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Mks1 in Concert with TOR Signaling Negatively Regulates *RTG* Target Gene Expression in *S. cerevisiae*

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Summary

The target of rapamycin (TOR) signaling pathway allows eukaryotic cells to regulate their growth in response to nutritional cues [1, 2]. In *S. cerevisiae*, TOR controls the expression of genes involved in several nutrient-responsive biosynthetic pathways [3–7]. In particular, we have demonstrated that TOR negatively regulates a concise cluster of genes (termed *RTG* target genes) that encode mitochondrial and peroxisomal enzymes required for de novo amino acid biosynthesis [7]. TOR acts in part by regulating the subcellular localization of the Rtg1/Rtg3 transcription factor complex. Nuclear entry of this complex requires the cytoplasmic protein Rtg2, whose precise function has remained ill defined. Here we establish that the likely role of Rtg2 is to antagonize the activity of another protein, Mks1, which we demonstrate is itself a negative regulator of *RTG* target gene activation. Results of epistasis analyses suggest that Rtg2 and Mks1 act downstream of TOR and upstream of Rtg1 and Rtg3. Moreover, we find that Mks1 phosphorylation responds to TOR as well as to each of the Rtg1-Rtg3 proteins, indicative of complex regulation within this branch of TOR signaling. In addition to *RTG* target genes, microarray analysis reveals robust expression of lysine biosynthetic genes in *mks1*Δ cells, which depends on a functional *RTG* pathway. This latter result provides a molecular explanation for the previous identification of *MKS1* as *LYS80*, a negative regulator of lysine biosynthesis [8].

Results and Discussion

The *MKS1* gene encodes a cytoplasmic protein that has been implicated in a number of diverse biological processes, including *ras* signaling [9], the lysine biosynthetic pathway [8], and nitrogen metabolism [10]. The precise cellular function of this protein has remained elusive, however. We became interested in Mks1 following the report by Shamji et al. that this protein acts downstream of TOR as a positive regulator of *RTG* target gene expression [6]. This conclusion was based on results of global gene expression analyses, where the *RTG* target genes *CIT2* and *DLD3* failed to be induced in an *mks1*Δ strain treated with rapamycin, a specific inhibitor of the TOR kinases [6]. This is in contrast to wild-type cells, where rapamycin treatment results in strong expression of these genes [6, 7]. These authors also observed reduced basal expression of *CIT2* and *DLD3* when this *mks1*Δ strain was grown in rich media (YPD), a condition that is known to repress activation of the pathway [7, 11].

We decided to pursue these observations and disrupted *MKS1* in the common laboratory strain W303 [12]. To our surprise, however, both *CIT2* and *DLD3* were expressed at very high levels when these *mks1*Δ cells were grown in YPD, in comparison to wild-type cells (Figure 1A, compare lanes 1 and 2). Because the regulation of *RTG* target gene expression has been reported to vary in different strains of yeast [13], we reasoned one possible explanation for the difference between our results and those reported by Shamji et al. was our choice of strain background. We therefore deleted *MKS1* in two additional strain backgrounds: DBY8721 (Laboratory of David Botstein, Stanford University), a derivative of S288c [14]; and Σ2000 (Microbia, Cambridge, MA), a derivative of Σ1278 [15]. In each case, deletion of *MKS1* again resulted in strong expression of *CIT2* and *DLD3* (Figure 1A, lanes 3–6).

To compare our results directly with those of Shamji et al., we obtained and analyzed the same *mks1*Δ strain used in their study [6]. In agreement with their published data, we observed reduced basal expression of *CIT2* and *DLD3* in this strain (Figure 1A, lane 8). Furthermore, no increase in expression of these genes resulted following treatment of cells with rapamycin (data not shown). Interestingly, we also observed that this particular *mks1*Δ strain but not the *mks1*Δ strains we constructed required exogenous glutamine (or glutamate) for growth (data not shown). Glutamate (or glutamine) auxotrophy is a well-established phenotype associated with an impaired *RTG* pathway [11].

The *mks1*Δ strain used by Shamji et al. [6] was originally constructed in the laboratory of Evelyn Dubois [8]. In an effort to resolve the above discrepancies, we obtained and analyzed an original isolate of this *mks1*Δ strain, where strong expression of *CIT2* and *DLD3* was observed (Figure 1B). Moreover, this *mks1*Δ strain displayed no growth defect in the absence of glutamate or glutamine (data not shown). Based on these combined results, we conclude that Mks1 negatively regulates *RTG* target gene expression. We believe further that it is likely that the particular isolate of the Dubois *mks1*Δ strain used by Shamji et al. has acquired an additional mutation(s) that impairs activation of this pathway.

