RGA2, a putative Rho-type GTPase-activating protein, is regulated by the transcription factor Tec1 during filamentous growth of Saccharomyces cerevisiae

by

Yukiko Ueno

B. A. Biochemistry
Columbia University, 1993

Submitted to the Department of Biology
in Partial Fulfillment of the Requirements for the Degree of
Master of Science in Biology

at the

Massachusetts Institute of Technology

February 1999

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RGA2, a putative Rho-type GTPase-activating protein, is regulated by the transcription factor Tec1 during filamentous growth of *Saccharomyces cerevisiae*

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ABSTRACT

In the yeast *Saccharomyces cerevisiae*, the transcription factors Tec1 and Ste12 cooperatively regulate pathway-specific transcriptional activation of genes involved in filamentous growth (21). While the regulation of the filamentous growth process has been studied in detail, the nature of its downstream effectors has remained unclear. We therefore searched for potential effector genes by identifying genes that require Tec1 for transcriptional activation with a randomly inserted *Tn3::lacZ* transposon library (3). Using this strategy, we have identified *RGA2*, a novel Rho-type GTPase activating protein (GAP) homolog which is regulated by Tec1. We constructed deletion strains of *rga2* and of its homolog *rga1* and characterized their phenotypes in filamentous growth: the double mutant *rga1 rga2* is synthetically defective for haploid invasive growth, whereas *rga1/rga1* and *rga2/rga2* are hyperactive for diploid filamentous growth.

Thesis Supervisor: Gerald R. Fink
Title: Professor of Biology
INTRODUCTION

In the yeast *Saccharomyces cerevisiae*, elements of the pheromone response MAP kinase pathway are also required for filamentous growth in diploids and invasive growth in haploids (17, 26) (See also Figure 1). Upon starvation for nitrogen in the presence of glucose, diploid cells undergo a dimorphic switch from a yeast form to a filamentous, pseudohyphal form (10, 14, 23). Mutations of a subset of the pheromone pathway signaling components, specifically loss-of-function alleles of *CDC42*, *STE20*, *STE11*, *STE7*, and *STE12*, block the filamentous and invasive growth responses (17, 24, 26). That multiple developmental programs, mating and filamentous/invasive growth, employ parts of a single MAPK cascade raises the question of signaling specificity: how do shared signal transduction components distinguish the different upstream signals and couple them to the correlating transcriptional outputs (13)(14, 24)? One of the determinants for establishing specificity is likely to be at the level of the transcription factor; there must exist some mechanism by which the activation of the proper targets is ensured.

One model is that of combinatorial control: the transcription factor Ste12 associates with pathway-specific transcription factors to enable the transcription of pathway-specific targets. Ste12 has been shown to bind to the DNA sequence termed the pheromone response element (PRE) (6, 7, 12, 32). Ste12, by forming homomultimers or heteromultimers with Mcm1, regulates transcriptional activation of mating-specific genes. On the other hand, Ste12 also interacts via cooperative DNA binding with Tec1 (21, 22). TEC1 was originally isolated as a gene product required for full activation of the yeast retrotransposon Ty1 (15). Loss-of function alleles of TEC1 block filamentous and invasive growth (8) but do not exhibit any mating defects (23). Tec1, a TEA/ATTS family transcription factor, binds to the TEA/ATTS consensus sequence (TCS) (21). Madhani and Fink (1997) showed that Tec1 acts as a filamentous growth pathway-specific partner of Ste12, recruiting Ste12 to filamentation-responsive elements (FRE), which consists of a Tec1-binding site (TCS) and a Ste12-binding site (PRE) (21) (See also Figure 2).
While this regulatory paradigm has been characterized in detail, the identity of the actual filamentous/invasive growth downstream target genes that are regulated by Ste12 and Tec1 has remained unclear. The experimental data are consistent with the model that, upon the activation of the filamentous/invasive growth signaling pathway, Ste12 associates preferentially with Tec1, which recruits Ste12 to filamentation-specific promoters via binding to the FRE (Figure 3): the FREs studied to date are that of a pathway reporter construct (FG(TyA)::lacZ) (24), and that of TEC1 itself (21) (See also Figure 2). The model predicts that there are a collection of effector genes required for filamentous/invasive growth that contain FREs in their regulatory sequences.

