Stb3 Plays a Role in the Glucose-Induced Transition from Quiescence to Growth in *Saccharomyces cerevisiae*

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ABSTRACT

Addition of glucose to quiescent *Saccharomyces cerevisiae* cells causes the immediate induction of ~1000 genes. These genes include ribosomal proteins (RP) and non-RP genes needed for ribosome production and other growth processes. RRPE sequence elements are commonly found 5' of non-RP growth gene ORFs, and Stb3 has recently been identified as an RRPE binding protein. Stb3 overexpression (Stb3OE) produces a slow growth phenotype that is associated with reduced expression of non-RP genes and a drop in the rate of amino acid incorporation. Genes affected by Stb3 are associated with a TGAAAAA motif. Stb3 is restricted to the nucleus in quiescent cells and is immediately released into the cytoplasm after glucose repletion. The Stb3OE slow growth phenotype is reversed by loss of Hos2 histone deacetylase activity, consistent with the idea that repression involves histone deacetylation. *SCH9* overexpression or *PPH22* deletion, mutations that activate target of rapamycin (Tor) nutrient sensing pathways, also reverse the Stb3OE phenotype. Inhibition of Tor signaling makes the phenotype more severe and restricts Stb3 to the nucleus. The results support a model in which Stb3 is one of the components that repress a large set of growth genes as nutrients are depleted. This repression is ended by glucose.

GLUCOSE is the preferred carbon and energy source for the yeast, *Saccharomyces cerevisiae*. Multiple systems for sensing environmental glucose allow yeast to quickly adapt to glucose depletion and glucose repletion (JAGADISH and CARTER 1977; SCHNEPER *et al.* 2004). Addition of glucose to quiescent *S. cerevisiae* cells alters the expression of almost a third of the genome in only a few minutes (MARTINEZ *et al.* 2004; WANG *et al.* 2004; RADONJIC *et al.* 2005; SLATTERY and HEIDEMAN 2007). This includes the downregulation of ~1000 genes and the upregulation of ~1000 genes by twofold or more (JORGENSEN *et al.* 2004; WANG *et al.* 2004; SLATTERY and HEIDEMAN 2007).

The majority of the glucose-induced genes are involved in protein synthesis and growth. Of the set of ~1000 upregulated genes, a subset of 116 genes encodes the ribosomal proteins (RPs), 571 genes encode ribosome biogenesis genes (often referred to as RiBi genes), and the substantial remainder are involved in other aspects of protein translation and cellular growth (WANG *et al.* 2004; SLATTERY and HEIDEMAN 2007; ZAMAN *et al.* 2008). The regulation of RP genes has been well studied, and it is thought that RP genes are

regulated by a mechanism that is distinct from the regulation of the other growth genes induced by glucose (SHORE 1994; JORGENSEN *et al.* 2004; MARTIN *et al.* 2004; RUDRA *et al.* 2005; SANTANGELO 2006). It is therefore convenient to divide this set of 1000 glucose-induced genes into the RP and non-RP growth genes.

RP genes are regulated by the Rap1 transcription factor. In addition, RP gene regulation also requires the transcription factor Fhl1 and the opposing activities of the coactivator Ifh1 and the corepressor Crf1 (MARTIN *et al.* 2004; SCHAWALDER *et al.* 2004; RUDRA *et al.* 2005). The transcription factors Hmo1 and Sfp1 also bind to the promoters of RP genes, and both proteins positively regulate the binding of Fhl1/Ifh1 to RP promoters (SHORE 1994; JORGENSEN *et al.* 2004; WADE *et al.* 2004; RUDRA *et al.* 2005; HALL *et al.* 2006; SANTANGELO 2006). An additional level of control is provided by the Rap1 cofactor Gcr1, which appears to regulate the nuclear location and, consequently, the expression of RP genes (SANTANGELO 2006).

In contrast, the non-RP growth genes do not appear to have binding sites for Rap1 or Fhl1/Ifh1. Instead, computational work has identified two motifs, RRPE and PAC elements, upstream of the non-RP growth genes (FINGERMAN *et al.* 2003; WANG *et al.* 2004; SLATTERY and HEIDEMAN 2007). The PAC (polymerase A and C) element (GATGAG), was first identified upstream of genes encoding protein subunits of RNA polymerase I and III (DEQUARD-CHABLAT *et al.* 1991); the RRPE (rRNA

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processing element; AAAWTTTT), is highly overrepresented upstream of genes involved in ribosomal RNA processing (HUGHES *et al.* 2000). RRPEs are able to confer glucose regulation when linked to a reporter gene (FINGERMAN *et al.* 2003; SLATTERY and HEIDEMAN 2007). We recently showed that the Stb3 protein binds specifically to RRPEs (LIKO *et al.* 2007).

Nutrient sensing pathways: Two growth-signaling pathways, PKA and TOR, are responsible for most of the immediate transcriptional response to glucose; transport and metabolism of glucose also produces signals, but these play a much smaller part in the overall transcriptional response to glucose response (WANG *et al.* 2004; SLATTERY *et al.* 2008).

Glucose activation of the cAMP/PKA pathway promotes protein synthesis and cell division while repressing stress responses (SCHNEPER *et al.* 2004; WANG *et al.* 2004). Upstream of PKA lies Gpr1, a G-protein-coupled receptor that senses glucose. This activates the Ras-dependent adenylyl cyclase to produce cAMP production that in turn stimulates the activity of protein kinase A (PKA) (MATSUMOTO *et al.* 1982; BOUTELET *et al.* 1985; YUN *et al.* 1997; SANTANGELO 2006).

