Transcript and proteomic analyses of wild-type and *gpa2* mutant *Saccharomyces cerevisiae* strains suggest a role for glycolytic carbon source sensing in pseudohyphal differentiation†

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In response to limited nitrogen and abundant carbon sources, diploid *Saccharomyces cerevisiae* strains undergo a filamentous transition in cell growth as part of pseudohyphal differentiation. Use of the disaccharide maltose as the principal carbon source, in contrast to the preferred nutrient monosaccharide glucose, has been shown to induce a hyper-filamentous growth phenotype in a strain deficient for *GPA2* which codes for a Gα protein component that interacts with the glucose-sensing receptor Gpr1p to regulate filamentous growth. In this report, we compare the global transcript and proteomic profiles of wild-type and Gpa2p deficient diploid yeast strains grown on both rich and nitrogen starved maltose media. We find that deletion of *GPA2* results in significantly different transcript and protein profiles when switching from rich to nitrogen starvation media. The results are discussed with a focus on the genes associated with carbon utilization, or regulation thereof, and a model for the contribution of carbon sensing/metabolism-based signal transduction to pseudohyphal differentiation is proposed.

Introduction

A clear understanding of how biological systems can utilize multiple redundant signal transduction pathways to produce radically different physiological responses is important to not only basic biology, but also to applications such as understanding and treating disease processes.[1,2] The baker's yeast *Saccharomyces cerevisiae* has proven itself to be among the best organisms available to study these processes for over 100 years and as a result an ever-growing plethora of sensing/signaling pathways have been elucidated in this model eukaryotic system.[1,2] These include nutritional pathways such as those that respond to carbon or nitrogen sources in the environment, those involved with mating type, response to heat shock or stress, and even aging to name but a paltry few.[3–9] It has become evident that many of the signal transduction pathways in yeast utilize common elements that 'cross-talk' to produce radically different outcomes. Even small differences in stimuli such as switching between two closely related carbon sources, *i.e.*, maltose to glucose, can result in genome wide changes in the expression of hundreds of genes and significantly different physiological outcomes.[10–13]

Filamentous growth or pseudohyphal differentiation is used as a model system to study the complex physiological responses of yeast to changes in environmental nutrient levels.[3,4,7,8,14–17] Carbon abundance and nitrogen source limitation stimulate diploid yeast cells to undergo a filamentous transition in cell growth, a process whereby cells become elongated with unipolar bud formation, are connected physically with a chain-like appearance and invade the growth media.[3,4,7,8,14-17] It has been hypothesized that this process may allow these cells to 'forage' for limited nutrients.[18,19] Two parallel signal transduction pathways have been demonstrated as regulators of this process including a cyclic adenosine monophosphate- (cAMP) dependent pathway and the pheromone responsive MAP kinase signal transduction cascade.[3,4,8,14,16,17] Lorenz *et al.*,[20] elegantly demonstrated that the G protein-coupled receptor Gpr1p is a nutrient sensor that regulates pseudohyphal differentiation *via* the cAMP pathway and independent of the MAP kinase cascade. *GPR1* is induced by glucose and other structurally related sugars and its expression is also regulated by nitrogen starvation. Gpr1p functions primarily through its downstream Gα protein Gpa2p and deletion of *GPA2* abolishes Gpr1p function.[20] Growth of a diploid wild-type strain on maltose under nitrogen starvation conditions induced significantly more filamentation relative to growth on glucose and growth of the *gpa2Δ* strain on maltose under nitrogen starvation conditions induced what appeared to be a hyper-filamentous phenotype (Fig. 1).[20] This, in combination with other observations,[3,14,20] have suggested that carbon source sensing/metabolism may contribute to the regulation of pseudohyphal differentiation.

In this report we focus on the role of carbon source sensing in pseudohyphal differentiation. Transcriptome and proteome analyses were performed on wild-type (WT) and *gpa2Δ* strains in both rich and nitrogen starvation media. Comparison of the data between the two strains revealed a modulation of almost 600 genes in the *gpa2Δ* strain when switching from rich to nitrogen starved maltose media as compared to only 124 genes in the WT strain. The bulk of the differentially expressed genes

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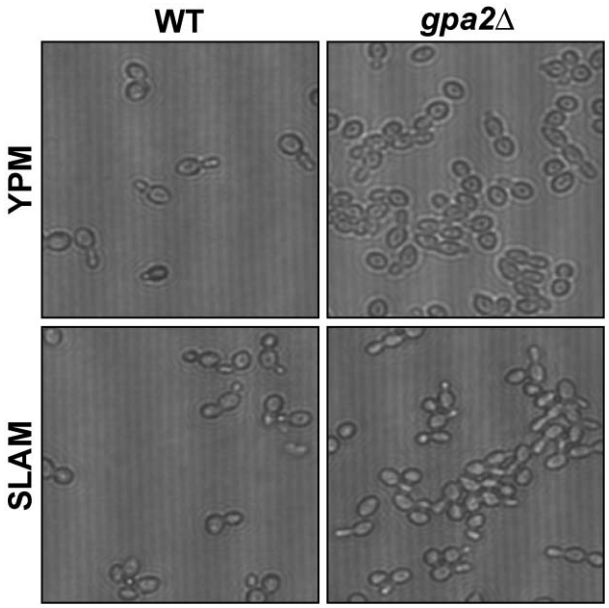

Fig. 1 Representative micrographs of the WT (MLY61) and *gpa2Δ* (MLY132) strains grown overnight in either rich maltose media (YPM) or after transfer and 2 hours of growth in synthetic low ammonium maltose media (SLAM). Note that the *gpa2Δ* cells are elongated with unipolar buds, are physically connected and have a chain-like appearance.

were correlated with protein biosynthesis, RNA metabolism, cellular organization/biogenesis, and nucleotide metabolism. More modest changes between the two strains during the media switch were noted at the protein level. The results are discussed with a focus on genes associated with carbon utilization and a model for the contribution of carbon sensing/metabolism to pseudohyphal differentiation is proposed.

Results and discussion

Growth conditions and comparisons

The construction and use of the diploid MLY61 WT and MLY132 *gpa2Δ* strains has been previously described.[20] Both strains were able to ferment maltose indicating the presence of a complete *MAL* locus.[21] Both the WT and *gpa2Δ* strain were grown in YPM media and harvested, or filtered and transferred to SLAM media for another 2 hours of additional growth and then harvested. The latter process is referred to hereafter as the media switch. The collected cell pellets were then subjected to microarray-based transcript analyses and mass-spectrometry-based proteomic analyses as described in the Experimental section. Samples from three growth replicates, each isolated separately were subjected to analysis on three separate microarray chips or alternatively mass-spectral analysis. The resultant transcript and protein expression data were then compared between each growth condition or strain and the significantly different genes/proteins were classed by common biological processes using a previously described hierarchy[22] (Fig. 2, Table 1 and Supplementary Tables I,II†). Due to overlap in structure/function, some ribosomal proteins were classed under the closely related protein biosynthesis or nitrogen source utilization processes.

