Effects of Abolishing Whi2 on the Proteome and Nitrogen Catabolite Repression-Sensitive Protein Production

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ABSTRACT

In yeast physiology, a commonly used reference condition for many experiments, including those involving Nitrogen Catabolite Repression (NCR), is growth in Synthetic Complete (SC) medium. Four SC formulations, SC⁰, SC⁰⁰⁰⁵, SC⁰⁰⁰⁴, and SC⁰⁰⁰⁵, have been used interchangeably as the nitrogen-rich medium of choice (Cold Spring Harbor Yeast Course Manuals, SC⁰, and a formulation in The Methods in Enzymology SC⁰). It has been tacitly presumed that all of these formulations support equivalent responses. However, Chen et al. (2018) concluded that: (i) TorC1 activity is down regulated by the lower concentration of primarily leucine in SC⁰ relative to SC⁰. (ii) The Whi2-Psr1/2 complex is responsible for this down regulation. TorC1 is a primary nitrogen-responsive regulator in yeast. Among its downstream targets is control of NCR-sensitive transcription activators Gln3 and Gat1. They in turn control production of catabolic transporters and enzymes needed to scavenge poor nitrogen sources (e.g., Proline) and activate autophagy (ATG14). One of the reporters used in Chen et al. was an NCR-sensitive DAL80-GFP promoter fusion. This intrigued us because we expected minimal if any DAL80 expression in SC medium. Therefore, we investigated the source of the Dal80-GFP production and the proteomes of wild type and whi2Δ cells cultured in SC⁰ and SC⁰⁰⁰⁵. We found a massive and equivalent reorientation of amino acid biosynthetic proteins in both wild type and whi2Δ cells even though both media contained high overall concentrations of amino acids. Gcn2 appears to play a significant regulatory role in this reorientation. NCR-sensitive DAL80 expression and overall NCR-sensitive protein production were only marginally affected by the whi2Δ. In contrast, the levels of 58 proteins changed by an absolute value of log₂ between 3 and 8) when Whi2 was abolished relative to wild type. Surprisingly, with only two exceptions could those proteins be related in GO analyses, i.e., GO terms associated with carbohydrate metabolism and oxidative stress after shifting a whi2Δ from SC⁰ to SC⁰⁰⁰⁵ for 6 hours. What was conspicuously missing were proteins related by TorC1- and NCR-associated GO terms.

INTRODUCTION

Free living yeast cells face constantly changing nutritional environments. In response, they have evolved sophisticated mechanisms to successfully exploit times when nutrients are plentiful and tolerate those when they are not. In Saccharomyces cerevisiae, one of the principal mechanisms for sensing and responding to varying environmental conditions depends on the global nutrient-responsive protein kinase Target of Rapamycin Complex 1 (TorC1) (Beck and Hall 1999; Cardenas et
TorC1 Regulation

TorC1’s responses to changes in the nitrogen supply controls cell growth, and the downstream pathways associated with it. For example, when TorC1 is active, ribosome production and the proteins needed to support translation as well as many other requisites for cell division are upregulated. Simultaneously, pathways that have evolved to deal with nutrient deprivation, e.g., nitrogen scavenging, autophagy and vacuolar nutrient mobilization, are severely down-regulated (Broach 2012; Gonzalez and Hall 2017; Sutter et al. 2014; Ljungdahl and Daignan-Fornier 2012). When TorC1 is active, the PP2A and PP2A-like Sit4 phosphatases interact with TorC1-bound Tor-Associated Protein Tap42 (Figure 1) (Di-Como and Arndt 1996; Jiang and Broach 1999; Wang et al. 2003; Yan et al. 2006; Rohde et al. 2008). In this form the phosphatases are inactive. As a result, Gln3 and Gat1, the NCR-sensitive transcriptional activators, are sequestered in the cytoplasm bound to the negative regulator protein Ure2. (Courchesne, W. E., and B. Magasanik 1988; Coffman et al. 1994; Blinder et al. 1996; Cunningham et al. 2000; Kulkarni et al. 2001; Carvalho and Zheng 2003). Cytoplasmic Gln3 sequestration not only requires Ure2, but also the unbound forms of Sit4 and PP2A and the Gln3 dephosphorylations they mediate in nitrogen-rich medium (Tate et al. 2018; Tate et al. 2019).

The detailed molecular mechanisms through which amino acids control TorC1 activity are being increasingly understood (Figure 1) (Binda et al. 2009; Binda et al. 2010; Bonfils et al. 2012; Sutter et al. 2013; Panchaud et al. 2013a, 2013b; Stracka et al. 2014; Peli-Gulli et al. 2015; Ukai et al. 2018; Hu et al. 2019; Hatakeyama and De Virgilio 2019a, 2019b; Hatakeyama et al. 2019a,b). In amino acid excess, active GTP Exchange Factor (GEF) Vam6 converts the Gtr components of the Gtr-Ego complex to their active Gtr1\textsuperscript{GTP}-Gtr2\textsuperscript{GDP} form. This activated Gtr-Ego complex, in turn physically interacts with and activates TorC1 at the vacular membrane. Leucine tRNA synthetase (LeuRS) complexed with leucine also promotes Gtr1-GTP formation (Figure 1, left panel). TorC1 is also up regulated by Pib2 during high glutamine conditions. Further, glutamine, interacting with Pib2, activates TorC1 in a purified in vitro system (Tanigawa et al. 2021).