Another glucose sensing pathway is the target of rapamycin (Tor) pathway. The Tor pathway plays a role in both nitrogen and glucose signaling. The Tor1 and Tor2 kinases are members of the PIK-related family of kinases (LOEWITH *et al.* 2002; WEDAMAN *et al.* 2003; WULLSCHLEGER *et al.* 2006). Tor1 is associated with the TORC1 protein complex that controls several nutrient responses including nitrogen catabolite repression (NCR), the retrograde response (RTG), and the general amino acid control response (GAAC) (CRESPO and HALL 2002; HINNEBUSCH 2005; ROOSEN *et al.* 2005). Inhibition of Tor by rapamycin produces a starvation phenotype (JORGENSEN *et al.* 2004; MARTIN *et al.* 2004; ZURITA-MARTINEZ and CARDENAS 2005).

Downstream effectors of TORC1 include Sch9, an ACG family protein kinase (JIANG and BROACH 1999; URBAN *et al.* 2007). TORC1 phosphorylation and activation of Sch9 promotes induction of ribosome biogenesis genes, repression of stress genes, and degradation of the growth inhibitory kinase Rim15 (SWINNEN *et al.* 2006; URBAN *et al.* 2007). Sch9 overexpression can bypass mutations that block PKA activity (TODA *et al.* 1988).

In *S. cerevisiae*, activation of Tor1 causes phosphorylation of Tap42. The phosphorylated Tap42 in turn binds to and inactivates the Pph21 and Pph22 catalytic subunits of yeast PP2A phosphatases. This Tor-dependent inhibition of PP2A activity opposes the dephosphorylation and activation of Maf1, a negative regulator of RNA polymerase III. Thus the Tor pathway promotes events that favor protein translation and inhibits responses normally induced by stress (SHAMJI *et al.* 2000; CHERKASOVA and HINNEBUSCH 2003; DUVEL *et al.* 2003; SANTHANAM *et al.* 2004; LEE *et al.* 2009).

Gene regulation: *S. cerevisiae* has five class I histone deacetylases (HDACs): Rpd3, Hda1, Hos1, Hos2, and Hos3. HDAC activity is generally associated with gene repression (KURDISTANI and GRUNSTEIN 2003), and HDAC activity is thought to play an important role in the repression of growth-promoting genes during quiescence (TSANG *et al.* 2003; HUMPHREY *et al.* 2004). While the different HDACs affect overlapping gene sets, the Hos2 deacetylase is thought to be involved in controlling the RP and non-RP growth genes (PIJNAPPEL *et al.* 2001; ROBYR *et al.* 2002; WANG *et al.* 2002).

In this report we further characterize Stb3 using overexpression of the wild-type (WT) allele of *STB3*. This produces a slow growth phenotype associated with reduced protein synthesis and decreased expression of glucose-induced genes. Pursuing a model in which Stb3 acts to repress growth genes during quiescence, we find that overexpression of Sch9 rescues the growth phenotype caused by Stb3OE; deletion of *SCH9* exacerbates the slow growth phenotype. In addition, loss of the Hos2 HDAC restores normal growth in Stb3OE cells. These results and others support a model in which Stb3 is confined to the nucleus to repress a set of growth genes during post-log phase and is released from the chromatin when glucose is added.

MATERIALS AND METHODS

Yeast strains, plasmids, and growth conditions: Cells were grown in either YPD, (1% yeast extract, 2% bacto peptone, and 2% glucose), synthetic complete medium (SC) containing 6.7 g/liter yeast nitrogen base (Difco) supplemented with adenine, uracil, and amino acids, with 2% glucose or 2% galactose. Strains used in this study are listed in Table 1.

Incorporation of [³⁵S]methionine: Determination of the rate of [³⁵S]methionine incorporation into protein was as previously described (BOUCHERIE 1985). Triplicate 250-μl samples containing equal numbers of cells for each time point were labeled in microfuge tubes at 30° with 5 μCi of Trans³⁵S-Label (Amersham) for 2 min. Incorporation was stopped with ice-cold 5% final concentration of trichloroacetic acid (TCA). Incorporation was determined to be linear throughout the 2-min assay.

Construction of plasmids: Plasmid pYES 2.1 (Invitrogen) was used to overexpress Stb3. To make a plasmid overexpressing Stb3 (pDL10), the Stb3 ORF was amplified from BY4742 using *Pfu* polymerase (Invitrogen), per manufacturer's instructions, and primers 5'-TGT AAT ACG ACT CAC TAT AGG GAA TAT TAA GCT CGC CCT TAT GTC AGA AAA CCA AAA GGA G-3' upstream and 5'-GGC TTA CCT TCG AAT GGG TGA CCT CGA AGC TCG CCC TTT TAA GAT TTT AAG CTC ATT AAT-3' downstream. These primers contain a 40-bp overlap with the pYES2.1 plasmid for insertion via homologous recombination. To make a plasmid overexpressing TAP-tagged Stb3 (pDL11), the ORF containing STB3-TAP was amplified from the strain YDR169C-TAP using primers 5'-TGT AAT ACG ACT CAC TAT AGG GAA TAT TAA GCT CGC CCT TATG TCA GAA AAC CAA AAG GAG-3' upstream and 5'-GGC TTA CCT TCG AAT GGG TGA CCT CGA AGC TCG CCC TTT CAC TGA TGA TTC GCG TCT AC-3' downstream. Both plasmids were confirmed via sequencing.