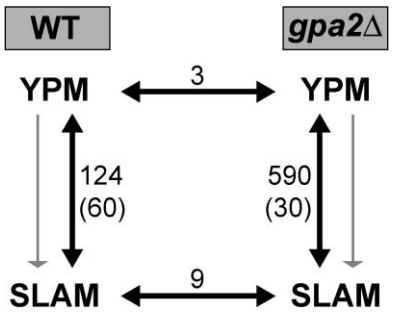

Fig. 2 Comparisons performed between the WT strain and the *gpa2Δ* strain. Samples were grown in rich YPM conditions and then harvested for analysis or transferred to nitrogen starvation SLAM media (gray arrows) and then harvested. Boldfaced lines indicate a comparison and the adjacent unbracketed values denote the number of significantly modulated transcripts while the bracketed values found underneath denote the number of significantly modulated proteins for that same comparison. The YPM to SLAM media switch had 44 transcripts and 11 proteins in common between the two strains. Note that the protein levels were not measured for comparisons between the two strains.

Quality of yeast RNA processing and microarray results

From yeast cell pellets averaging about 100 μL in size, the RNA yield was 113 ± 128 μg (mean ± 3SD) and electropherograms showed good RNA quality had been isolated (data not shown). From 8 μg of starting total RNA, the cRNA adjusted yield was 40 ± 37 μg, and the spectrometric 260/280 ratio of the purified cRNA was 2.07 ± 0.13. For determining the quality of microarray results, the plotting of signals *versus* known concentration of *BioB*, *BioC*, *BioD*, and *Cre* cRNA 'spiked-in' controls showed a linearity of $r = 0.94$, confirming hybridization efficiency. The *BioB* control, whose concentration is at the limit of detection, was detected on all microarrays confirming low copy number sensitivity. The average background was 50 ± 15 arbitrary units, and the ratios of the 3′ to the 5′ signal of the actin transcript (YFL039C) were 1.7 ± 0.7, which was within the 20–100 background range and below a ratio of 3, respectively, suggested by Affymetrix guidelines. The percent present or detection rate was 84% ± 17%, suggesting that most of the transcripts probed by the arrays were detected. There were no differences noted in Scale Factors among the sample groups, suggesting that most transcripts remained unchanged, thus allowing the use of the RMA algorithm to calculate transcript quantities.[23] Only data with concordance among the three microarray chips for each condition were used. Cumulatively, these results indicate that the microarray measurements were of good quality and thus could be included in subsequent analyses.

Transcript analyses under different growth conditions

Wild-type *vs gpa2Δ* strain. The first comparison of transcript levels looked at the difference between the WT and *gpa2Δ* strains grown under either rich maltose media (YPM) or after induction of nitrogen starvation (SLAM) conditions (Fig. 2, Table 1). In the YPM media, only three transcripts (*TKL2*, *NDE2*, *PGM2*) associated with carbon source utilization were significantly different between the two strains and all were

Table 1 Comparison of over-represented functional clusters of genes and proteins from WT and *gpa2Δ* strains grown under different media conditions[a]

	WT				*gpa2Δ*				YPM				SLAM			
	YPM *vs* SLAM[b]				YPM *vs* SLAM[b]				*gpa2Δ vs* WT				*gpa2Δ vs* WT			
	Up[c]		Down[c]		Up[c]		Down[c]		Up[d]		Down[d]		Up[d]		Down[d]	
Biological process	T[e]	P[e]	T	P	T	P	T	P	T	P	T	P	T	P	T	P
Cellular organization and biogenesis		21 (1)	11 (6)	1		1 (1)	106 (6)								6[h]	
RNA metabolism		2	23 (5)				109 (5)									
Transcription							10									
Nucleotide/nucleobase metabolism		1		2 (2)			145	3 (2)								
Protein biosynthesis		4 (2)	36 (22)			6 (2)	137 (22)	1								
Carbon source utilization	1	9 (2)	2	3 (1)	16	4 (2)		1 (1)	3[g]						1[i]	
Nitrogen source utilization	15 (11)[f]	3	28	5	26 (11)	1	27	4								
Organelle biosynthesis/structure and function				4												
Signal transduction								1								
Other metabolic functions[k]	4		4	5	14	5		3							2[j]	
Totals	20 (11)	40 (5)	104 (33)	20 (3)	56 (11)	17 (5)	534 (33)	13 (3)	3 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	9 (0)	0 (0)

[a] See Supplementary Tables I and II for gene and protein identification lists. Only gene expression levels that met a significance of $p < 0.01$ were included for each comparison. [b] Comparison mass spectrometry protein data. [c] Up = higher in YPM compared to SLAM; Down = lower in YPM compared to SLAM. [d] Up = higher in *gpa2Δ* compared to WT; Down = lower in *gpa2Δ* compared to WT. [e] T = RNA transcripts; P = proteins. [f] Values in parenthesis are the number of transcripts or proteins common to both WT and *gpa2Δ* comparisons. [g] Three transcripts identified as *TKL2, PGM2* and *NDE2*. [h] Six transcripts identified as *GAR1, BRX1, RPL12B, DIM1, CGR1* and *EMG1*. [i] Transcript identified as *HXK2*. [j] Two transcripts identified as *PHD1* and *GPA2*. [k] Homeostasis, localization, biotin/sulfur compound biosynthesis, transport, ubiquitin, proteolysis.

up-regulated in the mutant relative to WT. *TKL2* is a transketolase, *NDE2* is a mitochondrial NADH dehydrogenase and *PGM2* is the major phosphoglucomutase in glycolysis. After induction of nitrogen starvation, nine significantly modulated transcripts were noted between the two strains and in this case all were down-regulated in the mutant relative to the WT strain. Six have roles in ribosome assembly or function and ribosomal RNA processing. Also noted was *HXK2*, the primary hexokinase in glycolysis during growth on glucose and which also functions in the nucleus to repress expression of *HXK1*/*GLK1* and to induce its own expression. A role for hexokinases in cAMP dependent induction of pseudohyphal differentiation has been suggested previously.[4] *GPA2* down-regulation serves to confirm its deletion in the mutant strain and also suggests that it is present somewhat above background in the WT strain. Lastly, *PHD1* which is known to activate *FLO11* as part of the pseudohyphal differentiation process independent of the PKA and MAP kinase pathways,[4] was also down-regulated in the mutant strain in SLAM media.

Wild-type media switch. The comparisons for each strain's induction to nitrogen starvation media from rich media displayed far more complex expression changes (Fig. 2, Table 1 and Supplementary Tables I,II†). Of the 104 WT transcripts down-regulated during this switch, 33 transcripts were shared in common with the same nutrient change in the mutant strain and 34 originate from cellular organizational functions and ribosomal/RNA metabolism (Table 1, Supplementary Table I†). A further 36 transcripts were associated with protein biosynthesis of which 22 or ~two-thirds were in common with the mutant strain. Twenty-eight transcripts associated with nitrogen source utilization are also noted, though none of these are common to the mutant strain. The combined total of 64 down-regulated transcripts drawn from these two related protein synthesis/nitrogen source processes is not surprising as the WT cell physiology is adapting to the induced nitrogen starved environment. Some of the down-regulation of ribosomal genes from the cellular organization class is probably directly correlated with this change as well. Interestingly, the two WT down-regulated transcripts associated with carbon source utilization include invertase (*SUC2*) and an invertase precursor (*SUC4*), both of which are required for sucrose metabolism. The remaining four transcripts were associated with other non-related metabolic functions. Of the 20 up-regulated genes, 15 were associated with nitrogen source utilization and 11 (~half), were shared in common with the mutant strain. Under carbon utilization only *PCK1* was noted coding for a phosphoenolpyruvate carboxykinase which is a key participant in gluconeogenesis. Glucose is known to repress *PCK1* transcription and accelerates its mRNA degradation. *PCK1* is also regulated by Mcm1p, a MADS-box transcription

factor, component of the protein kinase C-mediated MAP kinase pathway, and Cat8p, a putative transcription factor necessary for derepression of gluconeogenic enzymes.[24,25] The remaining four transcripts were again associated with other unrelated metabolic functions although *FYV10* is believed to be involved in proteasome-dependent catabolite inactivation of fructose-1,6-bisphosphatase.[26]