To date, the only characterized downstream target gene in filamentous/invasive growth is FLO11 (18, 19) FLO11, required for both filamentous and invasive growth, requires STE12 for its function and responds to the filamentous growth MAPK signaling pathway and also to a cAMP-dependent, PKA-mediated signaling pathway (18, 27, 28). The identification of multiplex signaling pathways in filamentous/invasive growth pathways raises another question of specificity. At least two parallel signaling pathways, the MAPK pathway and the cAMP pathway, as well as three sets of transcription factors, Ste12/Tec1, Phd1, and Sfl1, have been implicated in filamentous/invasive growth (10, 19, 23, 29, 31) As all previously identified regulators of filamentous growth contribute to the transcriptional regulation of FLO11 activation (28), it is presently not clear whether each signaling pathway govern a specific subset of the developmental process, or all signaling pathways converge upon regulation of the same target genes.

We therefore searched for potential downstream effector genes by isolating genes that require Tec1 for its transcriptional activation using a Tn3::lacZ-based transposon library. Identification of Tec1 target genes can verify the FRE regulation model; also, identification of genes in the filamentous growth process that requires Ste12 and Tec1 may help define the subset of the developmental process that is directly governed by the MAPK signaling pathway.
MATERIALS AND METHODS

Yeast Strains, Media, and Growth Conditions.

All yeast strains used in this study are described in Table 1 and are derived from the Σ1278b genetic strain background (17). Standard yeast genetic techniques and growth conditions were used (11). Synthetic low ammonia dextrose media (SLAD) for assaying filamentous growth was prepared as described (10).

Isolation and genetic analysis of Tec1 target genes.

Screen for TOT (Targets of Tec1) genes were performed using the Tn3::lacZ transposon-mutagenized yeast genomic DNA library (3)(4, 30). Strain YM120 (MATa tec1::HIS3 his3 leu2 ura3) carrying plasmid BHM256 (2μTEC1 URA3) was transformed with NotI-cleaved DNA from the yeast genomic library carrying random Tn3::lacZ::LEU2 insertions (3, 23). Approximately 10% of the 85,000 transformants selected on SC -6 medium expressed in-frame lacZ fusions. These transformants were replica-plated to either SC -6 medium to retain the TEC1 plasmid (genotype: TEC1), or to SC -leu +5-FOA medium to select for the loss of the TEC1 plasmid (genotype: tec1); they were subsequently screened for β-galactosidase production as previously described (3). Tec1 target genes (TOT) were identified by screening for transformants with lacZ fusions that turned blue in the TEC1 background but were white in the tec1 background (Figure 4).

Putative TOT strains were retested by transforming BHM258 (CEN TEC1 URA3) into the TOT tec1 background. 43 lacZ fusions that displayed Tec1 dependence for expression by turning blue with CEN-TEC1 were chosen for further analysis. Genomic DNA immediately adjacent to Tn3::lacZ::LEU2 of the TOT strains were isolated as described (3). Sequencing of the flanking genomic DNA revealed that the transposon insertions were placed in a total of 3 ORFs: Ty1 (39 insertions), RGA2 (3 insertions), and YJR129c (1 insertion).

All three Tn3::lacZ::LEU2 insertions in RGA2 were identical and are hereby designated as transposon insertional allele rga2-101 (as per Mosch and Fink, 1997). Tetrad analysis of the cross of rga2-101 with tester strain 10560-1A (MATa his3::hisG leu2::hisG trp1::hisG) showed that LEU2 segregated in a 2:2
pattern and cosegregated with Tec1-dependent β-galactosidase expression, indicating that the rga2-101 strain carries only a single transposon insertion.