We wanted to confirm that increased expression of *CIT2* and *DLD3* observed in *mks1*Δ cells was mediated by *RTG* components rather than through activation of an alternative pathway. Accordingly, we monitored expression of these genes in strains deleted for *MKS1* as well as each of the three *RTG* genes *RTG1–3*. No expression of *CIT2* or *DLD3* was observed in *rtg1*Δ *mks1*Δ or *rtg3*Δ *mks1*Δ cells, confirming that expression of these genes in *mks1*Δ cells requires the Rtg1/Rtg3 transcription factors (Figure 1D, lanes 6 and 8). In striking contrast, *CIT2* and *DLD3* were expressed to the same extent in both *mks1*Δ as well as *rtg2*Δ *mks1*Δ cells,

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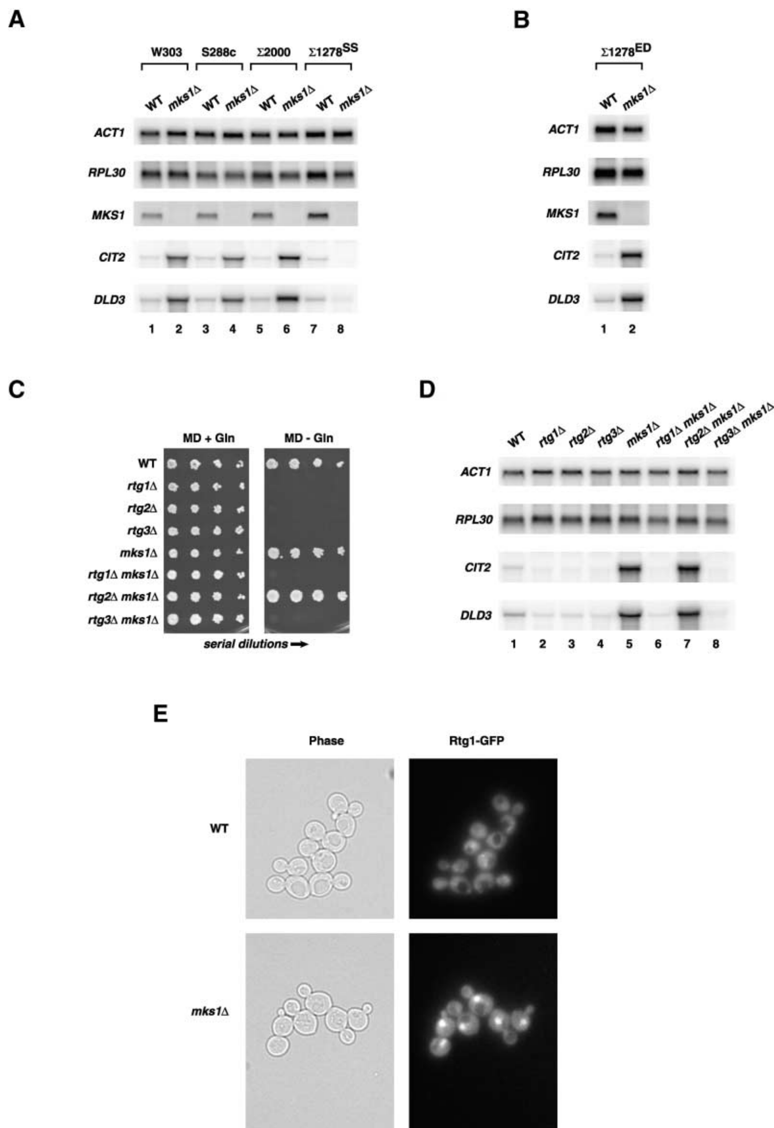