TorC1 activity is additionally regulated by three important complexes: SEACAT, consisting of Sec13, Seh1, Sea2-4; SEACIT, consisting of Im1, Npr2, and Npr3; and methylated PP2A (Figure 1, left panel). In excess nitrogen, SEACAT inhibits SEACIT. As a result, the inactive form of SEACIT is unable to regulate TorC1 activity. SEACIT is additionally inhibited by methylated PP2A, which occurs when methionine and hence S-adenosyl methionine concentrations are high.

As amino acid supplies dwindle, the GTPase Activating Protein (GAP) complex, Lst4-Lst7, converts the Gtr complex to its inactive Gtr1\textsuperscript{GDP}-Gtr2\textsuperscript{GTP} form (Figure 1, right panel). SEACAT is no longer able to inhibit the actions of SEACIT, which now down-regulates TorC1. Low leucine, methionine and glutamine additionally lowers the activities of the positive TorC1 regulators: LeuRS, PP2A and Pib2.

Nitrogen Catabolite Repression-Sensitive Regulation


NCR consists of shared regulation by both TorC1 and Gcn2, another well studied global amino acid-responsive kinase (Staschke et al. 2010; Tate et al. 2017). When TorC1 is active, the PP2A and PP2A-like Sit4 phosphatases interact with TorC1-bound Tor-Associated Protein Tap42 (Figure 1) (Di-Como and Arndt 1996; Jiang and Broach 1999; Wang et al. 2003; Yan et al. 2006; Rohde et al. 2008). In this form the phosphatases are inactive. As a result, Gln3 and Gat1, the NCR-sensitive transcriptional activators, are sequestered in the cytoplasm bound to the negative regulator protein Ure2. (Courchesne, W. E., and B. Magasanik 1988; Coffman et al. 1994; Blinder et al. 1996; Cunningham et al. 2000; Kulkarni et al. 2001; Carvalho and Zheng 2003). Cytoplasmic Gln3 sequestration not only requires Ure2, but also the unbound forms of Sit4 and PP2A and the Gln3 dephosphorylations they mediate in nitrogen-rich medium (Tate et al. 2018; Tate et al. 2019).
As nitrogen supplies are depleted, Gcn2 is activated and TorC1 inhibited, resulting in release and thereby activation of the Tap42-phosphatase complexes from TorC1 (Figure 1, right panel). The Tap42-phosphatase complexes in turn dephosphorylate Gln3 permitting it to enter the nucleus and support NCR-sensitive transcription of genes encoding the transport and catabolic enzymes needed to scavenge poor nitrogen sources such as proline or allantoin. Gln3 also activates ATG14 expression required for autophagy (Chan et al. 2001).

Conditions That Influence Gln3 Regulation

There are five experimental conditions that elicit NCR-sensitive gene expression (Tate and Cooper 2013). Each condition has a specific requirement for the Sit4 and PP2A phosphatases: nitrogen limitation, i.e., growth in poor nitrogen sources, e.g., proline (Sit4); short-term nitrogen starvation (1-4 hours depending on the strain background) (Sit4); long-term nitrogen starvation independent of, but associated with, G-1 arrest (greater than 6 hours) (neither phosphatase); treating cells growing in nitrogen rich medium with the TorC1 inhibitor rapamycin (PP2A and Sit4); or the glutamine synthetase inhibitor methionine sulfoximine (Msx) (neither phosphatase).

Whi2-Mediated Regulation

When leucine, and other less defined amino acid, concentrations are low, a second amino acid-responsive protein complex, Whi2-Psr1/Psr2 has been reported to negatively regulate TorC1 activity in a SEACIT-Gtr- and PKA-independent manner (Figure 1, right panel). Whi2 was originally identified in a genetic screen of very small (wee) cells that continued to divide rather than G1 arrest as cultures transitioned into stationary phase (Carter and Sudbery 1980). Whi2, in association with the plasma membrane bound Psr1/2 phosphatases, were reported to dephosphorylate Msn2 thereby activating a general stress response (Kaida et al. 2002; Martínez-Pastor et al. 1996). This correlates with the observations that loss of Whi2 elicits hypersensitivity to heat, oxidative stress, and acetic acid.

In colonies growing on complete respiratory medium GM, Whi2 (together with Psr1 and Psr2) is involved in cell cooperation. The absence of Whi2 leads to competitive superiority via the mechanism of interference competition, likely due to increased production of an inhibitory metabolite. This Whi2-Psr/Psr2 function is not associated with TORC1, i.e., TorC1 is active in both wild type and whi2Δ cells under the conditions assayed. (Marsikova 2020).

Whi2 regulation in high and low amino acids

More recently, an exciting phenotype of whi2 mutants was discovered fortuitously when their growth was compared to that of wild type cells provided with either of two routine formulations of nitrogen-rich Synthetic Complete, SC medium: one published in the 1994 and 2005 Cold Spring Harbor manuals (SC<sub>CSH</sub>) and the other in Methods of Enzymology (SC<sub>ME</sub>) (Kaiser et al. 1994; Amberg et al. 2005; Guthrie and Fink 1991). The main difference between these media is the overall concentrations of amino acids, particularly leucine; SC<sub>ME</sub> medium has ~30% lower concentrations of amino acids and greater than 10-fold less leucine than SC<sub>CSH</sub>. The whi2Δ mutant cells were observed to grow much better than wild type in the lower amino acid medium. In a genome-wide survey of BY4741 knockout strains, npr2Δ and npr3Δ mutants were identified along with the whi2Δ based on this phenotype, i.e., mutants exhibiting limited growth during nitrogen starvation, but more robust growth in low amino acid medium (Teng et al. 2013, 2018, 2019). The identification of these three strains among the knockouts was important because, as noted above, Npr2 and Npr3 are components of the SEACIT inhibitor of TorC1 activity.