***gpa2Δ* media switch.** The same rich to nitrogen starvation media transcript comparison in the *gpa2Δ* mutant strain has the largest number of significantly different genes noted in this study (Table 1, Supplementary Table II†). From just the overview in Table 1, we note that the deletion of *GPA2* results in 590 significantly modulated transcripts. This is ~5× as many as in the WT, with 534 or 91% down-regulated similar to the ~88% ratio found down-regulated in the WT comparison. Whereas the WT comparison shares 35% of the transcripts in common with the mutant comparison, the large total here reduces this to only ~7.5% concordance with the WT. About 70% or 360 of the 534 down-regulated genes are associated with cellular organization/biogenesis, ribosomal function, RNA metabolism and nucleotide metabolism. A further 164 (~31%) correspond to protein biosynthesis and nitrogen source utilization although it should again be noted that many ribosomal functions maybe related to these two processes as well. The 10 transcripts associated with transcription all encode RNA polymerase subunits suggesting negative regulation, or alternatively loss thereof, of an upstream control element associated with transcript machinery. Up-regulated genes include 16 associated with carbon source utilization, 26 with nitrogen source utilization and 14 sundry metabolic functions.

The 16 genes up-regulated under carbon utilization include: *CAT8*, mentioned previously for its putative role as a transcription factor that derepresses *PCK1* and other gluconeogenic enzymes,[25] *GID1/7*, involved in proteasome-dependent catabolite inactivation of fructose-1,6-bisphosphatase,[27] *GLC8*, a regulatory subunit of Glc7p protein phosphatase 1,[28] *PFK26*, a phosphofructokinase whose transcriptional regulation involves protein kinase A,[26] *PIG2*, which encodes a putative type-1 protein phosphatase targeting subunit that tethers Glc7p to Gsy2p—a glycogen synthase,[26] *SHC1*, encoding a sporulation-specific activator of chitin synthase III which is required for the synthesis of the chitosan layer of ascospores[29] and *UBC8*, which encodes a ubiquitin-conjugating enzyme that negatively regulates gluconeogenesis by mediating the glucose-induced ubiquitination of fructose-1,6-bisphosphatase.[29] The up-regulated transcripts classed under other functions for this comparison include *ATG7/8*, coding an E1-like activating enzyme and precursor involved in the two ubiquitin-like systems required for cytoplasm to vacuole transport and autophagy[30] as well as several genes associated with fatty acid metabolism.

Promoter analyses

Up- and down-regulated transcripts from each class comparison were subjected to promoter analyses as previously described.[31,32] This method analyzes the genomic sequence occurring -800 to -1 before the start codons of genes identified as differentially expressed transcripts, identifies statistically significant over-represented sequences and then compares them to known transcription factor consensus binding sequences. Although this provides a method to look for potential factors involved in regulating the identified transcripts, it does not confirm a transcription factors active regulatory role or binding to that particular sequence. Table 2 presents the pertinent transcription factors identified from this analysis. From the WT media switch comparison, no transcription factors corresponding to down-regulated transcripts where noted while 13 factors are identified from up-regulated transcripts. Of these sites, six are common to both the WT and mutant strains during the media switch including a Gatap site. Gatap is a zinc finger transcription factor and Dal80p homolog that negatively regulates nitrogen catabolic gene expression by competing with Gat1p for Gatap site binding and whose function requires a repressive carbon source.[33] Other nitrogen source related transcription factor binding sites include those corresponding to Dal82p and Gln3p. Interestingly, Gal4p, the factor responsible for activation of the *GAL* genes is also identified. Of the remaining 10 promoter binding sites identified within the WT, most are also associated with nitrogen source utilization.

The promoter analysis of the mutant strain during the media switch identified 13 transcription factor binding sites that were present upstream of both up- and down-regulated transcripts (Table 2). Nine unique sites were identified

Table 2 Putative transcription factors identified from promoter analyses[a]

WT YPM *vs* SLAM		*gpa2Δ* YPM *vs* SLAM[d]	
Up-regulated	Down-regulated	Up-regulated	Down-regulated
GATA	None	**GATA**	MIG1
GAL4		**GAL4**	CSRE
DAL82		**DAL82**	MAL63
GLN3		**GLN3**	STE12
QBP		**QBP**	URS1ERG11
UASGATA		**UASGATA**	MATα2
UME6		CUP2	HAP1,2,3,4
URSINO		TcwTATA[e]	REB1
URS1HSC82		URSSGA	HSE, HSTF
HAP1			URSPHR
BUF			ADR1
RoCAR1[c]			RME1
UASGABA			BUF
			UIS
			PHO2,4
			UASCAR
			GA-BF
			ARC
			BAF1
			BAS1
			PRP1
			NBF
			UASPHR
			LEU3
			UASINO

[a] Transcription factor matches based on 6–8 bp identity in the consensus binding sites. [b] Boldface indicates transcription factors common to both WT and *gpa2Δ* strain comparisons. [c] Repressor of CAR1. [d] Common to *gpa2Δ* up- and down-regulated – SWI5, ABF1, HSE(HSTF), IRE, UASCAR, HsnHSE (heat shock), RAP1, DAL82, GAL4, RPO21, MCM1, UASH, MOT3. [e] Tc weak TATA.

upstream of up-regulated transcripts of which six are shared in common with the WT comparison. Twenty-five sites were identified upstream of down-regulated transcripts and, more importantly, four putative factors of interest were identified; namely Mal63p, Mig1p, Csrep and Ste12p. Mal63p is the maltose-activated transcriptional factor that controls expression of the *MAL* loci.[34] As there are many *MAL* loci present in different yeast strains, each expressing slightly different *MAL*-activator alleles, it is not clear that this is a unique Mal63p site; rather it most probably represents a consensus sequence bound by *MAL*-activator proteins in general. Mig1p is a glucose-responsive factor that represses transcription of the *MAL61,62* structural genes as well as the aforementioned *MAL63* gene.[35] Csrep is a transcriptional activator that binds to the carbon source-responsive element of gluconeogenic genes and is also involved in the positive regulation of gluconeogenesis and is regulated by the Snf1p protein kinase complex. Lastly, Ste12p is a MAP kinase signaling cascade factor that activates genes involved in mating or pseudohyphal/invasive growth pathways and cooperates with the Tec1p transcription factor to regulate genes specific for invasive growth.[16,17] Many of the remaining sites identified correspond to factors that regulate other aspects of nitrogen utilization and various different functions ranging from phosphate utilization to transcription itself.