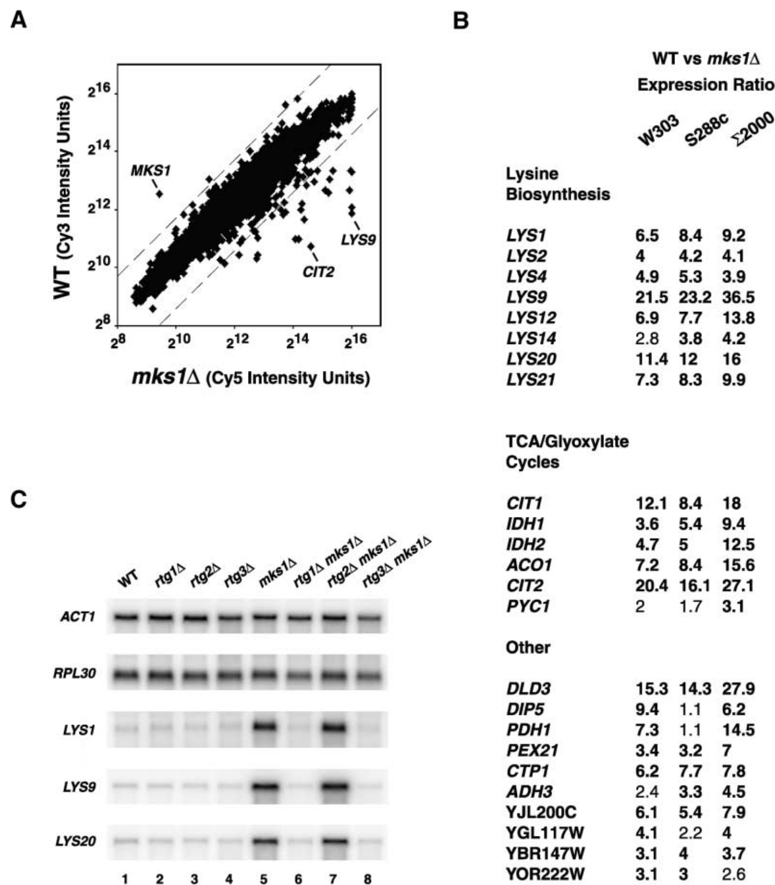
Figure 1. Mks1 Is a Negative Regulator of the *RTG* Pathway

(A and B) Northern blot analysis of specified mRNAs isolated from wild-type (WT) and *mks1*Δ cells, derived from several different strain backgrounds, as indicated. To delete *MKS1*, the entire coding region of this gene was replaced with the *LEU2* gene from plasmid pRS305 [17], using standard gene replacement techniques [18]. $\Sigma1278^{SS}$ denotes strains obtained from the Schreiber laboratory and used in the study by Shamji et al. [6]. $\Sigma1278^{ED}$ denotes strains obtained from the Dubois laboratory and used in the study by Feller et al. [8]. (C) Analysis of growth properties of strains on SCD agar plates that contained (left panel) or lacked (right panel) glutamine. Cells were grown in YPD to OD_{600} = 1.0, washed with sterile 50 mM potassium phosphate (pH 7.5), serially diluted and spotted out onto plates, and incubated for 3 days at 30°C. SCD media contained 0.8% yeast nitrogen base without amino acids (pH 5.5), 2% dextrose, and 0.2% glutamine where appropriate. In addition, to supplement the auxotrophic requirements of these strains, SCD media was also supplied with appropriate amino acids, adenine, and uracil as described [20]. (D) Northern blot analysis of specified mRNAs isolated from wild-type and mutant strains deleted for the indicated gene(s). In (A), (B), and (D), cells were grown in YPD (1% yeast extract, 2% peptone, 2% dextrose) to mid-log phase (0.5 OD_{600}/ml), and total RNA was isolated and analyzed as described [19]. (E) Localization of Rtg1-GFP in wild-type (WT) versus *mks1*Δ cells. Cells were transformed with plasmid pRTG1-GFP [7] and grown to mid-log phase (0.5 OD_{600}/ml) in SCD media that contained glutamine but lacked uracil (to select for plasmid maintenance). Images documenting GFP localization were collected on a Nikkon Eclipse E600 microscope using a 60× oil immersion objective (Nikkon) and recorded with a CCD camera (Hamamatsu) using an exposure time of 2 s. Image analysis was performed using Open Lab software (Improvision, Boston, MA). All strains in (C)—(E) were derived from the W303 background [12].

demonstrating that Rtg2 is not required for the expression of these target genes in the absence of Mks1 (Figure 1D, lanes 5 and 7). These expression patterns of *CIT2* and *DLD3* also correlated with the growth properties of the different mutants on selective solid media. Thus, whereas *mks1*Δ, *rtg2*Δ, and *rtg2*Δ *mks1*Δ cells all grew on agar plates containing ammonia and glutamine as nitrogen sources, only *mks1*Δ and *rtg2*Δ *mks1*Δ cells grew on plates that lacked glutamine (Figure 1C). Taken together, these results indicate that, in the absence of Mks1 activity, the *RTG* pathway is expressed constitutively, and Rtg2 becomes dispensable for growth, at least under these experimental conditions. As expected, deletion of *MKS1* failed to suppress the glutamine auxotrophy of either *rtg1*Δ or *rtg3*Δ cells (Figure 1C).

