is defective in the *lst8-1* mutant (114), a plausible explanation for *lst8-1* mutant phenotype is the defective targeting of the amino acid sensor, Ssy1p, homolog of functional amino acid permeases, to the plasma membrane. Taken together, these results not only indicate that Lst8p is a negative regulator of the RTG pathway, they also suggest that Lst8p acts at two sites, one upstream and the other downstream of Rtg2p (**Figure 1**).

Involvement of TOR signaling in the RTG pathway was also discovered through two microarray studies, which showed increased expression of *RTG*-target genes in cells treated with rapamycin (75, 123). Rapamycin, a fungicidal macrolide produced by bacteria, together with the immunophilin FKBP12, binds to and inactivates the TORC1 complex (144). In contrast, the TORC2 complex is insensitive to rapamycin inhibition. Activation of the RTG pathway via rapamycin treatment is strictly dependent on Rtg2p. It is not clear whether TOR is directly involved in sensing mitochondrial function. A relation between TOR and mitochondrial function has been described in mammalian cells in which perturbation of mitochondrial function leads to inhibition of TOR kinase activity (68). ATP, a product of mitochondrial respiratory activity, has been proposed to be an activator of TOR kinase (23). The signal generated from mitochondria that affects TOR kinase activity remains to be determined. Microarray experiments also revealed TOR signaling in nitrogen catabolite repression (NCR), a nutrient sensing pathway activated when a nonpreferred nitrogen source is utilized (13, 75, 123). When yeast cells switch from a preferred nitrogen source, like glutamine or asparagine, to a nonpreferred nitrogen source, like proline or urea, two GATA transcription factors, Gln3p and Gat1p, dissociate from a negative regulator Ure2p and translocate into the nucleus to activate target gene expression.

What is the role of Lst8p in TORC1 and TORC2? Rapamycin treatment and nitrogen starvation do not affect the integrity of the TORC1 and TORC2 complexes (87). In the TORC2 complex, Lst8p binds to the C-terminal kinase domain of Tor2p, independently of Avo1/2/3p (145). Lst8p depletion destabilizes the interaction between Tor2p and Avo2p or Avo3p. Lst8p is required for full Tor2p kinase activity, and its depletion leads to defective polarization of actin cytoskeleton, similar to *tor2* mutations (87, 145). In some *lst8* mutants, cell wall integrity is compromised similar to that observed in a temperature-sensitive *tor2* mutant (17). The role of Lst8p in TORC1 is less clear. First,

it is difficult to reconcile that Lst8p acts both upstream and downstream of Rtg2p, whereas rapamycin treatment only affects the RTG pathway upstream of Rtg2p (75, 84). Second, different *lst8* mutations only affect a subset of TOR-regulated pathways: Mutations in *lst8-2∼5* activate Rtg1/3p without Rtg2p, whereas the *lst8-1* mutation does not (84); although *lst8-1∼5* mutations activate Gln3p, none activate Gat1p (44); some *lst8* mutant alleles have a large effect on Rtg1/3p target gene expression, whereas others have a large effect on Gln3p target genes (17). Third, loss of mitochondrial functions activates the RTG pathway but not the NCR pathway, suggesting that retrograde regulation of Rtg1/3p is not via TOR and that the RTG pathway and TOR signaling act in parallel to converge on Rtg1/3p (44).

It is difficult to sort out the differential effects observed in *lst8* mutants. One possibility is that Lst8p uses different blades of its β-propeller structure to interact with different effectors. Thus, different mutant alleles would affect only a subset of downstream pathways mediated by TOR. This possibility remains to be tested.

TOR is involved in nutrient sensing, in particular, nitrogen sensing (144). Glutamine has been proposed to signal to TOR to inhibit Rtg1/3p and Gln3p. This was based on the observation that glutamine starvation abolished glutamate repression of Rtg1/3p and Gln3p, but not Gat1p and Msn2/4p (22). To exclude the possibility that the effect is specific to glutamine starvation but not to a general amino acid starvation response, the effect of histidine starvation on glutamate repression of Rtg1/3p and Gln3p was examined (44). The results were that histidine starvation abolished glutamate repression of *CIT2*, an Rtg1/3p target gene, but not of *GLN1*, a Gln3p target gene. This starvation response requires Rtg2p but is independent of Gcn2p and Gcn4p, two factors mediating the general amino acid control. Differential effects on activation of transcription factors elicited by starvation of two different amino acids suggest that the mechanism by which TOR senses nutrients is complex.