Proteomic analysis under different growth conditions

Mass-spectral analysis of the WT strain media switch identified 797 proteins with a UnProScore >1.3 (797 proteins were identified from at least a single peptide with 95% confidence) of which 64 had a p value <0.01. The same analysis in the *gpa2Δ* strain resulted in 614 proteins with an UnProScore >1.3 of which 34 had a p value <0.01. Only those proteins determined to have a significant change in level between conditions were included, reducing the number of proteins to slightly less than 100. The relative ratio metric changes in protein levels determined are provided in Supplementary Table I and II†. The majority (>95%) of the proteins were identified with UnProScores >4 which corresponds to ⩾99.99% confidence in the identification. Four proteins were identified with scores of 2 and one with a score of 3 which corresponds to 99 and 99.9% confidence, respectively. Further, these five proteins are not involved in the derived model (see below). Mass-spectral data was searched only against the *Saccharomyces* database and, in conjunction with these scores, this allows us to place very high confidence in the protein identifications.

The largest increase in protein level was an ~3 fold change noted for Rps17ap along with a similar magnitude decrease for Leu1p both of which originate from the WT media switch. The media switch to nitrogen starvation in the WT strain resulted in the significant modulation of 60 proteins; 20 were down-regulated, 40 were up-regulated and eight (~10%) were shared in common with the corresponding change in the mutant strain (Table 1 and Supplementary Table I†). The three down-regulated proteins associated with carbon utilization include Tdh3p, a glyceraldehyde-3-phosphate dehydrogenase involved in glycolysis, Tal1p, a transaldolase enzyme in the non-oxidative pentose phosphate pathway and Tpi1p, a triose phosphate isomerase (also present for the same change in the mutant strain). The seven down-regulated proteins associated with nitrogen utilization include Pep4p, the predominant vacuolar proteinase. Under organelle function Qcr2p, a subunit of the ubiquinol cytochrome-c reductase complex and a component of the mitochondrial electron transport chain and whose transcription is regulated by Hap1p, Hap2p/Hap3p, and heme is noted.[24] The remaining down-regulated proteins include heat shock proteins and superoxide dismutatases. Of the 40 up-regulated proteins, 28 or 70% are associated with ribosomal function, protein biosynthesis and nitrogen source utilization including Cdc33p, a cytoplasmic mRNA cap binding protein functional during translation,[36] Asc1p, involved in translation regulation and required for repression of Gcn4p activity in the absence of amino-acid starvation, Cct5p, a putative GroEL chaperonin and several translational/elongation factors. The seven unique proteins associated with carbon utilization here include Cdc19p an allosteric pyruvate kinase that regulates glycolysis, Pfk2p, a phosphofructokinase that is another key regulatory enzyme in glycolysis, Pdc1p, the major pyruvate decarboxylase which is key in alcoholic fermentation and is subject to both glucose-/ethanol- regulation and is involved in amino acid catabolism along with other enzymes required for cell wall synthesis and several aldolases and enolases.[37]

Proteomic analysis of the media switch in the *gpa2Δ* strain results in 30 differentially regulated proteins of which seven (~23%) are common with the WT strain (Table 1 and Supplementary Table I†). The 10 unique down-regulated proteins include Rnr2p, a ribonucleotide-diphosphate reductase,[33] Cdc33p, the translation associated mRNA cap which was up-regulated during the same switch in the WT along with other mitochondrial proteins and several thioredoxin peroxidases.[36] Under carbon utilization, the 13 unique up-regulated proteins noted included Tdh1/3p, two glyceraldehyde-3-phosphate dehydrogenases. Organelle structure/function includes three up-regulated proteins with important roles in the yeast stress response. These include Hsp104p, a stress responsive heat shock protein that functions to refold aggregated or denatured proteins, Trx2p, another thioredoxin isoenzyme which protects cells against both oxidative and reductive stress and Ubi4p, or the ubiquitin protein itself which is conjugated to proteins marking them for selective degradation *via* the ubiquitin-26S proteasome system and which is essential for the cellular stress response.[37,38]

Correlation between transcript and protein levels

Looking at the correlation between protein and transcript levels, several interesting facts are noted. In the WT media switch, the *ASC1* transcript is down-regulated while its protein level is up-regulated. In the mutant media switch, the *ASC1* transcript is again down-regulated but the protein level is not significantly changed. This protein functions as a repressor of Gcn4p, the transcriptional activator of amino acid biosynthetic genes in the absence of amino acid starvation, and so this difference is counter-intuitive and suggests a complex regulation process. In this same WT comparison the Leu1p level is reduced but the transcript is up-regulated. Beyond this

we find no other direct gene–protein correlations, although many of the proteins that are differentially expressed in both comparisons have similar functions to the significantly regulated transcripts within their same class, *i.e.* functional cluster coincidence.

There are many prominent studies that have monitored genome-wide transcriptional changes in *Saccharomyces* in response to a variety of growth, nutrient or stress conditions.[22,39–41] However, significant comparisons cannot be drawn between many of these studies due to differences in growth conditions, strain background and physiology. For example, switching just the carbon source from maltose to glucose is responsible for a global change in gene/protein expression of hundreds of genes due to glucose repression alone.[11–13] Further, the current strain is diploid and is directly compared to a single gene knockout derivative diploid strain while undergoing nitrogen deprivation whereas many of those reported on in other studies are in a haploid state. (It should also be noted that these same issues apply to comparisons in the levels of protein modulation across different studies). Nevertheless, some generalities can be drawn about the expression results. In overall terms, the number of differentially expressed genes reported here, ~600, is comparable to the ~500 reported for growth of yeast under a single growth macronutrient limitation, *i.e.* carbon or nitrogen source.[39] Additionally, it was not surprising to see involvement of *HXK2*, *MIG1* and even *PHD1* in response to the growth limiting conditions due to their multiple putative roles in myriad biophysical processes (see below). In contrast, the metabolic response of galactose grown yeast cells to highly stressful lithium toxicity resulted in the modulation of almost 1400 open reading frames.[22] Within this same study, only 48 proteins, 3.5% of the number of genes *versus* ~15% for the current study, were found to be differentially modulated and only 6 proteins (0.4%), all of which were inhibited, matched a corresponding change in mRNA level. Given this example of an extremely small concordance between mRNA and protein changes with more than twice as many reported differences in mRNA, our result above does not seem particularly unusual.