Subsequent studies of the whi2Δ led to the conclusions that Whi2: (i) is required to dampen TorC1-dependent cell growth and division as amino acid nitrogen decreases, but is not exhausted, and (ii) is a highly conserved inhibitor of TorC1 in response to low amino acids, particularly leucine (Teng
et al. 2018, 2019). However, low concentrations of leucine alone are insufficient to elicit Whi2-mediated TorC1 inhibition, low concentrations of other amino acids are required as well for inhibition to occur. Whi2 regulation of TorC1 appears to be restricted to amino acid sensing as the loss of Whi2 did not affect TorC1 inhibition elicited by low glucose (Chen et al. 2018).

Three widely-used assays were employed to assess the effect of Whi2’s ability to inhibit TorC1 activity in SC<sub>CSH</sub> and SC<sub>ME</sub> media: (i) suppressed phosphorylation of the TorC1 target small ribosomal subunit protein Rps6, (ii) suppressed phosphorylation of Npr1 protein kinase, and (iii) expression of an NCR-sensitive reporter construct (Chen et al. 2018). The reporter employed was a DAL80-GFP promoter fusion plasmid in which GFP expression was driven by a DAL80 promoter. The latter reporter was also used by Neklesa and Davis (2009) to identify npr2 and npr3 deletions (Rousselet et al. 1995). A Gat1-GFP assay was also used, along with a rapamycin control in recent studies investigating the regulation and dynamics of the expansion of papillae that arise during colony aging (Marsikova 2020).

Present Study
The DAL80-GFP results obtained in the Whi2 study were surprising to us because both SC media contain highly repressive amounts of nitrogen. NCR-sensitive regulation has never, to our knowledge, been compared in SC<sub>CSH</sub> and SC<sub>ME</sub> media because these media, when used, are the negative control conditions for NCR-sensitive expression experiments. Therefore, our objective was to obtain a greater understanding of what occurred at the protein level when wild type and whi2Δ cells were grown in or downshifted from the higher amino acid containing SC<sub>CSH</sub> to lower containing SC<sub>ME</sub> medium.

The data obtained demonstrate the major change in the proteome when cells are transferred from SC<sub>CSH</sub> to SC<sub>ME</sub> medium is a dramatic shift in amino acid metabolism, including many proteins that participate in amino acid biosynthesis even though SC<sub>CSH</sub> and SC<sub>ME</sub> media contain a high concentration (0.5%) of ammonia and 0.12% or 0.176% amino acids, respectively. Further, Whi2 presence or absence had only a marginal effect on overall NCR-sensitive gene expression. The DAL80 (Dal80-GFP) expression, observed by Chen et al. (2018) likely derived from the fact DAL80 transcription is strongly activated by Gat1 whose production is autogenously regulated and somewhat insensitive to nitrogen catabolite repression. In contrast to Dal80, there were far stronger effects on the levels of many other proteins.

MATERIALS AND METHODS

Strains, plasmids and culture conditions
The <i>S. cerevisiae</i> strains and the plasmids we used in this work are in Table 1. This strain background was selected for analysis so that data from the present study can be directly compared with past and future data. Transformants, prepared by the lithium acetate method (Ito et al. 1983), were used as soon as possible after transformation (5 or less days).

 Cultures (50 ml) were grown to mid-log phase (A<sub>600 nm</sub> ~ 0.5) in Yeast Nitrogen Base (YNB, without amino acids or ammonia; VWR Life Science AMRESCO) minimal medium containing the indicated nitrogen source (final concentration, 0.1%). Leucine (120 μg/ml), histidine (20 μg/ml), and lysine (40 μg/ml) were added as needed to cover auxotrophic requirements. SC cultures were grown in either Synthetic Complete Cold Spring Harbor (SC<sub>CSH</sub>) or Synthetic Complete Methods in Enzymology (SC<sub>ME</sub>) to the A<sub>600 nm</sub> indicated in the figures. Cells were treated with 200 ng/ml rapamycin (Sigma) for 15 or 20 minutes or 2 mM methionine sulfoximine (Msx; Sigma) for 30 minutes (Georis et al. 2011).
Gln3-Myc\textsubscript{13} and Gat1-Myc\textsubscript{13} localization and image processing

These methods are reproduced from Tate et al. (2021) with permission of the publisher. Cell collection and Gln3-Myc\textsubscript{13} [and Gat1-Myc\textsubscript{13}] visualization by indirect immunofluorescence microscopy were performed as described (Feller et al. 2013; Tate et al. 2019). Microscopic images for presentation were prepared using Adobe Photoshop and Illustrator programs. Level settings (shadow and highlight only) were altered where necessary to avoid any change or loss in cellular detail relative to that observed in the microscope; changes were applied uniformly to the image presented and were similar from one image to another. Mid-tone, gamma settings were never altered. These processed images were used for illustrative presentation only, NOT for scoring Gln3-Myc\textsubscript{13} intracellular distributions.

Determination of intracellular Gln3-Myc\textsubscript{13} or Gat1-Myc\textsubscript{13} localization

These methods are reproduced from Tate et al. (2021) with permission of the publisher. Gln3-Myc\textsubscript{13} intracellular localization was manually scored in 200 or more cells for each data point. Unaltered, primary .zvi image files viewed with Zeiss AxioVision 3.0 and 4.8.1 software were exclusively used for scoring purposes. Cells were classified into one of three categories: cytoplasmic (cytoplasmic fluorescent material only, red histogram bars), nuclear-cytoplasmic (fluorescent material appears in both the cytoplasm and co-localizing with DAPI-positive material, DNA, yellow bars), or nuclear (fluorescent material co-localizing only with DAPI-positive material, green bars). Representative “standard” images and detailed descriptions of these categories appear in Figure 2 of Tate et al. 2009. The precision of our scoring has been repeatedly documented (Tate et al. 2006; Tate et al. 2010; Rai et al. 2013; Rai et al. 2014). Standard deviations of data from independent experiments appear as error bars. Greatest variation was observed when Gln3-Myc\textsubscript{13} was significantly localized to more than one cellular compartment.