The plasmid pDG 108, overexpressing Stb3 from the *TEF2* promoter, was constructed by PCR amplification of the *STB3*

TABLE 1
Strains used in this study

Strain	Genotype	Source or reference
BY4742	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0</i>	Invitrogen
DLY10	BY4742 carrying pDL10 (<i>GAL-STB3</i>)	This study
DLY11	BY4742 carrying pDL11 (<i>GAL-STB-TAP</i>)	This study
DLY12	BY4742 carrying pYes 2.1/lacZ (<i>GAL-lacZ</i>)	This study
DGY108	BY4742 carrying pDG108 (<i>TEF2-STB3</i>)	This study
DGY109	BY4742 carrying pDG109 (<i>TEF2-lacZ</i>)	This study
W303	<i>MATα ade2 can1 his3 leu2 trp1 ura3-52</i>	D. Stillman
DLY13	W303 carrying pDL10 (<i>GAL-STB3</i>)	This study
DLY14	W303 carrying pYes2.1/lacZ	This study
DY150	W303 <i>rpd3Δ::LEU2</i>	D. Stillman
DLY27	DY150 carrying pDL10 (<i>GAL-STB3</i>)	This study
DLY28	DY150 carrying pYes2.1/lacZ	This study
15347	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 hda1Δ::KanMX</i>	Invitrogen
DLY25	15347 carrying pDL10 (<i>GAL-STB3</i>)	This study
DLY26	15347 carrying pYes2.1/lacZ	This study
15487	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 hos1Δ::KanMX</i>	Invitrogen
DLY23	15487 carrying pDL10 (<i>GAL-STB3</i>)	This study
DLY24	15487 carrying pYes2.1/lacZ	This study
14561	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 hos2Δ::KanMX</i>	Invitrogen
DLY29	14561 carrying pDL10 (<i>GAL-STB3</i>)	This study
DLY30	14561 carrying pYes2.1/lacZ	This study
MCY690	<i>MATα his3-11,15 leu2-3,112 lys1 lys2 ura3-52 trp1Δhos2::KanMX</i>	This study
MCY669	MCY690 with pDG109 (<i>TEF2-lacZ</i>) and pRS414	This study
MCY670	MCY690 with pDG108 (<i>TEF2-STB3</i>) and pRS414	This study
MCY671	MCY690 with pDG109 (<i>TEF2-lacZ</i>) and pRS414- <i>HOS2</i>	This study
MCY680	MCY690 with pDG108 (<i>TEF2-STB3</i>) and pRS414- <i>HOS2</i>	This study
MCY681	MCY690 with pDG109 (<i>TEF2-lacZ</i>) and pRS414- <i>hos2</i> (H195,196A) WANG <i>et al.</i> (2002)	This study
MCY682	MCY690 with pDG108 (<i>TEF2-STB3</i>) and pRS414- <i>hos2</i> (H195,196A) WANG <i>et al.</i> (2002)	This study
12136	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 hos3Δ::KanMX</i>	Invitrogen
DLY31	12136 carrying pDL10 (<i>GAL-STB3</i>)	This study
DLY32	12136 carrying pYes2.1/lacZ	This study
yMT2129	<i>MATα ade2 can1 his3 leu2 trp1ura3-52 sch9::kanR-GAL1-SCH9</i>	JORGENSEN <i>et al.</i> (2004)
DLY17	yMT2129 carrying pDL10 (<i>GAL-STB3</i>)	This study
DLY18	yMT2129 carrying pYes2.1/lacZ	This study
13886	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 pph22Δ::KanMX</i>	Invitrogen
DLY691	13886 carrying pDL10 (<i>GAL-STB3</i>)	
DLY692	13886 carrying pYes2.1/lacZ	
yMT2422	<i>MATα ade2 can1 his3 leu2 trp1ura3-52 sfp1::kanR-GAL1-SFP1</i>	JORGENSEN <i>et al.</i> (2004)
DLY15	yMT2422 carrying pDL10 (<i>GAL-STB3</i>)	This study
DLY16	yMT2422 carrying pYes2.1/lacZ	This study
KMY6	<i>MATα ade2 can1 his3 leu2 trp1ura3-52 sch9Δ::HIS3</i>	MORANO and THIELE (1999)
DLY19	KMY6 carrying pDL10 (<i>GAL-STB3</i>)	This study
DLY20	KMY6 carrying pYes2.1/lacZ	This study
15312	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 sfp1Δ::KanMX</i>	Invitrogen
DLY21	15312 carrying pDL10	This study
DLY22	15312 carrying pYes2.1/lacZ	This study
YDR169C-TAP	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 HIS3::STB3-TAP</i>	Open Biosystems
95700	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 met15Δ0 ura3Δ0 STB3-GFP</i>	Invitrogen
DS10	<i>MATα his3-11,15 leu2-3,112 lys1 lys2 ura3-52 trp1Δ</i>	BOORSTEIN and CRAIG (1990)
MCY689	DS10 carrying pMC1205 (<i>Stb3-GFP::TRP1</i>)	
TW60	<i>MATα leu2-3,112 ura3-52 trp1-1 his3-532 his4 cyr1Δ0ura3Δ0 cam</i>	RUSSELL <i>et al.</i> (1993)
MCY688	TW60 carrying pMC1204 (<i>Stb3-GFP::URA3</i>)	This study

ORF and inserting this into a pRS425 plasmid into which the *TEF2* promoter and terminator sequences had been cloned (a gift from Trey Sato). The *Stb3* ORF was inserted downstream of the *TEF2* promoter and upstream of the terminator by homologous recombination. Upstream primer: 5'-GCT AGG ATA CAG TTC TCA CAT CAC ATC CGA ACA TAA ACA ACC CCG GAT CGG ACT ACT AGC-3'. Downstream primer: GAC AAG TTC TTG AAA ACA AGA ATC TTT TTA TTG TCA GTA CGA GGG CGT GAA TGT AAG CGT. The control plasmid pDG109 was constructed in an identical manner, amplifying *lacZ* and inserting into the pRS245-*TEF2* plasmid.

Plasmid pMC1204, expressing GFP-tagged *Stb3*, was constructed by PCR amplification of the region encompassing *STB3* upstream sequence, the *STB3* ORF, the in-frame GFP tag, and the *ADH1* terminator sequences using chromosomal DNA from Invitrogen's *Stb3*-GFP-tagged strain (catalog no. 95700, ORF YDR169C) as the template. Upstream primer: 5'-GCG AAT TGG GTA CCG GGC CCC CCC TCG AGG TCG ACG GTA TAA TAC GGC CAA AAT TAT GAA-3'. Downstream primer: 5'-AGC TCC ACC GCG GTG GCG GCC GCT CTA GAA CTA GTG GAT CGG CCG GTA GAG GTG TGG TCA-3'. This fragment was cloned by homologous recombination into pRS416 (*URA3*) cut with *Clal* and *Bam*HI. Plasmid pMC1205 was made in the identical fashion except the fragment was inserted into pRS414 (*TRP1*).