Glycolytic sensing in pseudohyphal differentiation

Many of the key components of pseudohyphal differentiation signal transduction have been mapped and two parallel pathways have been demonstrated as the primary regulators of this process (Fig. 3).[3,4,20] In the cAMP-dependent pathway, low nitrogen and abundant glucose signal *via* Gpr1p/Gpa2p and the protein kinase A (PKA) components Tpk2p/Bcy1p to regulate Flo8p and Sfl1p which act upon the promoter region of *FLO11* (*MUC1*).[3,4,20] *GPR1* expression is induced by nitrogen starvation and then signals abundant extracellular sugar suggesting it may function as a dual sensor for both conditions.[20] Flo11p is the key glycosylphosphatidylinositol-(GPI) anchored cell surface glycoprotein required for diploid pseudohyphal formation and haploid invasive growth and is known to be regulated from a large and complex promoter region (~3 kb).[42] *FLO11* expression is a good measure of filamentous growth signaling and of a cell's capacity to undergo pseudohyphal differentiation under different conditions.[3,4,20] The second pathway involves a well defined pheromone-responsive MAP-kinase cascade that regulates the promoter region of pseudohyphal differentiation genes through components that are also common to the mating and osmoresponsive pathways.[8] However, the upstream origin of the signal that utilizes this pathway has not been clearly identified.[8] In addition, the transcription factor Phd1p contributes to filamentous growth along with several other genes whose transduction pathways are also undefined.[3,15] Extracellular nitrogen conditions are also sensed and transduced in part by the ammonium permease Mep2p as well as many other factors, although this sensing mechanism is not as clearly defined as the cAMP and MAP-kinase pathways.[3,17]

A further regulatory pathway has been postulated to originate from the sensing or metabolism of carbon sources.[3] This carbon sensing pathway includes Hxk2p the predominant glycolytic hexokinase. Evidence exists that *FLO11* may be repressed through the main glucose repression pathway which requires Hxk2p as a key mediator.[3,43–45] Glucose repression originates, in part, from the hexose transporter homologues Rgt2p and Snf3p which sense and transduce the presence of high and low extracellular glucose concentrations, respectively.[3,10,46] The exact sensing mechanism and nature of the signal generated by these two membrane proteins still remain to be elucidated and the cAMP pathway has already been excluded as a mediator. Another key downstream component of the glucose repression pathway is the Snf1p protein kinase complex which, in part, regulates transcription through the inhibition of repressors such as Mig1p or the stimulation of activators such as Cat8p.[11–13] Snf1p functionality is also partially determined by interaction with the protein phosphatase Glc7p and has further been shown to regulate transcription of *FLO11*.[47]

In comparison to the complexity of pseudohyphal differentiation and its regulation, the components and function of genes involved in maltose metabolism are far simpler. To ferment maltose, yeast cells require an intact *MAL*-locus which consists of three genes. Overall control is elicited by the maltose-responsive transcription factor, the *MAL*-activator, which induces expression of the maltose permease and maltase genes at a bi-functional promoter in the absence of repressor(s). The permease is responsible for maltose uptake from the growth media while maltase cleaves maltose into two glucose molecules allowing them to enter the first step of glycolysis.[21] Glucose repression directly inhibits expression of the *MAL* genes at the transcriptional level along with rapidly inactivating the permease post-translationally and this signal originates, in part, from the hexose transporters, Hxtp's, along with the glucose sensors Rgt2p and Snf3p.[48] Addition of glucose to maltose grown cells induces the rapid phosphorylation and inactivation of the maltose permease which is followed by its ubiquitination, endosomal internalization and vacuolar degradation.[49,50] The glucose-sensitive Mig1p repressor has also been shown to regulate *MAL* gene expression downstream of the glucose signal mediated by the Snf1p complex.[35] As stated previously, growth of the *gpa2Δ* strain on maltose under nitrogen starvation conditions induced what

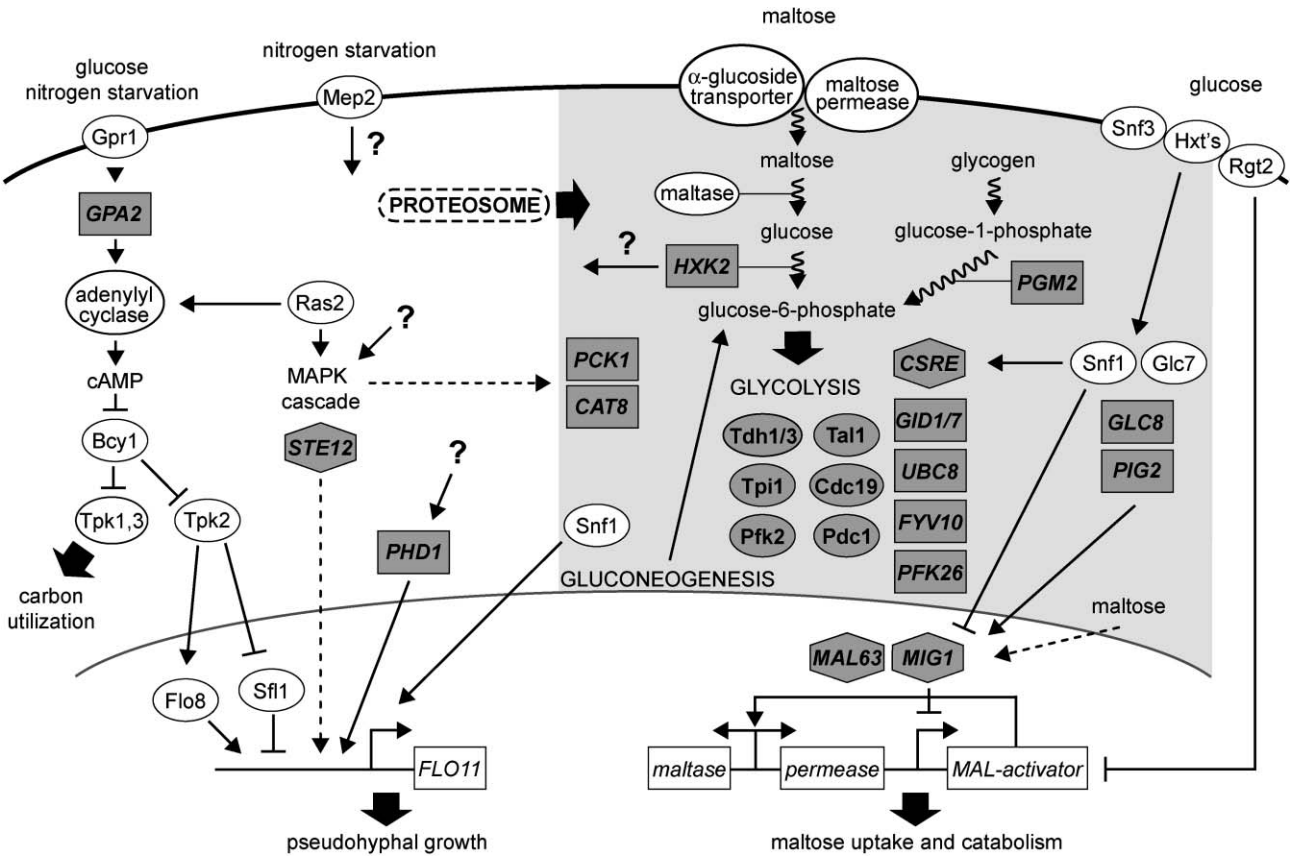

Fig. 3 Schematic model depicting known participants in pseudohyphal differentiation, signal transduction and maltose regulation/metabolism. Gray-colored shapes represent significantly modulated transcripts (rectangles), significantly modulated proteins (ovals) and putative transcription factors (hexagons) identified in this study. Glycolytic enzymes along with regulators thereof in the current model are found within the gray backdrop. Solid thin black arrows indicate positive regulation, solid black lines indicate negative regulation and dashed black arrows indicate speculative interactions. Question marks indicate signals of unknown origin. The *PHD1* transcript was identified in our expression profiling analyses and is thus presented in a rectangle despite its function as a transcription factor.

appeared to be a hyper-filamentous phenotype as compared to glucose.[20] Taking this into account along with the well known and highly complex global role of glucose repression in yeast,[11–13] we opted to evaluate RNA and protein expression of the WT and *gpa2Δ* strains in both rich and nitrogen-starved media with maltose as the sole carbon source; *an approach that allows us to remove many of the complicating effects of glucose repression from the results*.