Images accompanying the histograms were chosen on the basis that they exhibited intracellular Gln3-Myc\textsubscript{13} distributions as close as possible to those observed by quantitative scoring. However, identifying a field that precisely reflected the more quantitative scoring data was sometimes difficult unless the tagged protein was situated in a single cellular compartment.

Cell collection for western blot or qRT-PCR analyses

These methods are reproduced from Tate et al. (2021) with permission of the publisher. Cultures to be analyzed were grown to mid-log phase (A\textsubscript{600nm} = 0.4–0.5) as described above. Once the desired A\textsubscript{600nm} was reached, or following treatment, the cells were harvested by filtration (using type HA, 0.45 mm Millipore filter), quickly scraped from the filter, placed in a sterile 1.5-ml microcentrifuge tube, and flash-frozen by submerging the microcentrifuge tube and cells in liquid nitrogen for 20–30 sec. The total time for cell harvest to the point of submersion in liquid nitrogen was 25–35 sec. The tube, still containing liquid nitrogen, was then quickly transferred to -80°C until further processing of the cells was performed.

Protein extraction and western blot analyses

These methods are reproduced from Tate et al. (2021) with permission of the publisher. Extracts for western blots were prepared following the method of Liu et al. (2008). Total protein was extracted by lysing cells in a solution of 0.3 N NaOH, 1.2% β-mercaptoethanol (final concentrations), on ice for 10 min. Protein was then precipitated with trichloroacetic acid (TCA) at a final concentration of 8%, for an additional 10 min on ice. Precipitated protein pellets were then resuspended in 1x sodium dodecyl sulfate (SDS) loading buffer and the extract neutralized with 1 M unbuffered Tris. Crude extracts were then boiled, protein resolved by SDS-PAGE (6 or 7% polyacrylamide) and transferred to nitrocellulose membrane (Bio-Rad) in non-SDS containing buffer.

Membranes were blocked for 1 hr at room temperature with 5% Carnation milk in 1x TTBS (20...
mM Tris-HCl pH 7.5, 0.05% Tween20, 0.5 M NaCl). Membranes were then incubated overnight at room temperature with 9E10 (c-Myc) monoclonal antibody (sc-40; Santa Cruz Biotechnology) at a dilution of 1:1000 in 1x TBS (20 mM Tris-HCl pH 7.5, 0.5 M NaCl) plus 0.25% gelatin. Membranes were washed with 1x TBS and incubated with goat anti-mouse IgG (H+L)-horseradish peroxidase conjugate antibody (Bio-Rad) at a dilution of 1:10,000 for 1 hr in 1x TBS containing 0.005% Tween20 and 0.25% gelatin. Membranes were washed with 1x TBS containing 0.005% Tween20 buffer. Immuno-reactive species were detected using the SuperSignal West Pico Chemiluminescent Substrate kit (ThermoScientific) following the manufacturer instructions and results recorded on Classic blue autoradiography Film BX (Midwest Scientific).

**qRT-PCR analyses**

These methods are reproduced from Rai et al. (2015) with permission of the publisher. Total RNA was extracted using the RNeasy Mini Kit (Qiagen), following the manufacturer’s instructions for purification of total RNA from yeast—mechanical disruption of cells. Two modifications were made to this protocol from our previous report: (i) cells were broken with glass beads (0.45 µm) using a BeadBug homogenizer (Benchmark Scientific): 4000 rpm, 4° for 30 sec intervals followed by 30 sec in an ice bath, and (ii) on-column RNase-free DNase I treatment was performed for 1 hr instead of 40 min. Quality of the total RNA was analyzed on an Agilent 2100 Bioanalyzer using the Agilent RNA 6000 Nanochip by the University of Tennessee Health Science Center (UTHSC) Molecular Resource Center. Complementary DNAs (cDNAs) were generated using the Transcriptor First Strand cDNA Synthesis Kit (Roche) following the manufacturer’s recommended protocol using both Oligo(dT),8 and random hexamer primers (provided with the kit) for synthesis. Samples were prepared for quantification with LightCycler 480 SYBR Green I Master Mix (KAPABiosystems) using the manufacturer’s protocol. Quantification and subsequent analysis of cDNAs were performed on a Roche LightCycler 480 Real Time PCR System using LightCycler 480 software version 1.5.

**Proteomic analyses**

Comparison of proteomes was performed by nano LC-MS/MS analysis. Harvested cells were disrupted with glass beads (five times for 20 s in Fast-Prep, Thermo Savant) in 100 mM triethylammonium bicarbonate (TEAB), 10 mM Tris(2-carboxyethyl)phosphine, 50 mM chloroacetamide buffer containing 2% sodium deoxycholate; after the first two runs, samples were heated at 95 °C for 5 min. Protein aliquots (30 µg per sample; determined by bi-quinoninic acid assay, Sigma) were used for MS sample preparation. Samples were further processed with SP3 beads according to Hughes et al. (2019). Briefly, 5 µl of SP3 beads were added to 30 µg of proteins in lysis buffer and diluted to 50 µl with 100 mM TEAB. Protein binding was induced by adding ethanol to a final concentration of 60% (v/v). Samples were mixed and incubated for 5 min at lab temperature. Beads were washed twice with 180 µl of 80% ethanol and then samples digested with trypsin (trypsin/protein ratio 1/30), acidified with TFA to 1% final concentration. Peptides were desalted with C18 disks (Empore). Peptides (2 µg) from each sample were separated on nano-reversed-phase columns (EASY -Spray column a 50-cm x 75 mm ID, PepMap C18, 2 µm particles using a 1-h elution gradient and analyzed in DDA mode on an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific).