**Deletion of RGA1 and RGA2.**

A disruption allele of RGA1 (rga1::URA3) was created by one-step gene replacement of strain L5792 (MATα/α his3::hisG/his3::hisG leu2::hisG/leu2::hisG ura3-52 ura3-52) with HindIII-digested pSL2601 (rga1::URA3) (30) (See also Appendix 3). pSL2601 deletes the amino-terminal third of the RGA1 ORF but displays phenotypes indistinguishable from the full deletion allele in assays by Stevenson et. al (30). The heterozygote, the chromosomal deletion of which was verified by PCR, was dissected to generate MATα and α rga1::URA3 strains.

A deletion construct of RGA2 was created by first amplifying the flanking sequences at the 5′ and 3′ ends of RGA2 ORF by PCR: primers T1K1, 5′-GAGGAGATAAGTCTATATTTTTG-3′, and T1K2, 5′-GGATCCGAAACGCCAAGTATGCAAAGATG-3′, were used to amplify the 5′ flanking fragment corresponding to -828 to -118 nts in respect to the RGA2 coding region; primers T1K3, 5′-GGATCCGAATTATCTGGAATTTTAC-3′, and TIK4, 5′-CGACAAATGCTGCGATGACCCT-3′, were used to amplify the 3′ flanking fragment corresponding to +2968 and +3765 nts. Both flanking fragments contain BamHI sites, introduced by the T1K2 primer and the T1K3 primer, respectively. Both flanking fragments were ligated into the pGEM-T vector (Promega); subsequently a 2.5kb BglII fragment from YEp13 containing the LEU2 ORF was inserted into the BamHI site to generate BYU47 (rga2::LEU2; see Appendix 1 and 2). The orientation of the insertions was verified by restriction mapping. The BYU47 construct removes the entirety of the RGA2 ORF.

A deletion allele of RGA2 (rga2::LEU2) was created by one-step gene replacement of strain L5792 (MATα/α his3::hisG/his3::hisG leu2::hisG/leu2::hisG ura3-52 ura3-52) with NcoI/PstI-digested BYU47 (rga2::LEU2). The heterozygote, the chromosomal deletion of which was verified by PCR, was dissected to generate MATα and α rga2::LEU2 strains. YUY100 (MATα rga1::URA3 his3 leu2) and YUY97 (MATα rga2::LEU2 his3
ura3) were crossed and dissected to generate the tetratype tetrad YUY88 (MATα rga2::LEU2 his3 leu2 ura3), YUY89 (MATa his3 leu2 ura3), YUY90 (MATa rga1::URA3 his3 leu2 ura3), and YUY91 (MATα rga1::URA3 rga2::LEU2 his3 leu2 ura3); these strains were used for the haploid invasive growth assays (See also Appendix 4). Homozygous diploids YUY111 (MATa/α rga1::URA3 rga2::LEU2/rga2::LEU2 his3/his3 leu2/leu2 ura3/ura3), YUY112 (MATa/α rga1::URA3 rga1::URA3 his3/his3 leu2/leu2 ura3/ura3), YUY113 (MATa/α rga2::LEU2 rga2::LEU2 his3/his3 leu2/leu2 ura3/ura3), and congenic wild-type diploid YUY114 (MATα/α his3/his3 leu2/leu2 ura3/ura3) were created by crossing spores generated from the crosses described above.

All the plasmids used and constructed in this study are described in Table 2.

Construction of rga2-102.

Genomic DNA containing the RGA2 ORF was isolated by colony hybridization from the Rose library. The 5.6kb Ncol-Eagl fragment containing RGA2 ORF was ligated into SmaI/NotI-digested pRS316 to generate BYU56 (pRS316-RGA2; see Appendix 5). rga2-102, a promoter mutation allele, was generated with the QuikChange site-directed mutagenesis kit by Stratagene. Primers R2TM25, 5'-TATGAAATTTGGCAAAACCTTGGAGTTTGTTAGCCAGAGATCG -3', and R2TM23, 5'-CGATCTTCTGGCTAACAAACTCCAAGGTTTGCCAAATTTCATA -3', were used to alter the putative Tec1-binding site sequence (TCS) from CATTCY to CAAACY (See Appendix 6).

Haploid invasive growth and diploid filamentous growth assays.

Haploid invasive growth assay (26) and diploid filamentous growth assays (25, 10) were performed as previously described.

Northern Analysis.