An overview of the results *in toto* allow some general points to be made. The differences between the WT and *gpa2Δ* mutant strains when under either rich or nitrogen starvation conditions are minor. The bulk of the changes arise within each strain as they undergo the nutrient switch to low nitrogen conditions and these are found predominantly clustered within similar cellular functions (Table 1). This includes such processes as RNA metabolism, cellular organization/biogenesis and nucleotide metabolism with the bulk of the remaining genes associated with various aspects of protein biosynthesis—something that is expected due to the loss of adequate nitrogen sources. However, the *gpa2Δ* strain exhibits almost 5× as many significantly modulated genes with almost 90% of them down-regulated. This result along with the promoter analysis presented in Table 2 strongly argues that although *GPA2* is primarily an upstream glucose sensor, its loss can lead to a global deregulation of many processes, mostly at the transcription level initially, during the physiological switch to filamentous growth.

One method of regulating the switch to filamentous growth may be to target many of the proteins involved with other processes that are no longer required for specific degradation and a recent report has suggested that proteosome degradation may be required to regulate filamentous growth.[40] This would be accomplished, in part, by targeting key cellular proteins for either endosomal internalization and/or proteosomal degradation through their ubiquitination. The upregulation of *UBC8*, a ubiquitin conjugating enzyme which negatively affects gluconeogenesis and *ATG7/8* along the high level of ubiquitin (Ubi4p) provides evidence to support this assertion and suggests that some of the involved proteins may be regulated by their targeting for proteosomal degradation.[38] Additionally, many of the glycolytic enzymes along with maltose permease and *FYV10/GID1/7* which were identified in this study are known to be involved and regulated by this process.[38]

The identified genes and proteins that are associated with carbon source sensing/metabolism or otherwise pertinent to

the current focus are shown in gray in the Fig. 3 signal transduction schematic, and almost all are found associated with glycolysis, gluconeogenesis and glycogen metabolism. The associated transcription factors identified from upstream putative binding sites are also shown as hexagons. Table 3 provides the corresponding comparison from which this data originates along with ratio metric data on the relative expression levels. Transcripts of particular genes of interest in Fig. 3 include *HXK2*, *PCK1*, *CAT8*, *GLC8*, *PIG2, PHD1, UBC8, FYVIO, GID1/7, ATG7/8, PGM2*, and *PFK26* along with the transcription factors encoded by *MIG1*, *STE12* and *CSRE* due to their putative roles in carbon sensing/metabolism and/or transduction of pseudohyphal differentiation. *MAL63* is generally understood to function more exclusively with *MAL* gene induction.[21,34] Differentially expressed proteins include Tdh1/3p, Tal1p, Tpi1p, Cdc19p, Pfk2p and Pdc1p. Interestingly, a recent report suggests that *PDC1* transcription may be regulated by multiple glucose sensing-independent pathways.[51] As their putative function, if any, in regulating pseudohyphal differentiation signal transduction remains to be elucidated, the genes/proteins shown in Fig. 3 are not assigned a positive or negative role. However, these results, in combination with earlier work,[20] allow a general model of the contribution of carbon source sensing/metabolism to pseudohyphal differentiation signal transduction to be posited.

Maltose sensing in pseudohyphal differentiation

In the WT background, growth on maltose induces modest filamentous growth and this effect is exacerbated in the *gpa2Δ* strain[20] (see Fig. 1). Besides removing the Gpr1p/Gpa2p pseudohyphal differentiation signaling pathway and the change in carbon source, the common element being compared between the two strains is the removal of glucose repression. In the absence of the glucose repressing signal originating from Snf3p/Rgt2p *via* the Snf1p complex, growth on maltose alone allows significant amounts of internal glucose to enter glycolysis. This strongly suggests that an upstream pseudohyphal differentiation signal component may originate from internal glycolytic flux. When the Gpr1p pathway is intact this effect is somewhat repressed or modulated, either from cross-talk between the pathways or a different signal. However, removal of the Gpr1p/Gpa2p pathway in conjunction with non-repressing conditions allows a strong pseudohyphal differentiation-inducing signal to originate from internal glucose sensing/metabolism or glycolytic flux. *HXK2*, the catalyst of first enzymatic step in glycolysis and a known participant in carbon source signal transduction,[3] demonstrates the largest ratio metric change between the WT and *gpa2Δ* strains under SLAM conditions in Table 3. Its myriad functions in early glycolysis along with this result strongly argue for a role in this signal transduction pathway. Several possibilities as to how this signal is transduced can be postulated around the function of Hxk2p which may: (i) signal into the existent *GPR1* pseudohyphal differentiation pathway as has been previously suggested,[3,14] (ii) signal *via* the Snf1p kinase complex or (iii) directly interact with appropriate promoter cis-elements. It is likely that the Hxk2p-mediated signal does not involve cAMP, or enters the *GPR1* pathway downstream, as cAMP was not stimulated after addition of maltose to carbon source starved cells including the *gpa2Δ* strain.[20] The implication of roles for *PCK1* and *CAT8* suggest the possibility of an alternative downstream pathway that may lead into the MAP-kinase cascade and stimulate *via* Ste12p/Tec1p. However, unpublished data suggests that Flo8p is required for maltose-induced pseudohyphal differentiation but not *STE12* which argues against a role for the MAP-kinase cascade (personal communication, C. Michels, Queens College CUNY, NY). A third mechanism suggested by Lorenz *et al.*[20] is that a signal may arise from transport by the *MAL*-permease itself similar to Snf3p/Rgt2p function, however, to date no evidence for this has been found. Additionally, constitutive *MAL*-activator alleles do not induce filamentous growth that also suggests that the signal does not arise from the *MAL* system (personal communication, C. Michels, Queens College CUNY, NY). A further possibility is that an unidentified protein(s) can transduce a signal from glycolysis through an existing or novel pathway. As its differential expression was noted with the second largest ratio metric change noted in Table 3 between the WT and *gpa2Δ* strains under SLAM conditions, *PHD1* may very likely be a downstream effector in this pathway. Phd1p is known to induce pseudohyphal differentiation but its upstream regulation still remains unclear, however, the current model suggests a role for coupling glycolytic sensing to *FLO11* regulation and expression. Lastly, many of the key components/enzymes involved in this signaling pathway may be regulated by their targeted proteosomal degradation.