Hos2 HDAC inactive strain: Plasmids expressing the mutant and wild-type form of *Hos2* (a generous gift from M. Grunstein) were transformed into strain 14561, in which *HOS2* has been deleted.

Protein preparation and Western blotting: Cellular lysates were made as described (MILLER and CROSS 2000). Western blotting was performed as described previously (HALL *et al.* 1998). Anti-TAP antibody (Open Biosystems) was used as per manufacturer's instructions.

Flow cytometry: Cells were fixed in 70% ethanol, treated with 0.25 mg/ml RNase A in 50 mM sodium citrate, and stained with 2 mM SYTOX green (Molecular Probes) in 50 mM sodium citrate. Flow cytometry was performed using a Becton Dickinson FACScan.

Fluorescence microscopy: Fluorescence microscopy was performed with a Zeiss Axioplan 2 microscope with a $\times 63$ oil immersion objective with differential interference contrast (DIC) as described previously (LAABS *et al.* 2003). DAPI was used to stain the nuclei at a 0.01 μ g/ml concentration.

Labeled cRNA preparation and microarray hybridization: cDNA and labeled cRNA were generated from total yeast RNA using the GeneChip One-Cycle Target Labeling kit (Affymetrix) according to the manufacturer's protocol. First strand cDNA was generated using a T7-Oligo(dT) primer and SuperScript II reverse transcriptase. Second strand cDNA synthesis was performed using *Escherichia coli* DNA ligase, *E. coli* DNA polymerase I, and RNase H, followed by incubation with T4 DNA polymerase. After cleanup of cDNA, biotin-labeled antisense cRNA was generated using the IVT labeling kit. Cleanup and fragmentation of labeled cRNA were performed using the GeneChip Sample Cleanup Module. Labeled cRNA was then mixed with hybridization controls and hybridized to a Yeast Genome 2.0 Array (Affymetrix) at 45° with rotation (60 rpm) for 16 hr. Microarrays were then washed and stained with streptavidin-phycoerythrin using a GeneChip Fluidics Station 400.

Microarray data analysis: Affymetrix Yeast Genome 2.0 arrays were scanned using an Agilent GeneArray Scanner and Microarray Suite 5.0. The MAS generated .CEL files were then analyzed using DCHIP 1.3 (LI and WONG 2001). Intensity values were normalized across 12 independent microarrays using DCHIP's invariant set normalization method (LI and WONG 2003). Model-based analysis, including log₂ transfor-

mation of expression indices using the perfect match only model, was performed using values from two independent microarray experiments for each time point/condition. Overrepresented DNA motifs were extracted using the oligonucleotide analysis pattern discovery program in Regulatory Sequence Analysis Tools (VAN HELDEN 2003).

Viability test: To determine chronological lifespan, cells were diluted to early exponential phase in S-galactose medium. Cells were grown at 30° with shaking without replacing the growth medium throughout the experiment. At intervals throughout the growth period equal numbers of cells, as measured with a Coulter Counter Model Z2 using a 70-mm aperture, were plated on YPD agar media. Colonies were counted and plotted as percentages of total number of plated cells. Each experiment was performed in triplicate. Error bars show standard deviation.

Growth assays: Strains were grown overnight and diluted to 0.25 OD₆₀₀. Serial dilution of strains (10⁴, 10³, 10², and 10) were plated in the appropriate medium and grown for 2–3 days at 30°.

RESULTS

Overexpression of *Stb3* slows growth: Despite the fact that RRPEs are expected to play an important role in growth, deletion of *Stb3* produces no obvious growth phenotype. This could be explained if the loss of *Stb3* is compensated for by the actions of redundant components. However, while redundancy can mask the absence of a protein, redundancy cannot mask the effects of its overabundance. We tested this by overexpressing *Stb3* from the *GAL1* promoter on the pYES2.1 vector. In our hands this vector produces low but measurable transcription in cells grown on glucose, with rapid induction of high-level expression in galactose (Figure 1A).

Stb3 overexpression (*Stb3OE*) produced a marked growth defect that was not observed under noninducing conditions in glucose (Figure 1B). A control strain expressing the bacterial *lacZ* gene from the same plasmid grew normally on both glucose and galactose. We observed similar results when *Stb3* was overexpressed from the *TEF2* (Figure 1C) and *TDH3* promoters (not shown).

Stb3 binds to the RRPEs associated with non-RP growth genes. These genes are repressed during quiescence and induced by glucose depletion. Therefore, it is possible that *Stb3* normally represses growth genes during post-log quiescence. In such a case, *Stb3OE* might slow growth by continuing to turn down genes needed for protein translation even when a fermentable carbon source such as galactose is present. This would be expected to reduce protein production and slow growth. However, we also considered that the slow growth of *Stb3OE* cells could be due to a cell cycle defect or a morphological problem. *Stb3OE* might also reduce cell viability, slowing growth of the culture as a whole.

Microscopic examination of *Stb3OE* cells ruled out obvious effects on cell shape or morphology compared to controls (Figure 2A). We saw no evidence of cell lysis, nor did we see aberrations in the forms of growing or dividing cells.

FIGURE 1.—Stb3 overexpression slows cellular growth. Yeast cells expressing TAP-tagged Stb3 driven from the *GAL* promoter on pYES2.1 (DLY11) and control cells expressing *lacZ* from pYES2.1 (DLY12) were compared. (A) Cells were grown in YPD and switched to YPGal medium at time 0. Samples were collected at the indicated times for Western blotting with an anti-TAP antibody. The arrowhead indicates the Stb3-specific band. (B) Cells were plated from serial dilutions in 10- μ l drops at the indicated numbers on either SD or SGal medium. (C) Cells carrying plasmids expressing either *STB3* from the *TEF2* promoter (DGY108) or *lacZ* (DGY109) as a control were plated onto SD dropout medium.