Haploid strains were grown in SC complete or SC -ura liquid medium at 30°C to an OD600 of ~0.8. Diploid strains were grown in YNB liquid medium to an OD600 of ~0.8 and grown as a lawn on SLAD plates for 3 days.
Total RNA was harvested, and 20µg total RNA from each strain was analyzed by Northern blotting (1). An approximately 1kb-long PCR product, corresponding to +28 to +974nt of the RGA2 ORF, was used as a probe for RGA2 message; and an approximately 1kb-PCR product, corresponding to -21 to +989 of the RGA1 ORF, was used as a probe for RGA1 message. A 1.4kb PCR product internal to the ACT1 ORF was used as a probe for loading control.
RESULTS

Isolation of TOT (Target-of-Tec1) genes.

To identify potential filamentous growth effector gene that require Tec1 for transcriptional activation, we designed a Target-of-Tec1 (TOT) expression screen using the \textit{Tn3::lacZ} transposon library (3). This transposon-mutagenezied genomic library, carrying random \textit{Tn3::lacZ::LEU2} insertions in the yeast genome, were introduced by integrative transformation into YM120, a haploid (MATa) strain that is \textit{tec1A} in the chromosome and carries a high-copy \textit{TEC1} plasmid. The transformants expressing in-frame \textit{lacZ} fusions were screened for Tec1-dependent expression: transformants were replica-plated to media that selects either for the presence or for the loss of the \textit{TEC1} plasmid. Transformants that scored positive for β-galactosidase production in the \textit{TEC1} background but scored negative in the \textit{tec1} background were interpreted to be carrying \textit{lacZ} fusions that require Tec1 for transcriptional activation and were chosen for further study (see Figure 4 and MATERIALS AND METHODS).

The majority of the 43 retested TOT, or candidate Tec1-target fusions, were retrotransposon Ty1 elements. As \textit{TEC1} was originally isolated as a transcription factor required for Ty1-mediated transcriptional activation(15), and Ty1 elements contain a well-characterized FRE (16, 21), we expected that most of the \textit{lacZ} fusions would be Ty1 elements. However, we also identified \textit{lacZ} fusion in two genes that require Tec1 for expression: \textit{RGA2} and YJR129c. \textit{RGA2} was chosen for further analysis.

The expression of the \textit{lacZ} fusion in \textit{RGA2}, herein referred to as \textit{rga2-101}, is dependent on Tec1 and the filamentous growth MEK Ste7; also, \textit{lacZ} expression of \textit{rga2-101} increases upon increasing copy number of \textit{TEC1} (Figure 5). The \textit{RGA2} promoter sequence does not contain a FRE but contains tandemly-oriented putative Tec1-binding sites (Figure 6). The organization of two tandemly oriented TCS’s is seen in binding sites of other TEA/ATTS family transcription factors, namely that of \textit{abaA} in \textit{Aspergillus nidulans} (22, 1).
Filamentous/invasive growth phenotypes of rga2 and its homolog rga1.

RGA2 has a S. cerevisiae homolog, RGA1, with which it shares very strong sequence similarity (29) (See also Figure 7). Both Rga1 and Rga2 proteins show strong sequence homology to Rho-type GTPase activating proteins (GAPs) at the C-terminus (from aa’s 776-978 in Rga2) (2, 25, 30, 31), and also homology to the LIM domain consensus at the N-terminus (aa’s 13-132 in Rga2) (5, 30). Rga1 was identified as a negative regulator of the pheromone response pathway (30); because of its sequence homology and two-hybrid interaction with activated Cdc42, Rga1 has been proposed to be a Cdc42-GAP (30).

Because its homolog Rga1 has been implicated as a negative regulator and potential Cdc42-GAP of the pheromone response pathway, we hypothesized that perhaps Rga2 acts as a negative regulator and Cdc42-GAP of the filamentous/ invasive growth pathway (Figure 8). To address this possibility, we made deletion or disruption alleles of rga1 and rga2 and assayed for their filamentous/invasive growth phenotypes (See MATERIALS AND METHODS).