The WT and *gpa2Δ* strains have also been tested for pseudohyphal differentiation induction with galactose as a

Table 3 Ratio metric change in key carbon source utilization transcripts and proteins

Transcript	Comparison	Ratio[a]
HXK2	*gpa2Δ* SLAM *vs* WT SLAM	0.19
PCK1	WT SLAM *vs* WT YPM	9.81
CAT8	*gpa2Δ* SLAM *vs gpa2Δ* YPM	3.17
GLC8	*gpa2Δ* SLAM *vs gpa2Δ* YPM	2.45
PIG2	*gpa2Δ* SLAM *vs gpa2Δ* YPM	3.30
PFK26	*gpa2Δ* SLAM *vs gpa2Δ* YPM	4.02
PGM2	*gpa2Δ* YPM vs WT YPM	2.11
UBC8	*gpa2Δ* SLAM *vs gpa2Δ* YPM	3.58
PHD1	*gpa2Δ* SLAM *vs* WT SLAM	0.30
FYV10	WT SLAM *vs* WT YPM	2.47
GID1/7	*gpa2Δ* SLAM vs *gpa2Δ* YPM	2.36
ATG7	*gpa2Δ* SLAM *vs gpa2Δ* YPM	2.62
ATG8	*gpa2Δ* SLAM *vs gpa2Δ* YPM	2.87
Protein	Comparison	Ratio[b]
Tdh1p	WT SLAM *vs* WT YPM	2.70
Tdh1p	*gpa2Δ* SLAM *vs gpa2Δ* YPM	2.62
Tdh3p	WT SLAM *vs* WT YPM	0.84
Tal1p	WT SLAM *vs* WT YPM	0.87
Tpi1p	WT SLAM *vs* WT YPM	0.74
Cdc19p	WT SLAM *vs* WT YPM	1.16
Pfk2p	WT SLAM *vs* WT YPM	1.29
Pdc1p	WT SLAM *vs* WT YPM	1.24
Ubi4p	*gpa2Δ* SLAM *vs gpa2Δ* YPM	1.47

[a] Ratio of the average signal values of differentially expressed transcripts from three microarray experiments. [b] Ratio of the iTRAQ-generated differentially expressed protein abundance ratios.

carbon source and no significant filamentous phenotype was noted as compared to growth on maltose.[20] In contrast, it has been demonstrated that intracellular metabolic signals originating from a high level of glucose flux through glycolysis can induce inactivation of the maltose permease.[52] Overexpression of galactose permease was able to partially restore glucose transport and inactivate the permease in a strain deficient for glucose repression signaling from the primary hexose transporters and the other membrane glucose sensors. Use of galactose, considered a nonrepressing sugar, also resulted in inactivation of the maltose permease when the galactose transporter was overexpressed and the *GAL* genes fully induced. It is important to note that galactose entry into glycolysis *bypasses* the hexokinases. Maltose is also able to weakly signal inactivation of the maltose permease due to signal from glycolytic flux.[52] Taken together, these results (i) suggest that a high rate of metabolic flux may be necessary, (ii) provide more evidence that Hxk2p, or other hexokinase function in *early* glycolysis may be important to generating the necessary signal, and (iii) suggest that the signaling pathway is independent of those that regulate the *MAL*-system or inactivate the maltose permease. Interestingly, sugar transport and phosphorylation have been associated with glucose sensing in pancreatic cells suggesting that this mechanism may be evolutionarily conserved.[53]

Conclusions

The advent of transcriptome and proteomic expression analyses tools now allows for the cellular monitoring of global responses to various (extra)cellular conditions. This approach can help provide insight into the function of an entire signal transduction pathway. In this report, we have applied these tools to understand how growth on maltose induces a hyperfilamentous phenotype in a *gpa2Δ* diploid yeast strain. The abrogation of the *GPA2* signal pathway under non-repressing conditions in conjunction with the current results from growth on maltose under nitrogen starvation conditions and previous results[3,20,52] suggest that a signal for pseudohyphal differentiation may originate from an early sensing or metabolic step in glycolysis. Hxt2p or another hexokinase appears to be a likely candidate for this sensor. This pathway is not unexpected as multiple levels of nutrient sensing are involved with regulating the cellular commitment to this drastic physiological process.[3] Clearly, the complexity, overlap and possibility of multiple functions for each component will complicate the elucidation of such a pathway. This difficulty has already been encountered with the multiple carbon source sensing pathways in yeast and the pathway suggested here may incorporate many of the same components.[3] Additionally, the still unclear role(s) of nitrogen sensing cannot be excluded from the regulation of this signal transduction pathway. Future work will focus on testing key carbon source signaling components including *HXK2*, *SNF1* and *MIG1*. An understanding of how a biological system can transduce and physiologically respond to its environment will have impact not only on basic biology but also on understanding aberrant disease processes and *S. cerevisiae* still continues to provide a powerful and complex model system for this endeavor.

Experimental

Yeast strains, media and growth conditions

Diploid strains MLY61 **a**/α (*ura3-52/ura3-52 MAT**a**/α)* and MLY132 **a**/α (*Δgpa2::G418/Δgpa2::G418 ura3-52/ura3-52 MAT**a**/α*) were obtained from J. Heitman, Duke University.[16,20] Standard YPM media were used as described.[54] Synthetic low ammonium media consisting of SLAM (0.17% Yeast Nitrogen Base without ammonium sulfate or amino acids, 50 μM ammonium sulfate, 2% maltose) were used when switching to low nitrogen pseudohyphal inducing conditions.[16,20] Similar to a previously described method,[18] strains were grown to an OD_{600} of 1.0 in YPM media. Approximately 200 OD_{600} units were collected by filtering followed by resuspension in 10 mL RNAlater (Ambion) for <30 min, pelleted by centrifugation, flash frozen and stored at −80 °C. Strains were also grown to an OD_{600} of 1.0 in YPM media and approximately 200 OD were collected by filtration and transferred to SLAM media for 2 h at 30 °C prior to collection as above (media switch).

Expression profiling analyses

RNA isolation. The Ribopure™-Yeast kit (Ambion) was used to extract total RNA from yeast cells. Briefly, for each RNAlater-treated sample, cells were pelleted in a microtube and any remaining RNAlater removed. Then lysis buffer, 10% SDS, and 25 : 24 : 1 phenol : chloroform : isoamyl alcohol were used to resuspend the pellet, and the mixture was transferred to a screw cap microtube with Zirconia beads (BioSpec Products) to disrupt the yeast cells by vortexing. The lysate was centrifuged, and the upper aqueous layer transferred to 15 mL conical tubes with Binding Buffer and 100% ethanol. The mixture was drawn through a filter cartridge to capture the RNA, followed by washing and elution. To remove contaminating DNA, 50 μL of the eluted RNA was treated with 10 μL of 10× DNase I buffer and 4 μL of 8 units μL^{-1} DNase I. The reaction was mixed, incubated at 37 °C for 30 min, followed by addition of 0.1 volume DNase Inactivation Reagent. After 5 min at room temperature, the mixture was spun through a spin column (Qiagen) to remove the DNase binding beads in the DNase Inactivation Reagent. The quality/quantity of purified yeast total RNA was determined by separating 1 μL from each sample on a Bioanalyzer 2100 (Agilent).

Target preparation and hybridization to arrays. Eight μg of RNA were concentrated *via* ethanol precipitation as previously described.[55] All subsequent steps were as described in the GeneChip Expression Analysis Technical Manual version 701021 Rev. 3 (Affymetrix). Briefly, for each sample, the purified, concentrated RNA was reverse-transcribed using a poly-T primer with a T7 RNA-polymerase promoter sequence into double stranded cDNA followed by linear amplification *via in vitro* transcription (BioArray High Yield IVT kit, ENZO) to yield an antisense cRNA labeled with biotin. The purified biotinylated cRNA was fragmented by magnesium ions and heat and mixed with the hybridization cocktail that contained biotinylated cRNA controls with known concentrations to monitor the quality of the hybridization procedure.