Flow cytometry experiments showed normal distributions of cycling cells when grown on galactose (Figure 2B). Both Stb3OE and control strains had roughly equivalent G1 and G2/M peaks, indicating movement through all phases of the cell cycle. This shows that the growth retardation caused by Stb3OE is not caused by a cell cycle delay or arrest. These cells were also normal in size (not shown).

Continual loss of viability among individual cells would slow colony growth. To determine whether Stb3OE alters viability, Stb3OE cells were cultured over a 4-week period starting in SGal medium. Small samples were periodically plated to count viable cells (Figure 2C). Cells overexpressing Stb3 do not lose viability faster than controls. On the contrary, the *GAL-STB3* cells exhibited higher viability than the *lacZ* controls. We conclude that the reduction in growth rate is not due to loss of cell viability, defective morphology, or cell cycle blockade. Instead, the cells appeared to be normal except for their rate of growth.

Stb3OE inhibits the expression of a set of glucose-induced genes: Stb3 is thought to bind at regulatory sites upstream of non-RP growth genes that are induced by glucose repletion (LIKO *et al.* 2007). Stb3 could be a positive regulator, activating the genes when glucose is present, or Stb3 could be a repressor, turning these genes off when glucose is depleted. The slow growth

FIGURE 2.—Effect of Stb3 overexpression on cell morphology, progress through the mitotic cycle, and lifespan. Yeast cells expressing Stb3 driven from the *GAL* promoter on pYES2.1 (DLY10) and control cells expressing *lacZ* from pYES2.1 (DLY12) were grown on glucose or galactose medium for comparison. (A) DIC photomicrographs using a $\times 62$ objective were taken of cells growing in SD or SGal medium. (B) Samples of cells growing in SGal medium were collected and processed for Sytox green staining and flow cytometry. Positions of G1 and G2/M populations are noted in each histogram. (C) Cells were grown in galactose-containing media over the indicated period for viability testing by plating known numbers of cells onto YPD. The percentage of cells forming colonies is indicated.

FIGURE 3.—Stb3 overexpression down regulates a set of glucose-induced genes. Yeast cells expressing Stb3 driven from the *GAL* promoter on pYES2.1 (DLY10) and control cells expressing *lacZ* from pYES2.1 (DLY12) were grown for microarray experiments using Affymetrix S2.0 arrays. Saturated red indicates a $\log_2$ value of 3, and saturated green indicates a $\log_2$ value of -3 . (A) Heat map showing hierarchical clustering of three data sets. (Left) The wild-type (BY4742) response to glucose repletion after 10'. Glucose samples are compared to post-log samples as the baseline. (Middle) Fold change in transcript values in the Stb3OE strain compared to the *lacZ* control for cells growing in glucose medium. (Right) Comparison of the Stb3OE and *lacZ* strains from samples growing in galactose medium. Bar indicates overrepresented GO terms for a cluster of genes induced by glucose

produced by Stb3OE suggested repression of genes needed for protein translation.

To test this, we used microarrays to identify transcriptional changes caused by Stb3OE. The heat map in Figure 3A shows hierarchical clustering of three data sets. On the left is the fold change in transcript levels from glucose-repleted cells compared to post-log cells; red indicates glucose induction (Liko *et al.* 2007). The right column shows the comparison between *GAL-STB3* and *GAL-lacZ* in galactose medium, where Stb3 is highly expressed and produces a growth defect. In this case green means that transcript expression is reduced by Stb3OE compared to the *lacZ* control. The middle column is a control, comparing the *GAL-STB3* to the *GAL-lacZ* strain in glucose medium, where Stb3 is not overexpressed.

The most noticeable change caused by Stb3OE was the downregulation of a set of transcripts shown in the right column as green. This set is indicated by the bar and was enriched in genes involved in ribosome biogenesis and protein translation. It was also apparent that most of the genes affected by Stb3OE were also induced by glucose repletion.

To more clearly show the effect of Stb3OE on glucose-inducible genes, we selected a set of 1190 genes upregulated at least $2\times$ at 10 minutes after glucose repletion (Liko *et al.* 2007) and ordered this group of genes by their response to Stb3OE, with the transcripts most downregulated by Stb3OE at the top (Figure 3B). These genes are listed in Table S1. The response to glucose repletion is shown in the left column, and the response to Stb3OE is shown at right. This shows a strong connection between glucose induction and repression by Stb3OE.

Of the 429 genes downregulated $\geq 1.5\times$ by Stb3OE, 260 were also induced at least $2\times$ by glucose. While Stb3OE does not affect all of the glucose inducible genes, the group of 260 genes downregulated by Stb3OE makes up a substantial fraction of the 1190 genes upregulated by glucose (Figure 3C).

This diagram also shows the overlap with a set of genes affected by *STB3* deletion identified in previously published experiments (Liko *et al.* 2007). Upon reexamination of this data, we found that *stb3Δ* cells failed to properly repress a set of 307 genes during quiescence. Of these, 197 were contained in the glucose inducible

and repressed by Stb3OE. (B) Heat map limited to set of transcripts increased in expression by $\geq 2\times$ after glucose repletion. (Left) Wild-type (BY4742) glucose-repleted samples are compared to post-log samples as the baseline as above. (Right) Comparison of the Stb3OE and *lacZ* control strains from samples growing in galactose medium, as in A above. Samples are ordered by fold repression by Stb3OE, with the box indicating samples affected ≥ 1.5 fold. (C) Venn diagram showing overlap between genes affected by Stb3OE, compared to those induced ≥ 2 fold by glucose, or $\geq$ fold by *stb3Δ* (Liko *et al.* 2007). The *P*-values represent hypergeometric distribution.

set, and 136 were glucose inducible and affected by both *STB3* deletion and overexpression.