Surprisingly, the haploid invasive growth phenotypes suggest that Rga1 and Rga2 do not act as negative regulators, but, to the contrary, have a positive function in invasive growth. The rga1 single mutant is moderately reduced for invasive growth, whereas rga2 shows only a very slight defect (data not shown); the rga1 rga2 double mutant is synthetically defective in invasive growth (Figure 9). On the other hand, the diploid filamentous growth phenotype is consistent with the hypothesis that Rga1 and Rga2 are negative regulator of the process: both rga1/rga1 and rga2/rga2 are hyperfilamentous compared to wild-type (Figure 10); the phenotype is comparable to that of the hypermorphic MEKK allele STE11-4 (data not shown). The double mutant rga1/rga1 rga2/rga2 is not hyperfilamentous but displays some aberrant colony morphology, suggesting defects in bud selection. The phenotypes are summarized in Figure 11.
DISCUSSION / FUTURE EXPERIMENTS

I. Experiments in process.

Site-directed mutations in RGA2.

The most unusual feature of the rga1 and rga2 phenotypes in filamentous/ invasive growth is that the loss-of-function alleles have opposite phenotypes in different cell types (in haploid or in diploid). As Rga1 and Rga2 have homology to two well-characterized domains, one explanation is that perhaps each domain confers differential activity depending on the cell type: for example, the GAP catalytic activity may be required in diploids to act as a negative regulator, whereas the LIM domain, implicated in protein-protein interactions, may be required in haploids to activate the invasive growth process. Site-directed mutants in the LIM domain of RGA1, as haploids, show defects in axial budding (4). We therefore propose that, by constructing site-directed mutants of RGA2 and assaying for their filamentous invasive growth phenotypes, we may be able to identify whether the different domains contribute in the differential function of Rga2 upon differences in cell type.

The schematic representation of site-directed mutations in RGA2 are shown in Figure 12. In the GAP catalytic mutant, R928, corresponding to an arginine residue that is conserved among all GAPs (2, 25), is altered to an alanine (R928A). In the LIM domain mutant, the region corresponding to the LIM consensus sequence is deleted.

The construction of the third site-directed mutant, the TCS mutant, addresses whether the putative Tec1-binding sites (TCS’s) in the RGA2 promoter sequence is necessary for Rga2 function. The TCS sequence, CATTCY, is altered to CAAACY (this promoter mutant allele, rga2-102, has been constructed; see MATERIALS AND METHODS). If indeed Tec1 regulates RGA2 transcriptional activation via these two TCS sites, the site-directed TCS mutant would show a complete loss-of-function phenotype.
Regulation of RGA2.

The transcriptional regulation of RGA2 by Tec1 is inferred from the β-galactosidase activity of rga2-101; the regulation of wild-type RGA2 by Tec1 has not yet been addressed. Also, the question remains whether RGA2 also requires Ste12 for its transcriptional activation. While the RGA2 promoter sequence does not contain an ostensible match for a PRE, MATα-specific genes also do not contain PREs in their promoter sequences but still require Ste12 for their transcriptional activation (32).

Preliminary Northern analysis (Figures 13 and 14; see MATERIALS AND METHODS) does not provide conclusive evidence for either Tec1- or Ste12-dependent regulation of RGA1 and RGA2 transcripts. Hybridization of RGA2 probe with RNA from rga2 mutant suggest that there is significant cross-hybridization with, most likely, RGA1 (data not shown).

Overexpression of RGA2

Rga1 and Rga2, while showing strong sequence homology, seem to have overlapping yet distinct functions in the pheromone response (29). To address whether Rga1 and Rga2 are functionally redundant in the filamentous/invasive growth response, we propose to overexpress RGA2 and ask whether it can compliment the rga1 defect in haploid invasive growth (GAL-RGA2 has been constructed; see Appendix 7). Also, if Rga2 indeed acts as a negative regulator of diploid filamentous growth, the predicted phenotype of RGA2 overexpression is a defect in filamentation.