**Proteomic data analysis**

Three biological replicates were analyzed for each strain and condition. Raw files were processed in MaxQuant (v. 1.5.8.3) and checked against the latest version of the *S. cerevisiae* Uniprot database and the common contaminant database. Perseus (v.1.6.1.1) and Excel 2013 were used for further analysis. The significance of differences in protein abundance between two strains or conditions was determined using the unpaired two-tailed t-test. p-values of 0.05 or less were considered statistically significant. Functional categories enriched in specific proteome comparisons were identified using the GO term finder at SGD (https://www.yeastgenome.org/goTermFinder) (p-value 0.01). The accession number for the mass spectrometry proteomic data set used in this work is PXD0280004 and may be found at [http://www.ebi.ac.uk/pride/archive/projects/PXD028004](http://www.ebi.ac.uk/pride/archive/projects/PXD028004).
RESULTS

This study was initiated in response to observations made by Chen et al. (2018). They reported that: (i) GFP production supported by a DAL80-GFP plasmid was the same at zero, three, and six hours after a wild type transformant was transferred (down-shifted) from a richer SC_CSH (Synthetic Complete Cold Spring Harbor recipe, Table S-1) to SC_ME (Synthetic Complete Method in Enzymology recipe) medium, the latter having a lower amino acid content, and (ii) a similar result, but at a 2/3 lower level when the experiment was repeated with a whi2Δ recipient (Chen et al. 2018, Figure S-3). The conclusion derived from these observations was that Whi2 regulated NCR-sensitive gene expression via its negative control of TorC1 activity. However, two characteristics of the reported data attracted our attention: (i) there was easily detectable GFP production in both nitrogen-rich media. In contrast, we expected GFP production to be undetectable in both highly nitrogen, repressive media because its production was being driven by an NCR-sensitive DAL80 promoter, and (ii) in contrast to expectation, there was no successive increase in GFP production at 3 and 6 hours after a downshift in which the cells were transferred from richer SC_CSH to poorer SC_ME medium. Therefore, our initial objective was to understand these two unexpected observations.

Proteome in SC-grown wild type and whi2Δ cells

Our ability to explain the differences between our expectations and the results of Chen et al. (2018) was limited by the fact that very little is known about the response of NCR sensitive genes in such rich media for both wild type and whi2Δ strains. This is because nitrogen replete conditions, such as those used in the experiments cited above, are routinely used as the negative controls for rapamycin addition or other types of experiments involving NCR-sensitive gene expression or protein production. Therefore, to better understand the detailed behavior of NCR-sensitive protein production in nitrogen rich media, we characterized the proteomes of wild type and whi2Δ cells cultured in SC_CSH medium and then transferred to SC_ME medium for one or six hours. These growth protocols were analogous to those used in the previous reports (Chen et al. 2018). We obtained data for 2,261 proteins and used a change of an absolute log2 value equal to or greater than 1 as a significant change in protein levels. This criterion was not used in the gross comparison described in Figure 2 (Tables S-2 and S-4). In this figure, and its associated tables, a change in one or the other wild type and/or whi2Δ protein levels by an absolute log2 value equal to or greater than 1 was sufficient for it to be included.

The levels of only seven proteins significantly changed and did so similarly in the wild type and whi2Δ strains, one hour after transferring the cells from SC_CSH to SC_ME (Figure 2A and Table S-2). These proteins were highly enriched for the gene ontology (GO) processes associated with amino acid biosynthesis (P < 0.01) (Figure 2B and Table S-3). In fact, six of the seven proteins catalyze steps in the biosynthesis of arginine, methionine, or the aliphatic branched chain amino acids leucine, isoleucine or valine (Tables S-2 and S-3). This was remarkable because the cells were growing in amino acid rich media containing amounts of these amino acids normally added to minimal medium to cover arginine, leucine or methionine auxotrophs.

The seventh protein, Cha1, is a serine / threonine-inducible, catabolic threonine / serine deaminase. This enzyme produces ammonia and pyruvate and is a central component of serine, glycine and threonine catabolism. In this context, it is important to note that serine and threonine are the most abundant amino acids in SC_ME medium, i.e., up to six times higher concentrations than found in
SC$_{CSH}$ medium (Table S-1, Figure 2B). The high concentrations of these amino acids may account for changes in the deaminase levels when cells are transferred from SC$_{CSH}$ into SC$_{ME}$ medium.

Six hours after the downshift to SC$_{ME}$ medium, much more wide-spread changes occurred. The levels of 92 proteins differed in the two strains, including the seven whose levels increased at one hour after down-shift (Figure 2C and Table S-4, common proteins in Figures 2A and C are indicated with an asterisk). The highly enriched GO process terms that characterized these proteins were overwhelmingly associated with the biosynthesis of various amino acids (Figure 2D and Table S-5). This argued that shifting the cells from SC$_{CSH}$ to SC$_{ME}$ medium as well as nutrient utilization following the transfer elicited a dramatic reorientation of amino acid metabolism. In every case, proteins needed for amino acid biosynthesis increased. *A priori* one would have expected for some to increase while others decreased. Not only did the biosynthetic capabilities of the two strains change almost exclusively in a positive direction, but they also did so by nearly the same amounts whether in wild type or whi2Δ cells.