We have shown previously that the *RTG* pathway is controlled in part by regulated nucleocytoplasmic transport of Rtg1 and Rtg3, where these proteins become sequestered in the cytoplasm in the presence of glutamate and/or glutamine [7]. Constitutive expression of *RTG* target genes in *mks1*Δ cells suggested that the Rtg1/Rtg3 complex might be localized in the nucleus even under rich nutrient conditions. To test this prediction directly, we monitored the localization of an Rtg1-GFP fusion protein in *mks1*Δ cells grown in glutamine-containing rich media. Indeed, we observed that Rtg1-GFP was localized primarily in the nucleus in *mks1*Δ cells versus in the cytoplasm in wild-type cells (Figure 1E). We also observed that an Rtg3-GFP fusion protein was localized in the nucleus in *mks1*Δ cells under these same conditions (data not shown). Taken together, these results indicate that Mks1 is involved in retaining the Rtg1/Rtg3 complex in the cytoplasm in response to glutamine availability.

To extend the results of the above expression studies, we determined global transcriptional changes that re-



	WT vs *mks1*Δ Expression Ratio		
	W303	S288c	Σ2000
Lysine Biosynthesis			
LYS1	6.5	8.4	9.2
LYS2	4	4.2	4.1
LYS4	4.9	5.3	3.9
LYS9	21.5	23.2	36.5
LYS12	6.9	7.7	13.8
LYS14	2.8	3.8	4.2
LYS20	11.4	12	16
LYS21	7.3	8.3	9.9
TCA/Glyoxylate Cycles			
CIT1	12.1	8.4	18
IDH1	3.6	5.4	9.4
IDH2	4.7	5	12.5
ACO1	7.2	8.4	15.6
CIT2	20.4	16.1	27.1
PYC1	2	1.7	3.1
Other			
DLD3	15.3	14.3	27.9
DIP5	9.4	1.1	6.2
PDH1	7.3	1.1	14.5
PEX21	3.4	3.2	7
CTP1	6.2	7.7	7.8
ADH3	2.4	3.3	4.5
YJL200C	6.1	5.4	7.9
YGL117W	4.1	2.2	4
YBR147W	3.1	4	3.7
YOR222W	3.1	3	2.6

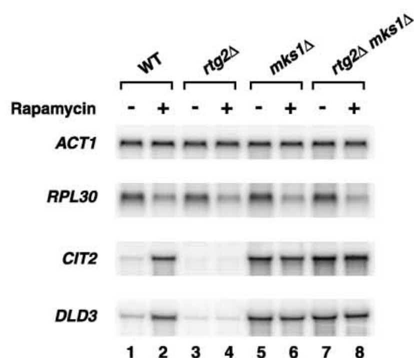
Figure 2. Mks1 Regulates the Activity of a Concise Number of Genes

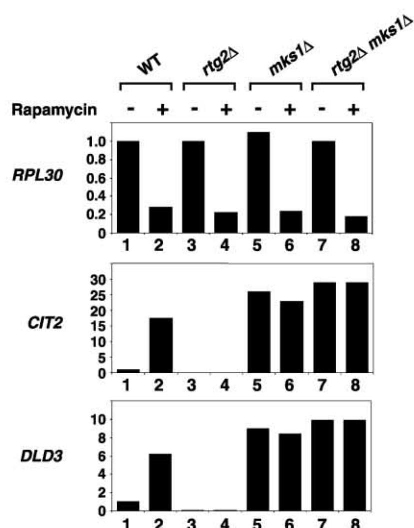
(A) Scatter plot analysis shows genome-wide differences in gene expression between WT and *mks1*Δ cells in the W303 background. The *y* axis depicts cDNA samples derived from WT cells and labeled with Cy3 dye, whereas the *x* axis depicts cDNA samples derived from *mks1*Δ cells and labeled with Cy5 dye. Dashed lines mark the boundaries of mean difference ratios in gene expression equal to 3.0 between the two strains. (B) Genes preferentially expressed in *mks1*Δ cells. Listed genes displayed a mean difference ratio of 3.0 or greater (log10 scale) in two of the three strain backgrounds (an exception is *PYC1*, known to be regulated in part by the *RTG* pathway [7], where the difference in expression in two of the strains is less than 3-fold). Similar results were observed in two independent experiments. (C) Increased expression of *LYS* gene expression in *mks1*Δ cells requires a functional *RTG* pathway. Northern blot analysis of specified mRNAs isolated from strains described in Figure 1D. In (A)–(C), cells were grown to $OD_{600} = 0.5$ in YPD. Preparation of fluorescently labeled cDNA and microarray analysis was performed exactly as described [7].