Stb3 overexpression reduces amino acid incorporation: If Stb3 acts to repress genes needed for protein synthesis, we would expect Stb3OE to reduce protein synthesis along with slowing growth. To test this, we cultured cells carrying the *GAL-STB3* plasmid in the poor, nonrepressing carbon source raffinose and at time 0 transferred the cells to galactose medium to allow fermentative growth and to induce overexpression of *STB3*. At the indicated times, small samples were removed from the culture and pulsed for 2 min with [³⁵S]methionine to measure the rate of amino acid incorporation into protein, as described in MATERIALS AND METHODS. In the control *GAL-lacZ* strain amino acid incorporation rates steadily increased over time as the cells responded to the fermentable carbon source (Figure 4). Cells carrying the *GAL-STB3* plasmid showed substantially reduced levels of amino acid incorporation in comparison. By 4 hr after the upshift, the rate of protein synthesis in the control culture was almost twice that of the Stb3OE strain.

Stb3 as a putative transcriptional repressor: Stb3 was originally isolated in a screen looking for Sin3 binding partners (KASTEN and STILLMAN 1997; KALKUM *et al.* 2001). This suggests that Stb3 might recruit Sin3/HDAC complexes to repress transcription of specific genes. If this model is true, then the phenotype produced by Stb3OE should be dependent on HDAC activity recruited to the chromatin by Stb3. To examine this more specifically, we tested the effect of deletion of each of the five known *S. cerevisiae* HDAC genes on the growth phenotype produced by Stb3OE. We predicted that deletion of one or more HDACs would improve growth.

In particular, none of our experiments manipulating Sin3 or Rpd3 matched our prediction. Cells carrying a deletion in *RPD3* grow poorly in both glucose and in galactose medium and grew even more poorly when this was coupled with Stb3OE (Figure 5A). This can be interpreted in more than one way: it may indicate that cells growing poorly in response to one deletion grow even more poorly in the presence of two, or it may indicate a genetic interaction in which loss of Rpd3 potentiates the effect of Stb3OE. Regardless, in experiments using *RPD3* deletions made in multiple strain backgrounds, the loss of Rpd3 clearly does not rescue cells from Stb3OE.

In contrast, deletion of *HOS2* rescued the cells from the effect of Stb3OE on growth (Figure 5A). This shows that the phenotype produced by Stb3OE is dependent on the presence of a specific HDAC, and is consistent with a model in which Stb3 and Hos2 repress a subset of glucose-inducible genes during post-log quiescence. None of the other HDAC gene deletions abolished the slow growth produced by Stb3OE.

To test the assumption that the HDAC activity of Hos2 is required for the Stb3OE slow growth phenotype, we

FIGURE 4.—Stb3 overexpression slows amino acid incorporation. Stb3OE (DLY10) and *lacZ* control (DLY12) strains were grown in SGal, and samples were collected for 2' pulse labeling with ³⁵S as described in MATERIALS AND METHODS. The average ³⁵S incorporation/2 min is shown as cpm for the indicated time points; error bars represent the standard deviation of triplicate samples.

overexpressed Stb3 in a strain carrying the Hos2 H195A, H196A point mutations that abolish HDAC activity (WANG *et al.* 2002). To do this we transformed into a *hos2Δ* background a plasmid expressing the HDAC-inactive Hos2. As controls we used the same plasmid expressing wild-type Hos2 and the empty vector. This produced strains expressing no Hos2, wild-type Hos2, and HDAC inactive Hos2. We then transformed each of these strains with the *TEF2-STB3* plasmid. Growth of these strains on SD medium is shown in Figure 5B. The cells expressing wild-type Hos2 were severely affected by Stb3OE, while the cells without any Hos2, and the cells carrying the HDAC-inactive Hos2 showed no growth defect in response to Stb3OE. Therefore the slow growth phenotype produced by Stb3OE is dependent on the HDAC activity of Hos2.

Glucose alters the intracellular location of Stb3: We expected to find Stb3 in the cell nucleus because of its identification as an RRPE-binding protein. To test this, we used a strain (95700) in which GFP is chromosomally inserted in-frame at the 3' end of the *STB3* ORF to create a GFP-tagged Stb3. In post-log cells Stb3 was concentrated in the nucleus, consistently corresponding to the location of DAPI staining (Figure 6A). However, after glucose addition we found Stb3 distributed throughout the cytoplasm. This demonstrates that glucose has an effect on Stb3 itself and suggests a mechanism by which post-log inhibition of gene expression by Stb3 could be relieved by glucose repletion. The GFP-labeled Stb3 was functional as measured with *in vitro* DNA-binding assays (not shown).

FIGURE 5.—Loss of *HOS2* restores normal growth to cells overexpressing Stb3. (A) Strains lacking each of the five recognized HDACs were transformed with the *GAL-STB3* or *lacZ* control plasmid and plated onto either glucose or galactose as described in Figure 1. The *rpd3Δ* mutant was made in the W303 background; the other mutants were in BY4742. (B) Stb3 was overexpressed from the *TEF2* promoter as in Figure 1 using the strain backgrounds indicated. Drop tests are shown on SD dropout medium.

To measure the time course of the response to glucose, we added glucose to post-log cells and captured fluorescence and DIC images over a 10-min time course (Figure 6B). The GFP signal remained in the nucleus at the 1' time point, but was partially into the cytoplasm by the next image at 5'. The signal is largely cytoplasmic in 7.5' and later images.

Glucose signaling pathways and Stb3: We hypothesize that specific signals produced by glucose turn off

FIGURE 6.—Stb3 moves from the nucleus to the cytoplasm in response to glucose repletion. (A) Cells expressing a GFP-tagged Stb3 (95700) were grown to post-log phase and glucose was added to a 2% final concentration. DAPI staining was used to locate the nucleus. Images show the original post-log cells and the glucose repleted cells after 10'. (B) Cells expressing Stb3-GFP (MCY689) were grown to post-log phase and 10 μ l of culture was placed on a microscope slide with cover slip. At 0', 2 μ l of 10% glucose was added at the edge of the cover slip. Images show cells near the edge of the coverslip at the indicated time after glucose addition.