The nature of the biosynthetic pathways involved was also informative. The changes were restricted to specific amino acids (Figure 2C and Table S-5). The most highly represented biosynthetic pathways in this experiment were those for arginine, histidine, lysine, serine, leucine, isoleucine, methionine and aromatic amino acids. These amino acids correlate very well with those whose concentrations were decreased in SC$_{ME}$ relative to SC$_{CSH}$ medium (Table S-1, highlighted in yellow).

**Absence of NCR-sensitive proteins in proteome**

Nitrogen/amino acid downshifts from rich to poor media result in the inhibition of TorC1 activity and corresponding increases in NCR-sensitive and autophagy gene expression (Tate et al. 2015; Tate and Cooper 2013; and Figure 1). Therefore, based on the Chen et al. report, we expected to see significant representation of NCR-sensitive and autophagy-related proteins in our experiments. However, only three of the 92 proteins observed in cells transferred from richer SC$_{CSH}$ to poorer SC$_{ME}$ medium for six hours derived from NCR-sensitive genes: Bat2, catabolic branched-chain amino acid aminotransferase; Gdh2, glutamate dehydrogenase catalyzing the conversion of glutamate to ammonia and α-ketoglutarate, and Yhi9, whose function is unknown but its loss results in a defective unfolded protein response (Figure 2C, green text).

One explanation for the paucity of NCR-sensitive proteins in the observed proteome might have been that we were overly stringent in defining this classification. This would not be surprising as it may have emanated from our and others experience with NCR-sensitive reporter genes where transcription data have been reported. Therefore, we generated a list of all known NCR sensitive genes (41 genes), plus those classified as putatively NCR-sensitive (44 genes) and additionally even those genes containing GATA sequences in their promoters (40 genes). Recall that GATA sequences are the cores of the NCR-sensitive transcriptional activator (Gln3, Gat1) binding sites (Figure 3A and Table S-6) (Rai et al. 1989; Bysani et al. 1991; Scherens et al. 2006; Godard et al. 2007; Kontos, et al. 2008; Georis et al. 2009; Ljungdahl and Daignan-Fornier 2012).

The highly enriched GO process terms associated with the 125 proteins we selected were those representative of nitrogenous compound transport, catabolism and cellular responses to nitrogen or nutrient levels (Figure 3B and Table S-7). Note that we enlarged the number of GO terms to include subcategories in hopes of finding terms shared with those reported in Table S-5. If nitrogen catabolic processes were represented in Table S-5, the GO analyses in Table S-7 should have identified them. There was, however, little if any commonality in the GO process terms derived from the data in Figure 2 and possible NCR-sensitive genes (Figure 3). In short, we saw many fewer than expected NCR-sensitive protein changes even in the 6-hour nitrogen downshifted cells we assayed.
The broadened classification did, however, increase the number NCR-related proteins in Figure 2C, but by only four: Arg4, argininosuccinate lyase, catalyzing the last step in arginine biosynthesis; \(\text{Cha1} \), threonine / serine deaminase; Ggc1, a mitochondrial GTP/GDP transporter; and Qdr2, a DHA1 family antiporter. Further, only three of 19 highly enriched GO process terms were associated with catabolic processes (Figure 2D). Those catabolic processes are involved in C-1 metabolism, interconversion of amino acids and/or their precursors (Tables S-4 and S-5). The involvement of C-1 metabolism was not surprising given the 2- and 4-fold increase of threonine and serine in SC\(_{\text{ME}}\) medium.

If we consider all of the experiments in the present work in toto, the levels of only 16 proteins out of the expanded classification of 125 NCR-related genes changed: four demonstrably NCR-sensitive genes, seven putative NCR-sensitive genes and five additional GATA containing gene promoters (Figure 3A, green text). Additionally, proteins encoded by 32 NCR-related genes were represented in our data set, but their levels did not change (Figure 3A, red text).

One of the proteins that remained unchanged in our experiments has often been used as an indicator of cytoplasmic nitrogen limitation. It is the NCR-sensitive \(\text{CAR1} \) gene, encoding arginase which catalyzes the first step in arginine catabolism. Growing cells accumulate large amounts of arginine in their vacuoles (Zacharski and Cooper 1978). During times of cytoplasmic nitrogen limitation these arginine reserves are mobilized so that dividing cells can reach their G1 and G0 phases (Sumrada and Cooper 1978). The value of this capability is that G1 cells are orders of magnitude more resistant to environmental insults than are dividing cells (Schenberg-Frascino and Moustacchi 1972; Elliott and Futcher 1993). Therefore, the onset of a nitrogen downshift would also lead one to expect increased levels of arginase and the urea degradative protein urea amidolyase encoded by \(\text{DUR1,2} \) (Sumrada and Cooper 1978).

Neither of these proteins exhibited significant changes in our experiments (Figure 2A and C, and Figure 3A). This result, however, can be viewed from another perspective. Mobilizing vacuolar arginine was unnecessary because arginine was present in both media at levels sufficient to cover a complete arginine auxotrophy. Further, it was being synthesized under the nitrogen replete conditions in SC\(_{\text{ME}}\) medium as evidenced by increases in arginine biosynthetic pathway proteins noted in Figure 2A and C. In sum, these data argue that NCR-sensitive protein production was not a major target of Whi2-control and hence TorC1-mediated regulation when cells were growing in nitrogen replete conditions or even after a six-hour downshift to a lower but still significant nitrogen presence.