RETROGRADE SIGNALING AND AGING

Aging in yeast can be measured by replicative life span, which is the number of daughter cells a mother cell can give rise to before senescence (96). Many factors contribute to the aging process in yeast, including the Ras-cAMP signaling pathway (61, 128), nutrient availability (63, 81), and extrachromosomal rDNA circles (ERC) (125). The literature on yeast aging is vast, and aging-related research has been reviewed extensively (54, 60, 67, 124). We focus our discussion on the involvement of retrograde signaling in aging.

The retrograde pathway was first implicated in yeast aging in a study by Kirchman et al., in which loss of mtDNA extends life span in some yeast strains (73). The life span-prolonging effect in yeast strains with reduced mitochondrial respiratory function correlates with the capacity to activate the retrograde response. Life span extension in ρ^0 cells is dependent on Rtg2p, which acts partly through inhibition of ERC formation (9). Loss of mitochondrial DNA increases the level of ERC (21), so one function of Rtg2p is to counteract the deleterious effect of ERC formation (9). Life span extension due to mitochondrial dysfunction is also observed in animal cells. For example, mutations in the *clk-1* gene, which is involved in ubiquinone biosynthesis, extend life span in both *Caenorhabditis elegans* and mice (34, 82). Three recent studies employing RNAi technology have strongly implicated mitochondrial function as a critical factor in determining life span in *C. elegans* (26, 48, 78). Long life span does not always correlate with reduced ROS (reactive oxygen species) production in long-lived worms with defective mitochondria, suggesting other pathways may be involved in prolonging life span. Life span extension due to mitochondrial dysfunction in both metazoans and yeast suggests a conserved role of mitochondria in aging. The

ERC: extrachromosomal rDNA circle

ROS: reactive oxygen species

underlying mechanism for life span extension in cells with mitochondrial dysfunction is likely to be a combination of increased resistance to ROS and a metabolic reconfiguration through active retrograde signaling pathways.

ROLES OF RTG2P IN THE NUCLEUS

Rtg2p is localized predominantly in the cytoplasm. However, two reports revealed potential nuclear functions of Rtg2p, as a component of the SLIK (SAGA-like) histone acetyltransferase complex and as a regulator of trinucleotide repeat (TNR) instability (5, 107). The SAGA (Spt-Ada-Gcn5) histone acetyltransferase (HAT) complex links transcriptional regulation to chromatin remodeling (45, 49). The SAGA and SLIK complexes share a common set of proteins, but contain Spt8p and Rtg2p as a specific component, respectively (107). The SLIK complex also contains a form of posttranslationally modified Spt7p. A mutation in *SPT7*, which is shared by both complexes, produces synthetic phenotypes more severe than those produced by a mutation in either *RTG2* or *SPT8*. When the *CIT2* gene is activated, the Rtg2p-specific SLIK complex, but not the SAGA complex, is recruited to the *CIT2* promoter region. In contrast, both complexes are recruited to the *GAL1-GAL10* promoter region when the *GAL10* gene is activated. These results suggest the SAGA complex and the Rtg2p-specific SLIK complex share overlapping but distinctive activities.

Rtg2p is implicated in TNR instability (5). Mutations in *RTG2* result in a modest increase in TNR expansion and a significant decrease of TNR retraction. This effect is specific to the stability of TNR since there are no changes in mutation rate for dinucleotide repeats or at the *CAN1* reporter gene in *rtg2* mutants. This feature is unique to Rtg2p because other mutations that affect TNR stability often lead to changes at other DNA sequences. The effect of Rtg2p on TNR stability is independent of the Rtg1/3p transcriptional complex. Because of Rtg2p's role in the SLIK histone acetyltransferase complex, which is also independent of the Rtg1/3p complex, it is tempting to link the role of Rtg2p in TNR instability to the SLIK acetyltransferase complex. Chromatin remodeling may play a role in mediating TNR instability.