FIGURE 7.—Rapamycin exacerbates the phenotype produced by Stb3OE. (A) Stb3OE (DLY10) and *lacZ* control (DLY12) strains were serially diluted as in Figure 1 and plated in 10- μ l drops onto glucose or galactose media with or without rapamycin (50 nM) as indicated and incubated at 30°. (B) Cells expressing Stb3-GFP (MCY689) were grown in SD dropout medium to mid-log phase and rapamycin (50 nM) was added to an aliquot of the cells at 0'. Images were taken at the indicated times.

Stb3 repression. If so, we can identify glucose signals controlling Stb3 by testing for genetic interactions with Stb3OE: increasing glucose signals to Stb3 should improve growth, while inhibiting glucose signals to Stb3 should exacerbate the slow growth phenotype.

Tor: To test this, we inhibited Tor with rapamycin in cells overexpressing Stb3 (Figure 7A). In galactose medium, cells carrying *GAL-STB3* were supersensitive to the presence of rapamycin and showed profound growth retardation. In glucose we could distinguish no

FIGURE 8.—*SCH9* activity controls the Stb3OE phenotype. (A) *GAL-STB3* and control *GAL-lacZ* plasmids were transformed into WT, *GAL1-SCH9*, and *sch9Δ* strains. Cells were serially diluted and plated on glucose or galactose plates and grown at 30° as described above.

difference between the growth of the *GAL-STB3* strain and the *GAL-lacZ* control when challenged with rapamycin. This shows a genetic connection between the Tor pathway and Stb3.

Exposing cells to rapamycin caused a rapid translocation of Stb3-GFP from the cytoplasm to the nucleus (Figure 7B). This is consistent with a model in which Tor activity is needed to keep Stb3 in the cytoplasm during growth and is also consistent with the inhibition of growth gene expression observed with rapamycin blockade of the Tor pathway.

Sch9 and Sfp1: The Sch9 protein kinase is an important downstream effector of Tor (URBAN *et al.* 2007). If nutrients alter the function or activity of Stb3 via Sch9, then deletion of *SCH9* should produce a phenotype similar to that produced by rapamycin. Overexpression of *SCH9* should do the reverse and rescue the cells from Stb3OE. This was confirmed in our experiments: *sch9Δ GAL-STB3* cells grew very poorly in galactose; Sch9OE almost completely rescued the growth defect produced by Stb3OE (Figure 8). This result indicates that Sch9 can turn off the growth inhibitory function of Stb3, and that normal Sch9 activity is necessary to prevent almost complete loss of growth.

PP2A protein phosphatase activity plays a role in halting growth when Tor is inactive (COX *et al.* 2004; SANTHANAM *et al.* 2004; WILLIS and MOIR 2007). It is possible that PP2A directly or indirectly switches Stb3 to a repressive state as one mechanism to slow growth. We found that deletion of *PPH22*, encoding a catalytic subunit of PP2A, partially rescued the phenotype produced by Stb3OE (Figure 9A). This shows another genetic connection between Stb3 and the Tor pathway.

Sfp1 is thought to lie even farther downstream of Tor, interacting with DNA binding proteins that regulate RP expression and playing a more poorly understood role in controlling non-RP growth genes (JORGENSEN *et al.* 2004; CIPOLLINA *et al.* 2008; LEMPIAINEN *et al.* 2009). Sfp1 is therefore a regulator of the growth response to glucose depletion. We found that the combination of Sfp1 loss and Stb3OE produced a severe growth defect

(Figure 9B). This is consistent with the combined overexpression of a repressor and loss of an activator.

As with Stb3, overexpression of Sfp1 produces a more profound growth defect than observed with Sfp1 deletion (JORGENSEN *et al.* 2004). We found that overexpression of Stb3 had little effect in rescuing the phenotype produced by Sfp1 overexpression.

cAMP/PKA: The cAMP/PKA and Tor pathways drive the transcriptional response to glucose (WANG *et al.* 2004; SLATTERY *et al.* 2008). In contrast to the clear connections between Tor and Stb3, we found no evidence linking Stb3 to cAMP/PKA. To test this, we transformed a cAMP-dependent *cyr1Δ* strain with a plasmid expressing the Stb3-GFP fusion shown earlier. In quiescent post-log cells, the Stb3-GFP signal was primarily in the nucleus (Figure 10). As expected, the GFP signal moved into the cytoplasm in response to addition of glucose and cAMP needed for growth. However, the GFP also moved into the cytoplasm in response to glucose without cAMP, a condition that does not support growth. Addition of cAMP without glucose did not cause the GFP to leave the nucleus, even after 60'. Therefore, the absence of cAMP did not keep Stb3 in the nucleus, and addition of cAMP did not move Stb3 into the cytoplasm.

In other experiments, we manipulated the cAMP/PKA pathway by expressing Val19 *RAS2* or deleting *BCY1*. This had no impact on the slow growth phenotype produced by Stb3OE (not shown). Thus, we could not detect a genetic connection between Stb3 and cAMP/PKA.

DISCUSSION

Working assumptions guiding the experiments: A subset of the genes induced by glucose is enriched with RRPE and PAC regulatory motifs. These motifs can confer glucose sensitivity to test promoters, especially when combined with other elements (FINGERMAN *et al.* 2003; WANG *et al.* 2004; SLATTERY and HEIDEMAN 2007).

FIGURE 9.—Pph22 and Sfp1 alter the slow growth phenotype produced by Stb3OE. (A) WT and *pph22Δ* cells carrying the *GAL-STB3* construct were grown and compared to cells carrying the lacZ control plasmid as described in Figure 1 (and *pph22Δ*). (B) The *GAL-STB3* and control *GAL-lacZ* plasmids were transformed into WT, *GAL1-SFP1*, and *sfp1Δ* strains. Cells were serially diluted and plated on glucose or galactose plates and grown at 30° as described above.