sulted from the loss of Mks1 function. To this end, DNA microarrays covering >95% of the yeast genome were probed with fluorescently labeled cDNAs prepared from wild-type and *mks1*Δ cells, using each of the three strain backgrounds described above. Of the more than 6200 genes examined, only a very small number of genes displayed significant differences in expression (3-fold or greater), and most were elevated in *mks1*Δ cells (Figures 2A and 2B). In addition to *CIT2* and *DLD3*, this list includes other known *RTG* targets, most notably, genes encoding enzymes associated with the early steps of the TCA cycle (Figure 2B) [7, 11]. Strikingly, genes involved in the lysine biosynthetic pathway were also strongly induced in *mks1*Δ cells (Figure 2B). This result is consistent with the previous identification of *MKS1* as *LYS80*, a negative regulator of lysine biosynthesis [8]. In agreement with our present findings, an analysis of *lys80* cells revealed an increase in intracellular levels of lysine as well as lysine biosynthetic enzymes [8].

We considered two possible explanations as to why genes involved in the *LYS* pathway are induced in *mks1*Δ cells: (1) Mks1 inhibits expression of these genes directly, or (2) expression of these genes is a consequence of constitutive activation of the *RTG* pathway. To distinguish between these possibilities, we used Northern blotting to examine the expression of three *LYS* genes in strains that were deleted for both *MKS1* and *RTG1–3*. Increased expression of these *LYS* genes was observed only under conditions where the *RTG* pathway was constitutively active, namely, in *mks1*Δ and *rtg2*Δ *mks1*Δ cells (Figure 2C). Indeed, the patterns of expression of these genes in the various mutant strains were virtually identical to the patterns observed for *CIT2* and *DLD3* (compare Figures 1D and 2C). Thus, we conclude that increased *LYS* gene expression is a secondary event that follows activation of the *RTG* pathway in cells lacking Mks1. Consistent with this conclusion, *mks1*Δ cells have increased levels of α-ketoglutarate, a product of the *RTG* pathway and an inducer of lysine biosynthesis [8]. In an extension of these results, microarray analysis of the different mutant strains showed that all changes in gene expression observed in *mks1*Δ cells could be attributed to constitutive activation of the *RTG* pathway (our unpublished data).

Having established that Mks1 negatively regulates *RTG* target gene expression, we wanted to determine the relationship between this protein and TOR signaling. Results from two independent lines of experimentation suggest that Mks1 functions as part of the TOR pathway. First, no change in expression of *CIT2* or *DLD3* was observed following addition of rapamycin to *mks1*Δ or *rtg2*Δ *mks1*Δ cells (Figures 3A and 3B, lanes 5 through 8). Such absence of any additive effect on *RTG* target gene expression when *both* TOR and Mks1 function is inhibited is consistent with each component acting within the same pathway. Second, Western blot analysis revealed a reproducible shift in the electrophoretic mobility of an epitope-tagged version of Mks1 ($Mks1\text{-}HA_3$),





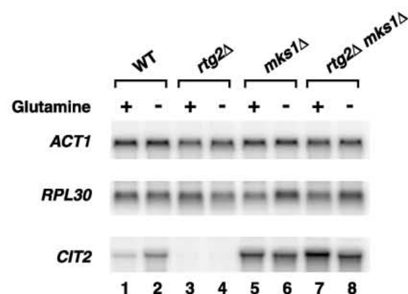


Figure 3. Testing the Relationship between Mks1, TOR Signaling, and Glutamine

(A) Northern blot analysis of specified mRNAs isolated from wild-type and mutant strains, as indicated. Cells were grown in YPD to $OD_{600} = 0.5$ and were treated with rapamycin (0.2 μg/ml final concentration) for 30 min, where indicated. All strains were derived from W303. To control for the effects of rapamycin on the expression of the *RTG* target genes *CIT2* and *DLD3*, the r protein mRNA *RPL30* (formally *RPL32*) was also analyzed, which displays reduced expression in the presence of drug, in agreement with our previous observations [19].

(B) Quantitation of data from (A), where the *y* axes indicate relative mRNA levels, normalized to *ACT1* levels.