EVOLUTIONARY CONSERVATION OF THE RTG PATHWAY

Rtg2p and Mks1p, two key regulatory factors in the RTG pathway, are conserved in multiple fungal species. Mks1p homologs have been detected in *Saccharomyces paradoxus*, *S. mikatae*, *S. bayanus*, *S. castellii*, *Candida glabrata*, *Kluyveromyces lactis*, *K. waltii*, and *Ashbya gossypii*. Conservation of Rtg2p among fungal species is more extensive. Besides those fungal species harboring an Mks1p homolog, Rtg2p homologs have also been detected in *S. kudriavzevii*, *S. kluyveri*, *Yarrowia lipolytica*, *Aspergillus oryzae*, *A. nidulans*, *A. fumigatus*, *Gibberella zeae*, *Neurospora crassa*, *Magnaporthe grisea*, and *Podospora anserina*. Some fungal species have multiple Rtg2p homologs, with three identified in *G. zeae*, two in both *A. fumigatus* and *A. oryzae*. The apparent lack of an Mks1p homolog in fungal species harboring an Rtg2p homolog is likely due to higher conservation of Rtg2p family members compared with Mks1p, which may have prevented identification of Mks1p homologs. For example, the sequence identity for Mks1p between *K. lactis* and *S. cerevisiae* is only 26%, whereas it is 69% for Rtg2p. Clones of *RTG2* and *MKS1* from several fungal species can complement *rtg2* and *mks1* mutations, respectively, when expressed in *S. cerevisiae* (Z. Liu, J. Thornton & R. A. Butow, unpublished results), suggesting that other fungal species have the RTG pathway and that the regulatory mechanism is likely to be conserved. Identification of two or three Rtg2p members in one fungal species suggests that Rtg2p function may have expanded in that species.

SUMMARY POINTS

1. The mitochondrial retrograde signaling pathway is mediated by four positive regulatory factors, Rtg1p, Rtg2p, Rtg3p, Grr1p, and four negative regulatory factors, Mks1p, Lst8p, and two 14-3-3 proteins, Bmh1p and Bmh2p.
2. Rtg2p-dependent dephosphorylation of Rtg3p correlates with Rtg3p's nuclear translocation and activation of the RTG pathway.
3. The critical regulatory step of the RTG pathway is the dynamic interaction between Rtg2p and Mks1p. Binding of Rtg2p to Mks1p leads to activation of the RTG pathway. Interaction of Mks1p with Bmh1/2p after its dissociation from Rtg2p leads to inhibition of nuclear translocation of Rtg1/3p. Grr1p-dependent degradation of free Mks1p ensures an efficient Mks1p switch between the Rtg2p-Mks1p complex and the Mks1p-Bmh1/2p complex and is required for induced expression of *CIT2*.
4. Lst8p is an integral component of TOR kinase complexes. It negatively regulates the RTG pathway at two sites, with one upstream and the other downstream of Rtg2p.
5. Mitochondrial dysfunctions result in proliferation of peroxisomes and the activation of the β-oxidation pathway and other anaplerotic pathways. These metabolic reconfigurations ensure the supply of α-ketoglutarate for the synthesis of glutamate.
6. Retrograde signaling is linked to aging, mtDNA maintenance, TOR signaling, and nutrient sensing pathways and is conserved in other fungal species.

FUTURE DIRECTIONS

1. One important question is how Mks1p inactivates Rtg1/3p. The N-terminal region of Mks1p harbors its activity domain. Isolation of *MKS1* mutations that map to the activity domain would be useful for designing multicopy suppressor screens to identify factors that may function with Mks1p to inhibit Rtg1/3p.
2. One critical regulatory step of the RTG pathway is a reversible interaction between Rtg2p and Mks1p. The ATP binding domain of Rtg2p is essential for its function. Biochemical analysis should be carried out to determine the putative ATPase activity of Rtg2p and an ATP-dependent regulation of interaction between Rtg2p and Mks1p.
3. Rtg3p and Mks1p are subject to phosphorylation modification. Their phosphorylation status correlates with the RTG pathway activity. A future focus will be the identification of kinase(s) for these two proteins and their roles in RTG pathway regulation.

ACKNOWLEDGMENTS

The authors' research on this subject was supported by NIH grants GM-22525 and GM33510 and by grant (I-0642) from The Robert A. Welch foundation. We thank the members of Butow laboratory for critical comments.

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Annual Review of Genetics

Volume 40, 2006

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