II. Future experiments.

Regulation by RGA2

If Rga2 can indeed act as a filamentous growth pathway Cdc42-GAP, the induction of RGA2 by Tec1 establishes a negative feedback loop, in which case the filamentous growth MAPK pathway output may be modulated. To address how Rga2 and Rga1 affect the filamentous/invasive growth pathway signaling and output, the activities of the MAPK pathway reporter construct
(FRE::lacZ) and the transcript level of the bona fide filamentous growth effector gene FLO11 can be measured.

**Is Rga2 a Cdc42-GAP?**

Three *S. cerevisiae* genes, Bem3, Rga1, and Rga2, have been proposed to act as Cdc42-GAPs (29). Of the three, the GTPase-activating protein activity of Bem3 has been demonstrated in vitro with HsCdc42 (33). The claim that Rga1 and Rga2 are Cdc42-GAPs is based on genetic, two-hybrid, or sequence homology evidence (29, 30). An in vitro Cdc42 GTPase assay using labeled GTP would establish unequivocally whether Rga2 is indeed a Cdc42-GAP.
15. Laloux, I., E. Dubois, M. Dewerchin, and E. Jacobs. 1990. TEC1, a gene involved in the activation of Ty1 and Ty1-mediated gene expression in
Figure 1. The pheromone response and filamentous / invasive growth pathways in *Saccharomyces cerevisiae* utilize an overlapping set of signaling components (Cdc42, Ste20, Ste11, and Ste7) and transcription factor (Ste12) (I).
1. **FRE-lacZ reporter**

FRE is an upstream regulatory sequence that confers filamentous-growth pathway-specific transcription (TCS, Stel2-binding site and a TCS, Tec1-binding site) in flexible spacing and orientation. FREs of the filamentous growth reporter construct FG(TyA::lacZ) and TEC1, characterized in Madhani and Fink (1997), are shown.

2. **TEC1**

Figure 2. Filamentation-responsive elements (FRE). FRE is an upstream regulatory sequence that confers filamentous-growth pathway-specific transcription (TCS, Ste12-binding site and a TCS, Tec1-binding site) in flexible spacing and orientation. FREs of the filamentous growth reporter construct FG(TyA::lacZ) and TEC1, characterized in Madhani and Fink (1997), are shown.
Figure 3. Searching for downstream target genes that are regulated by Ste12 and Tec1.
Haploid *tec1Δ* strain with *TEC1* on high-copy plasmid 

(MATa *tec1::HIS3 his3 ura3 leu2 <2µ TEC1-URA3>)

Introduce random *lacZ* insertions with transposon library

(*Tn3::lacZ, LEU2*)

Retain plasmid; *TEC1*  
(SC - 6)  

Select for plasmid loss; *tec1Δ*  
(SC - leu + 5-FOA)

Blue  
β-galactosidase expression  
(assayed on filters, SC -leu)  

White

Figure 4. Scheme of Target-of-Tec1 (TOT) screen.
Figure 5.

A.  
lacZ expression of rga2-101 is dependent on Tec1 and MEK Ste7.  
Haploid strains YUY42 (rga2-101), YUY43 (rga2-101 tec1::HIS3), and (rga2-101 ste7::URA3) were patched onto a SC complete plate; filter lacZ assays were performed as previously described.

B.  
lacZ expression of rga2-101 in varying copy numbers of TEC1.  
Haploid strains YUY60 (rga2-101 tec1::HIS3), YUY58 (rga2-101), YUY62 (rga2-101 tec1::HIS3 <CEN-TEC1>), YUY64 (rga2-101 tec1::HIS3 2μ-TEC1), and diploid strains YUY46 (rga2-101/rga2-101) and YUY47 (rga2-101/rga2-101 tec1::HIS3/tec1::HIS3) were patched onto SC -ura plates; filter lacZ assays were performed as in part A.  

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Figure 6. The RGA2 promoter sequence contains tandemly oriented Tec1-binding sites. From -185 to -170 (in relation to RGA2 ATG as +1) the sequences CATTCC and AGATTG, placed 4 basepairs apart, are both perfect matches to the consensus Tec1-binding sequence (TCS): CATTCCY (Y=pyrimidine).
Figure 7. Alignment of Rga2 and Rga1; Rga2 protein sequence is shown on top, and Rga1 protein sequence on bottom. Identities are represented in solid black boxes, and similarities are shown in shaded boxes.