There was a second puzzling observation in the proteomic data. Gdh2, NAD glutamate dehydrogenase, which catalyzes the conversion of glutamate to ammonia, \(\alpha\)-ketoglutarate and NADH, appeared in both wild type and \(\text{whi2Δ} \) samples (Figure 2C). Why would the cells require ammonia production when the medium they were growing in contained 0.5% ammonia? Speculatively, one possibility is that ammonia production by Gdh2, under the conditions of our experiments, was required to maintain the cell’s redox balance by transferring reducing equivalents from NADPH to NAD, whereupon Gdh1 would quickly recycle the NADH, \(\alpha\)-ketoglutarate and ammonia back to glutamate. Alternatively, one may question to what extent would the MEP genes, encoding the ammonia transporters, be expressed in such nitrogen-rich (amino acids plus ammonia) media (Marini et al. 1994; Airoldi et al. 2016; Dubois and Grenson 1979)?

**Wild type and \(\text{whi2Δ} \) cells respond indistinguishably in standard protocols assessing NCR-sensitive regulation**

Since less than half of the total number of \(S. \text{cerevisiae} \) proteins were represented in the data we obtained, one could argue that the Mep proteins escaped isolation or identification by the methods we used. Nonetheless, if Whi2 was down-regulating TorC1, we had expected to see changes in many of
the NCR-sensitive proteins. This was not the case. Cautious about deriving conclusions based on negative observations, we moved directly to investigate the transcription activators responsible for NCR-sensitive protein production, Gln3 and Gat1. In our standard nitrogen replete YNB-glutamine medium, Gln3-Myc13 was highly cytoplasmic in both the wild type and \( \text{whi}2\Delta \) (Figure 4A and B). Cytoplasmic Gln3-Myc13 partially migrated into the nuclei of rapamycin-treated cells yielding a tripartite distribution of Gln3-Myc13 in all three of the scoring categories in both strains. Again, there was no detectable difference between wild type and \( \text{whi}2\Delta \) cells.

To evaluate possible Whi2 participation across the spectrum of NCR regulation, we extended this experiment to increasingly derepressive conditions using ammonia or proline as nitrogen source (Figures 4A and B). Gln3-Myc13 responded only minimally in ammonia but similarly to rapamycin treated cells when proline was provided as the nitrogen source. Again, we were at a loss to convincingly argue that wild type and \( \text{whi}2\Delta \) cell responses were much different from one another. (Figure 4A and B). In the most derepressive condition, i.e., nitrogen starvation, wild type and \( \text{whi}2\Delta \) cells also responded similarly (Figure 4C). In sum, we observed no demonstrable difference in the responses of wild type and \( \text{whi}2\Delta \) cells to any of the experimental conditions normally used to assess NCR-sensitivity.

**Gln3 phosphorylation profiles are the same in wild type and \( \text{whi}2\Delta \) cells**

Gln3 is a highly phosphorylated protein whose intracellular localization and function are highly influenced by its phosphorylation (Cox et al. 2004; Tate et al. 2009, 2010; Georis et al. 2011; Rai et al. 2014; Tate et al. 2019). Further, protein phosphorylation (Rps6 and Npr1) was convincingly shown to be affected by deletion of \( \text{WHI}2 \) (Chen et al. 2018). Therefore, we followed Gln3-Myc13 phosphorylation under conditions where its phosphorylation profiles were known to change. The responses of Gln3-Myc13 phosphorylation to rapamycin and methionine sulfoximine in wild type cells were indistinguishable to those observed in the \( \text{whi}2\Delta \) (Figure 4D-G). The only possible differences we observed were perhaps slightly higher Gln3-Myc13 phosphorylation in unstarved wild type cells and those nitrogen starved for ten hours compared to those in the \( \text{whi}2\Delta \) (Figure 4I and J). The lack of Whi2-dependent alterations in Gln3-Myc13 phosphorylation contrasted markedly with those observed by rapamycin treatment or deletion of the \( \text{SIT}4 \) and \( \text{PPH}21/22 \) (PP2A) phosphatase genes (Beck and Hall 1999; Bertram et al. 2000; Cox et al. 2004; Tate et al. 2009, 2010; Georis et al. 2011; Rai et al. 2014; Tate et al. 2019).

**Gln3 localization in high and low amino acids is indistinguishable in wild type and \( \text{whi}2\Delta \) cells**

Concerned our results might derive from technical differences between our routine protocols and those previously reported, we repeated, as closely as we could, the conditions used in the Chen et al. (2018) report. First, we cultured wild type and \( \text{whi}2\Delta \) cells overnight in SCS\( \text{SH} \) medium to an \( A_{600 \text{ nm}} = 0.5 \). The culture was then split and transferred to either SCS\( \text{SH} \) or SC\( \text{ME} \) medium, as Chen et al. (2018) had used, and Gln3-Myc13 localization followed for an additional six hours (Figure 5A and B). Again, the results obtained with wild type and \( \text{whi}2\Delta \) were indistinguishable. Gln3-Myc13 was almost completely cytoplasmic throughout the experiment.