(C) Influence of glutamine deprivation on *CIT2* expression in wild-type and mutant strains, as indicated. Cells were grown in YPD to $OD_{600} = 0.5$. Each culture was then divided into two, and cells were then pelleted and resuspended in SCD media that either contained or lacked glutamine, as indicated. Cells were incubated for 30 min, harvested, and then processed for Northern blot analysis, probing for the specified mRNAs.

from a slower to a faster migrating form, following treatment of wild-type cells with rapamycin (Figure 4A, compare lanes 1 and 2). Because this shift to a faster mobility form could be mimicked by addition of phosphatase to cell extracts in vitro (Figure 4B, lane 1), we conclude that Mks1 is a phosphoprotein and, moreover, that its phosphorylation state responds to TOR signaling.

Western blot analysis also revealed that the phosphorylation state of Mks1 is influenced by the Rtg1-Rtg3 proteins (Figure 4A). In extracts prepared from either *rtg1*Δ or *rtg3*Δ cells, only the faster migrating form of Mks1-HA_3 was observed, indicating that Mks1 is constitutively hypophosphorylated in the absence of the Rtg1 or Rtg3 transcription factors (Figure 4A, lanes 3 and 7). In stark contrast, the mobility of Mks1-HA_3 shifted to a much slower and heterogenous form in *rtg2*Δ cells, suggesting Mks1 is hyperphosphorylated in the absence of Rtg2 (Figure 4A, lane 5). Whether these complexities in the pattern of Mks1 phosphorylation reflect physical interactions with one or more of the Rtg1–3 proteins remains to be determined.

We have demonstrated previously that rapamycin treatment mimics removal of glutamine from the growth media, with respect to *RTG* target gene activation [7]. Thus, to extend our present findings, we monitored *CIT2* mRNA levels in wild-type as well as in *rtg2*Δ, *mks1*Δ, and *rtg2*Δ *mks1*Δ cells in media that either contained or lacked glutamine (Figure 3C). Here the overall pattern of *CIT2* expression observed was very similiar to the pattern described above when rapamycin was added to cells grown in rich media (compare Figures 3A and 3C). In particular, high levels of *CIT2* expression was observed in both *mks1*Δ and *rtg2*Δ *mks1*Δ cells in the presence of glutamine; moreover, these levels did not increase following removal of glutamine from the growth media (Figure 3C, compare lanes 5 through 8). Therefore, we conclude that, in the absence of Mks1 activity, *RTG* target gene expression is largely uncoupled from regulation by glutamine. By contrast, wild-type cells showed normal glutamine-dependent inhibition of *CIT2* expression (Figure 3C, compare lanes 1 and 2).

We also wanted to verify the physiological significance of rapamycin-induced changes in the phosphorylation state of Mks1. We reasoned that if dephosphorylation of Mks1 is relevant to the mechanism of *RTG* target gene activation, then a similar effect should be observed when cells are grown in media lacking glutamine. Indeed, we observed a reproducible increase in the mobility of Mks1-HA_3 when wild-type cells were treated with rapamycin or grown in glutamine-deficient media (Figure 4C, compare lanes 1 through 3). Glutamine depletion in combination with rapamycin addition produced an additive effect on the mobility of Mks1-HA_3, confirming that both conditions contribute to Mks1 dephosphorylation (Figure 4C, lane 4).