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Figure 8. Rga2 may function as a negative regulator of Cdc42 in the filamentous growth pathway. Its homolog Rga1 has been demonstrated to be a negative regulator and putative Cdc42 GAP for the pheromone response pathway.
Figure 9. Haploid invasive growth phenotypes. Haploid strains YUY89 (wild-type), YUY91 (rga1::URA3), YUY88 (rga2::LEU2), and YUY91 (rga1::URA3, rga2::LEU2) were patched onto a YPD plate and grown at 30°C for 3 days and at room temperature for 1 day. The plates were photographed before and after gentle washing of cells on the surface of the agar.
Figure 10. Filamentous growth phenotypes. Homozygous diploid strains YUY114 (wild-type), YUY112 (rga1::URA3/rga1::URA3), YUY113 (rga2::LEU2/rga2::LEU2), YUY111 (rga1::URA3/rga1::URA3 rga2::LEU2/rga2::LEU2) were streaked onto SLAD plates and grown at 30°C for 6 days. The colonies were photographed before and after washing of the cells on the surface of the agar.
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<td>+/-</td>
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<td>++</td>
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Figure 11. Summary of the phenotypes of *rga1*, *rga2*, and *rga1* *rga2* mutants in invasive and filamentous growth. The phenotypes imply that Rga1 and Rga2 have: a positive function in invasive growth; and a negative regulatory function in filamentous growth.
1. wild-type *RGA2*

**RGA2 promoter:**

Tec-1-binding Sites (TCS)

**RGA2 gene:**

"LIM domain" catalytic GAP domain

2. TCS mutant

**CATTCCY -> CAAACY**

Tec-1-binding Sites (TCS)

"LIM domain" catalytic GAP domain

3. GAP catalytic mutant

R928A

Tec-1-binding Sites (TCS)

"LIM domain" catalytic GAP domain

4. "LIM domain" mutant

(deletion)

Tec-1-binding Sites (TCS)

"LIM domain" catalytic GAP domain

Figure 12. Schematic representation of site-directed mutants in *RGA2.*
Figure 13. RGA2 transcript levels.

A. MATa cells: 10560-2B (his3::hisG leu2::hisG ura3-52), YM120 (MATa tec1::HIS3 his3 leu2 ura3), L5793 (ste12::LEU2 his3 leu2 ura3), L5968 (ste7::URA3 his3 leu2 ura3), and YUY69 (tec1::HIS3 his3 leu2 ura3 <2μTEC1 URA3>), were grown in SC complete to OD600 ~0.8.

B. MATa/α cells: L5437 (ura3-52/ura3-52 <URA3>), YUY68 (tec1::HIS3/tec1::HIS3 his3/his3 ura3/ura3 <URA3>), L5533 (ura3-52/ura3-52 <STE11-4 URA3>) were grown on SLAD plates, 3 days.
Figure 14. RGA1 Transcript Levels.
MATα/α cells: L5437 (ura3-52/ura3-52 <URA3>), YUY68 (tec1::HIS3/tec1::HIS3 his3/his3 ura3/ura3 <URA3>), L5533 (ura3-52/ura3-52 <STE11-4 URA3>) were grown on SLAD plates for 3 days.
### Table 1. *S. cerevisiae* strains

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<tr>
<td>L5437</td>
<td>MATα/α ura3-52/ura3-52 &lt;pRS316&gt;</td>
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Source: H. Madhani, Fink lab collection, This study
Table 2. Plasmids.

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<th>Plasmid</th>
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<td>BHM258</td>
<td>TEC1  CEN URA3  AmpR pUCori</td>
<td>H. Madhani</td>
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<tr>
<td>BHM256</td>
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<td>rga1::LEU2  AmpR pBRori</td>
<td>George Sprague, Jr.</td>
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<td>pRS315</td>
<td>LEU2 CEN  AmpR pUCori</td>
<td>Sikorski and Hieter</td>
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<td>pRS316</td>
<td>URA3 CEN  AmpR pUCori</td>
<td>Sikorski and Hieter</td>
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