The cocktail was hybridized onto the GeneChip® Yeast Genome S98 (YG_S98) microarray (Affymetrix) which contains probe sets for 6,400 genes of the *S. cerevisiae* S288C strain identified in the *Saccharomyces* Genome Database (SGD) as of December 1998 and 600 additional probe sets for putative open reading frames (ORFs) identified by SAGE analysis, mitochondrial proteins, TY proteins, plasmids, and a small number of ORFs for strains other than S288C. Hybridization was carried out for 16 h at 45 °C with rotation at 60 rpm. The microarrays were then washed and stained using the Affymetrix GeneChip Fluidics Station 450 and scanned using the GeneChip Scanner 3000.

Data acquisition, integration, and analysis. GeneChip® Operating Software 1.2 (GCOS 1.2) (Affymetrix) was used to acquire and process array images, determine the quantity and presence or absence of a transcript using the Microarray Suite 5.0 (MAS5) algorithm, and generated the Report files summarizing the quality of target detection for each microarray. We used the RMA (robust multi-chip analysis) transcript quantities because this method improved detection of differentially expressed transcripts compared to the MAS5 algorithm.[23] However, MAS5 present and absent calls were still used for the filtering of probe sets. Arraytools 3.3.0 developed by Simon and Lam (http://linus.nci.nih.gov/BRB-ArrayTools.html) was used to calculate RMA transcript quantities, integrate transcript data with experimental information such as yeast mutant strains and growth conditions, and to determine differentially expressed transcripts *via* ANOVA (analysis of variance) and the random variance model.

JMP software (SAS) was used to join various data sets together, perform statistical quality control, and determine relations among variables. For joining transcript and protein data, probe set identifiers were mapped to SwissProt identifiers *via* NetAffx[56] and subsequently matched and joined to protein identifiers from mass spectrometry data output by Pro Group.[57] DAVID 2.0[58] was used to convert Affymetrix probe set identifiers and Pro Group SwissProt protein identifiers to yeast open reading frames (ORFs) or S*accharomyces* Genome Database (SGD) identifiers for input into the SGD GO Term Finder tool (http://db.yeastgenome.org/cgi-bin/GO/goTermFinder) to group up-regulated or down-regulated transcripts or proteins into biological processes. The yeast ORFs derived from DAVID 2.0 were also used for input into the Retrieve Sequence (http://rsat.ccb.sickkids.ca/) tool to obtain sequences for promoter analysis. Up-regulated or down-regulated transcripts from each class comparison were input into the promoter analysis tool.[31,32] Promoter analysis was performed on −800 to −1 sequence before the start codons of genes expressing differential transcripts. Sequential putative oligomer or dyad sequences were searched against known transcription factor consensus binding sequences using the Search Putative Sites (http://rulai.cshl.edu/SCPD/search-putative.html) and the Search Existing Motif tools (http://rulai.cshl.edu/SCPD/searchmotif.html) from The Promoter Database of *S. cerevisiae* (http://rulai.cshl.edu/SCPD/). Matches consist of 6 to 8 basepair identity in a transcription factor's known/consensus binding site.

Mass spectrometry analyses

Preparation of soluble protein fractions. A 100 μL cell pellet of each sample was resuspended in 700 μL of lysis buffer (8 M urea, 2 μg mL^{-1} pepstatin A, 50 mM triethylammonium bicarbonate, pH 8.5, 20 mM EDTA). A 400 μL volume of glass beads was added to each sample and vortexed for 5 min and then cooled on ice for 5 min. This cycle was repeated four more times. Samples were centrifuged at top speed using a tabletop centrifuge for 10 min and the supernatant was drawn off and ultracentrifuged at 150 000 × g for 1 h to further clarify the soluble fraction. The supernatant was drawn off and the protein concentration was determined using the DC protein assay (BioRad).

Protein digestion and iTRAQ labeling. Cysteine residues were reduced using 6 mM DTT for 2 h at room temperature and then blocked by incubating the protein mixture with 14 mM iodoacetic acid for 30 min in the dark. 100 μg of each sample was diluted in 1 M urea with 50 mM triethylammonium bicarbonate and digested with trypsin overnight using an enzyme : protein ratio of 1 : 50. Each of the samples was labeled with the individual iTRAQ reagents according to manufacturer specifications. Samples were combined and evaporated to dryness and excess iTRAQ reagents and salts removed using reverse phase C18 TopTips (PolyLC).

Offline hydrophilic interaction chromatography (HILIC) HPLC peptide separation. The samples were resuspended in HILIC mobile phase A (85% acetonitrile, 15 mM ammonium formate, pH 3) in preparation for HILIC HPLC prefractionation before LC-MS analysis. Peptides were separated using a 200 × 4.6 mm polyhydroxyethyl A column (PolyLC Inc., Columbia, MD). The flow rate was set at 1 mL min^{-1} and samples were collected in 1 min increments with a gradient of 0% mobile phase B (5% acetonitrile, 15 mM ammonium formate, pH 3) for 10 min, 0–20% B for 50 min, 20–50% B for 10 min, 50–100% B for 1 min, 100% B for 5 min, 100–0% B for 1 min, and 0% B for 23 min. A total of 80 fractions were collected and dried down in a speed-vac in preparation for LC-MS analysis.

LC-MS, protein identification and relative quantification. Samples to be compared were measured simultaneously in the same experiment. HPLC fractions were dried down to remove acetonitrile and resuspended in mobile phase A. Peptides were separated and analyzed using an Ultimate nanoLC System (Dionex) with a 5 mm PepMap100 C18, 5 μm, 100 Å pre-column and a 15 cm × 75 μm I.D. PepMap100 C18, 3 μm, 100 Å column (Dionex) coupled to a QStar Pulsar I mass spectrometer (Applied Biosystems). Here, mobile phase A consisted of 97.4% water, 2.5% acetonitrile, 0.1% formic acid, and mobile phase B consisted of 99.9% acetonitrile, 0.1% formic acid. Peptide separation was performed using a 60 min gradient of from 3–35% mobile phase B. Selected fractions from experiments were rerun to confirm instrument reproducibility which confirmed identifications in all cases.

Protein identification was performed using the Analyst 1.1 integrated software suite with Bioanalyst extensions and

ProQuant 1.1 (Applied Biosystems) accessing the UniProt Knowledgebase Release 7.6 consisting of the SwissProt/TrEMBL *Saccharomyces* databases. The MS mass tolerance is 0.3 Da and the MS/MS mass tolerance −0.3 Da. Ions with charged states of +2 to +3 were chosen for CID and MS/MS analysis. Search parameters included carboxamidomethyl modifications of cysteine residues and oxidation of methionine residues. The ProQuant 1.1 software automatically performs searches uses CNBr, chymotrypsin, asp N, arg C, trypsin, lys C, glu C and acid cleavage as digest agents so no specific enzyme was indicated. Up to 1 missed cleavage was indicated in the search parameters. All other parameters were automatically selected by the software. Protein identifications with confidence scores of $\geqslant$ 95% were recorded and the iTRAQ reporter ion ratios were used to determine the protein abundance ratios between the different samples.

Note

The expression data set described in this manuscript will be made available at GEO (http://www.ncbi.nlm.nih.gov/geo/) under submission number GSE7820 or NCBI tracking system # 15284919 with the title "Transcript and Proteomic Analysis of Wild-type and GPA2 Mutant *Saccharomyces cerevisiae* Strains."

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