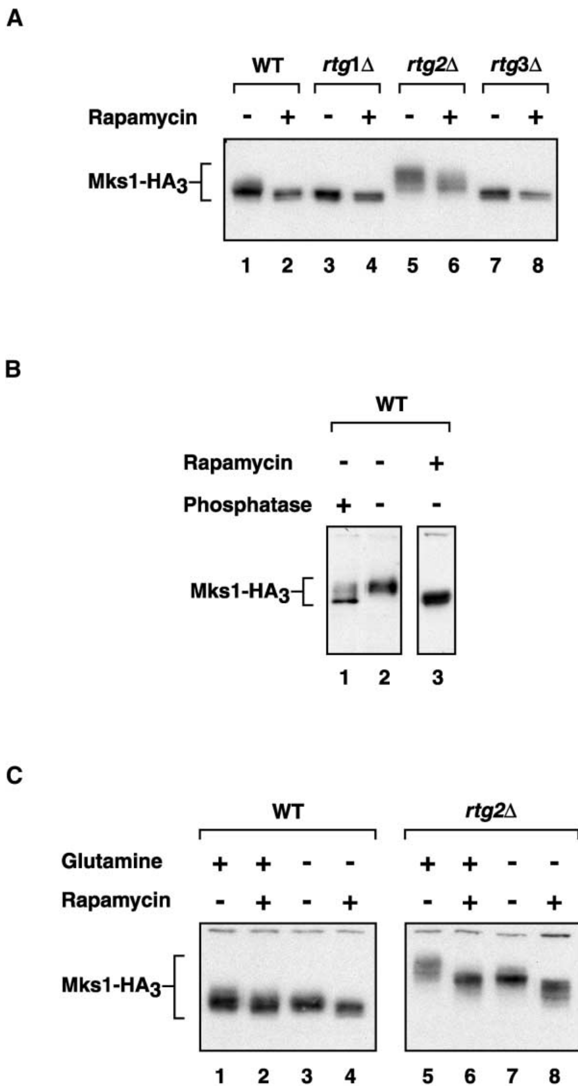


Figure 4. Demonstrating that Mks1 Is a Phosphoprotein Whose Phosphorylation State Responds to TOR Signaling, to Rtg1-Rtg3, and to Glutamine

(A) Wild-type and *rtg1*Δ-*rtg3*Δ strains expressing Mks1-HA_3 were grown in YPD to $OD_{600} = 0.5$ and were treated with rapamycin (0.2 μg/ml final concentration) for 30 min, where indicated. Extracts were prepared, and Western blot analysis was performed as described [7], using anti-HA monoclonal antibody (raw ascites fluid, 16B12, Babco) to detect Mks1-HA_3. Signal was detected using the Renaissance chemiluminescence detection system (New England Nuclear Life Sciences, Boston, MA). Three copies of the coding region for the hemagglutinin (HA) epitope were introduced at the C terminus of *MKS1*, using the PCR-based method described [21]. As template for PCR, plasmid pFA6a-3HA-HIS3MX6 was used [21], which contains the *S. pombe HIS3* homolog.

(B) Control experiment demonstrating that Mks1-HA_3 is a phosphoprotein. Extracts were prepared from wild-type cells and were either mock treated or treated with five units of calf intestinal alkaline phosphatase (CIP; Roche Molecular Biochemicals), according to the instructions of the manufacturer. For comparison, a rapamycin-treated extract was prepared from wild-type cells and loaded on the same gel, as indicated.

(C) Comparing the influence of rapamycin and/or glutamine deprivation on Mks1 phosphorylation. Wild-type and *rtg2*Δ strains expressing Mks1-HA_3 were grown in YPD to $OD_{600} = 0.5$. Each culture was then divided into two, and cells were pelleted and resuspended in SCD media that contained or lacked glutamine, as indicated. Each culture was then divided into two again, and rapamycin was added at a final concentration of 0.2 μg/ml to half of the samples, as indicated. Cells were incubated for 30 min and were processed for Western blot analysis.

Many of the functional interactions we have characterized can be described in terms of a formal pathway where TOR is linked to *RTG* target gene expression through interplay between Rtg2 and Mks1 (Figure 5A). According to this model, the primary function of Rtg2 is to inhibit Mks1, which, in turn, negatively regulates Rtg1 and/or Rtg3, most likely by preventing nuclear localization of the Rtg1/Rtg3 complex. This model can account for why Rtg2 is not essential for expression of *RTG* target genes in the absence of Mks1. Moreover, it can explain why these target genes are not induced when rapamycin is added to *rtg2*Δ cells (Figures 3A and 3B); thus, in the absence of Rtg2, Mks1 becomes a constitutive inhibitor whose activity is uncoupled from control by TOR and by nitrogen availability.

A number of additional observations exist, however, that cannot be reconciled with a simple linear pathway. Most significantly, we observed substantial dephosphorylation of Mks1 in rapamycin-treated and/or glutamine-depleted *rtg2*Δ cells, demonstrating that Mks1 remains responsive to both TOR and nutrient status in the absence of Rtg2 (Figure 4A, lanes 5 through 6, and Figure 4C, lanes 5 through 8). This result is reminiscent of our previously published observation that rapamycin-induced changes in the phosphorylation state of Rtg3 also persist in *rtg2*Δ cells [7]. Indeed, we have found that rapamycin-induced changes in Rtg3 phosphorylation persist in *mks1*Δ cells as well (our unpublished data). Taken together, these data suggest that additional functional interactions exist between TOR and individual components involved in *RTG* target gene regulation (summarized in Figure 5B). Understanding the significance of these interactions will require identifying the sites of phosphorylation in Mks1 and determining their importance to *RTG* target gene regulation.