Having identified Stb3 as a binding protein for RRPEs, we have worked under the assumption that Stb3 might play a role in regulating at least part of this gene set. The very modest phenotype produced by *STB3* deletion suggested a redundant system (LIKO *et al.* 2007). As with many redundant systems, Stb3 overexpression produced a much more profound phenotype than simple *STB3* deletion.

We had previously thought of Stb3 as a gene activator because loss of Stb3 reduces the fold induction by glucose for a group of genes (LIKO *et al.* 2007). However, reexamination of this data shows that in most cases the lost fold induction was due to a failure of the *stb3Δ*

mutant to turn off the gene in quiescence, rather than a failure to turn the gene on in response to glucose. This is illustrated in Figure 3. This explanation does not account for some of the Northern blots published in our previous study. These show not only failure to repress genes in quiescence, but also an apparent loss of activation in glucose for some specific genes. While the preponderance of our data suggests that Stb3 acts as a gene repressor, it remains possible that Stb3 also plays a role in gene activation.

Effect on growth genes: A large fraction of the genes affected by Stb3OE are part of the gene set induced by glucose repletion. Stb3OE decreased expression of these

FIGURE 10.—Stb3 movement out of the nucleus is not controlled by cAMP. Cells carrying a deletion in *CYR1*, which encodes the yeast adenylyl cyclase, were transformed with a plasmid expressing a GFP-tagged Stb3 (MCY688). Cells were grown to post-log phase and then transferred into synthetic medium containing combinations of glucose (2%) and cAMP (1 mM) as indicated. The cells require both cAMP and glucose for growth. Addition of rapamycin (50 nM) was used to arrest growth and to demonstrate localization of the Stb3-GFP to the nucleus.

genes, reduced amino acid incorporation, and reduced growth rate. Cells overexpressing Stb3 retained viability. In general they behaved as though they were growing on a poor medium rather than on the fermentable galactose medium surrounding them.

It might be expected that loss of *STB3* would produce cells that attempt to grow when they should be shutting down growth in quiescence. This might be expected to reduce viability, but in our experiments *stb3Δ* cells are able to survive starvation as well as wild-type cells (not shown).

Recent genome-wide experiments have shown that Stb3 is associated with the sequence TGAAAAA (ZHU *et al.* 2009). This sequence is similar to and partially overlaps the RRPE sequence. In our experiments we found that the genes affected by Stb3 were enriched for the TGAAAAA sequence. In particular we found that 124 out of the 136 genes at the intersection of the Venn diagram in Figure 3 had the TGAAAAA sequence in their promoter regions. This bolsters the results from ZHU *et al.* (2009) but calls into question whether Stb3 functions at the new sequence, RRPEs, or both.

Stb3 and Hos2: As a repressor, Stb3OE might be expected to recruit HDACs to deacetylate local histones and limit transcription. If such a model is true, then the phenotype produced by Stb3OE should be dependent on HDAC activity. *HOS2* deletion rescued the growth defect produced by Stb3OE. Hos2 is part of the Set3 HDAC complex, and has been implicated in regulating ribosomal genes (BERNSTEIN *et al.* 2000; ROBYR *et al.* 2002; WANG *et al.* 2002). In further experiments we found that the HDAC activity of Hos2 is necessary for the slow growth phenotype produced by Stb3OE. HDAC inactivating substitutions (H195A and H196A) in Hos2 (WANG *et al.* 2002) produced the same rescue of growth as complete *HOS2* deletion.

Altogether, this fits a model in which Stb3 moves into the nucleus during quiescence to slow transcription of target genes in a Hos2-dependent manner. Glucose would end repression by moving Stb3 out of the nucleus.

While the *hos2Δ* results are compelling, we expected to see similar results with *rpd3Δ*. Stb3 was originally identified as a binding partner for Sin3 (KASTEN and STILLMAN 1997), so our early hypothesis was that Stb3 would recruit Sin3 and the HDAC Rpd3 to slow growth. However, the combination of *rpd3Δ* and Stb3OE produces cells that grow very poorly, the opposite of our prediction. Beyond this, we cannot determine whether this indicates the combination of pleiotropic growth defects or an unexplained genetic interaction.

Genetic links between Stb3 and nutrient signaling: Stb3 is found in the nucleus in quiescent cells, and moves out into the cytoplasm when glucose is added. Rapamycin addition moves Stb3 into the nucleus despite the presence of glucose, indicating that at least one of the signals moving Stb3 out of the nucleus depends on Tor activity. In contrast, manipulation of the cAMP/

PKA pathway had no apparent effect on Stb3 or the phenotype produced by Stb3OE. This is consistent with a model in which Stb3 is a Tor-regulated protein. A recent genome-wide screen for Sch9 targets lists Stb3 as one of the proteins phosphorylated by Sch9 (HUBER *et al.* 2009). This strengthens the idea that Stb3 is a nuclear effector of the Tor pathway.

We speculate that the default form of Stb3, as first expressed, may be one that can enter the nucleus and repress growth genes. We further speculate that the Tor pathway can promote the movement of Stb3 into the cytoplasm. High Stb3 levels could conceivably overwhelm the capacity of Tor effectors such as Sch9, leaving enough Stb3 in the nuclear, repressive state to produce the phenotypes we observed.

This model accounts for the phenotype produced by Stb3OE, as well as the observed genetic interactions between *STB3* and Tor, Pph22, and Sch9. Increased Sch9 expression compensates for Stb3OE. On the other hand loss of Sch9, or the inactivation of Tor with rapamycin, dramatically worsens the slow growth produced by Stb3OE.

The presence of a single compound, glucose, is sufficient to trigger a profound change in the transcriptome of a yeast cell. There is ample evidence for redundancy and cross talk between the regulatory components carrying the signals generated by glucose. Our results indicate that Stb3 plays a role in connecting upstream signals with this transcriptional response.

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