How might Mks1 function, in mechanistic terms, to regulate *RTG* target gene expression? Clues to an answer come from previous studies where the intracellular localization of Rtg1 and Rtg3 was examined in different mutant backgrounds [7, 16]. In particular, Rtg3 is localized constitutively in the cytoplasm in *rtg2*Δ cells but is instead concentrated in the nucleus in both *rtg1*Δ and *rtg1*Δ *rtg2*Δ cells [7, 16]. These results indicate that Rtg1 plays an important role in holding the Rtg1/Rtg3 complex within the cytoplasm and, moreover, that this is a step regulated by Rtg2. Based on our results presented here, we suggest that Mks1 is also involved in this regulated nuclear transport step. Butow and coworkers have demonstrated that Rtg3 contains the nuclear localization signal (NLS) essential for nuclear import of the Rtg1/Rtg3 complex [16]. Thus, one attractive possibility is that Mks1 controls access to this NLS, for example, by regulating a conformational change within the Rtg1/Rtg3 complex. Alternatively, Mks1 may interact directly with the NLS to prevent access to nuclear transport factors. According to this model, the role of Rtg2 would be

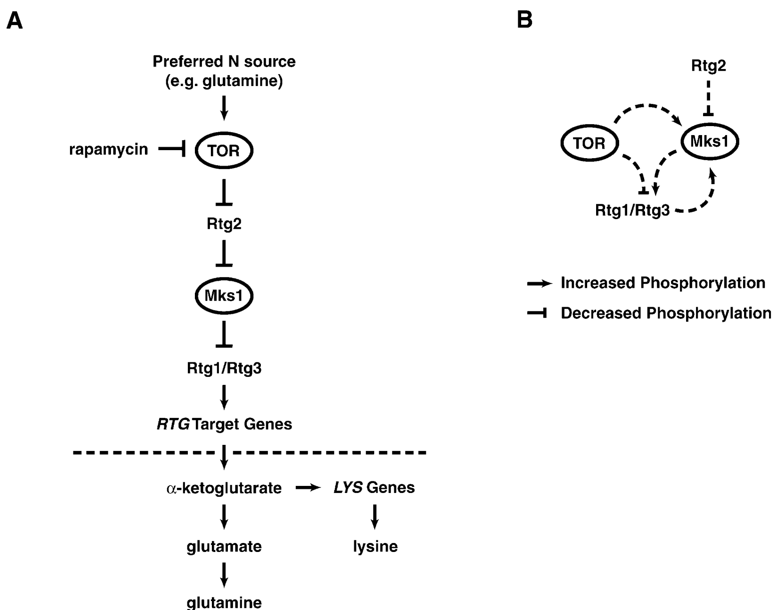


Figure 5. Architecture of the *RTG* Branch of the TOR Pathway

(A) Summary of functional relationships between components involved in *RTG* target gene regulation identified in this study. Inhibition of TOR signaling with rapamycin or loss of Mks1 function results in activation of *RTG* target gene expression. In contrast, loss of Rtg2 function alone results in constitutive inhibition of the pathway. Activation of the *RTG* pathway in *mks1*Δ cells leads to events denoted below the dotted line, including increased production of α-ketoglutarate and subsequent activation of the *LYS* pathway. In this respect, rapamycin treatment is more restricted, in that only events above the dotted line are likely to be affected. The explanation for this is that rapamycin treatment causes widespread changes in cellular physiology, including a sharp decrease in translational initiation [22], which is expected to prevent the actual synthesis of early TCA cycle enzymes.

(B) Relationships between *RTG* regulatory components, inferred from analyses of the phosphorylation states of Mks1 and Rtg3 under different conditions. Data for Mks1 phosphorylation is from Figure 4. Data for Rtg3 phosphorylation is from [7] as well as our unpublished data. We note that, in many cases, TOR-dependent effects appear to be antagonistic to those of other *RTG* regulatory components. The significance of these relationships remains to be determined.

to antagonize interactions between Mks1 and the Rtg1/Rtg3 complex. Such a central role for Mks1 in the regulation of the *RTG* pathway is in agreement with the complexities observed in the phosphorylation state of this protein. Moreover, the limited scope of genes whose expression is affected when Mks1 function is disrupted is consistent with this protein being dedicated primarily if not exclusively to this pathway. The challenge now at hand is to determine the precise molecular mechanism by which Mks1 inhibits the activity of the Rtg1/Rtg3 complex, as well as to understand how Mks1 itself is regulated by upstream events, including TOR signaling.

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