Additionally, Chen et al. (2018) pre-grew their cells to saturation in SCS\( \text{SH} \) medium, transferred them to fresh SCS\( \text{SH} \) medium for one hour and then transferred them a second time to either SCS\( \text{SH} \) or SC\( \text{ME} \) media. We could not ascertain the cell number used following the second transfer in Chen et al.’s experiments, and so we performed our experiments with both low (\( A_{600 \text{ nm}} = 0.1 \)) and higher (\( A_{600 \text{ nm}} = 0.5 \)) cell numbers. Behaviors of the two strains were again indistinguishable (Figure 5C and D). Histograms in these figures represent data with the low cell number, whereas the filled circles represent those obtained with the higher cell number.
**Gat1 NCR-sensitivity is indistinguishable in wild type and whi2Δ cells**

The above experiments with Gln3 lead us to conclude that Whi2 was not playing a demonstrable role in the regulation of its intracellular localization and hence its transcriptional function. There is, however, a second GATA-family transcription activator, Gat1, whose regulation differs somewhat from that of Gln3 (Kulkarni et al. 2006; Georis et al. 2008; Georis et al. 2011). For example, while Gat1 localization and function are nitrogen-responsive, its production is autogenous, its intracellular localization is not as NCR-sensitive as that of Gln3, even though it is more highly TorC1-regulated (rapamycin-responsive).

Therefore, we compared the responses of Gat1-Myc13 localization in wild type and whi2Δ transformants. In contrast with Gln3-Myc13, Gat1-Myc13 exhibited less cytoplasmic sequestration in nitrogen replete glutamine medium (Figure 6A). However, Wild type and whi2Δ cells again responded similarly. Gat1 also exhibited a much stronger response to rapamycin treatment than Gln3-Myc13, becoming largely nuclear in both wild type and whi2Δ cells (Figure 6A). The strong rapamycin response suggested the lack of Whi2 might have a stronger effect on Gat1-Myc13 localization as NCR was reduced. However, this was not observed. Gat1-Myc13 distributions in ammonia-grown wild type and whi2Δ cells did not differ greatly from those with glutamine (Figure 6A). There was a modest nuclear shift of Gat1-Myc13 in proline medium, but again wild type and whi2Δ cells did not yield convincingly different results. This indicated, importantly, that Gat1-Myc13, unlike Gln3-Myc13 localization was exhibiting little if any demonstrable NCR-sensitivity in this strain background even though it positively responded to rapamycin addition.

**Gat1 responds to shift from high to low amino acids at different rates in wild type and whi2Δ cells**

We again argued that our standard assay conditions might not yield the same results as those reported by Chen et al. Therefore, we performed two more experiments using Chen et al.’s protocols (Figure 7). The main difference between results with wild type and whi2Δ cells was the speed with which Gat1-Myc13 began migrating into the nucleus after the shift from SCCSH to SC-CSH medium (Figure 7A). A greater fraction of Gat1-Myc13 relocated to the nuclei of wild type cells at 3 hours after the shift. This did not occur in the whi2Δ mutant until 6 hours and even then, cytoplasmic Gat1-Myc13 remained higher than in the wild type. One could argue that these results are consistent with a loss of Whi2 diminishing TorC1 down-regulation. This, in turn, would result in higher Gat1-Myc13 cytoplasmic sequestration in the whi2Δ. Importantly, however, this difference did not occur when cells were shifted from the SC-CSH to SC-ME medium with its lower concentrations of amino acids and particularly leucine (Figure 7B). Wild type and whi2Δ cells responded indistinguishably to this transfer. Unfortunately, we can offer no speculation about why the shift into SC-ME medium for increasing amounts of time yielded largely cytoplasmic Gat1-Myc13 sequestration. A priori, we would have expected just the opposite, i.e., a greater response as cells spent increasing times in the SC-ME medium.

Finally, analogous to the experiment reported by Chen et al., we cultured wild type and whi2Δ cells to saturation in SC-CSH medium (Figure 7C and D), transferred them to fresh SC-CSH medium (FrSC) for one hour and then split each of the cultures and transferred them a second time to either SC-CSH or SC-ME medium (Figures 7C and D, respectively). Shifting the saturated cultures from overnight incubation in SC-CSH to fresh SC-CSH medium for one hour resulted in consistently more cytoplasmic Gat1-Myc13 sequestration in the whi2Δ than wild type (Figure 7C and D).
In the case of the SC_{CSH} to SC_{ME} shift, the intracellular distributions of Gat1-Myc_{13} in wild type and \( \text{whi2}\Delta \) cells were similar at the half hour time point (Figure 7D). In response to the SC_{CSH} to SC_{ME} shift, Gat1-Myc_{13} began translocating into the nucleus. However, this was a transient effect which ended at three hours after the transfer in wild type and one hour in the \( \text{whi2}\Delta \). By six hours Gat1-Myc_{13} was efficiently sequestered in the cytoplasm of both strains. This is opposite of what one would expect since the cells had been in the poorer of the two media downshifted for 6 hours. The expectation was that the Gat1-Myc_{13} would be more nuclear at six hours than earlier.

We are unable to speculate about the molecular mechanisms generating these unconventional results. We do, however, now have a possible explanation for the DAL80-GFP expression in Chen et al.’s experiments. DAL80-GFP production required the action of a GATA-family transcription activator. Gat1 provides this requirement. Further, Gat1 plays a very large role in DAL80 expression (Cunningham et al. 2000). One may then justifiably query why Gat1 was even being produced since we clearly demonstrated that Gln3-Myc_{13} was securely sequestered in the cytoplasm of all experiments in SC_{CSH} and SC_{ME} media? GAT1 expression like that of DAL80 is partially Gln3-independent. This partial independence occurs because GAT1 expression is autogenously activated and Gat1 significantly activates DAL80 expression (Coffman et al. 1996).

**Dal80 protein production vs. DAL80 gene expression**

Both our and the earlier experiments by Chen et al., rested on a presumption, i.e., Whi2 was a major negative regulator of TorC1 which in turn telegraphed its response to the downstream target genes. There was, however, an alternative way of viewing the data. What if NCR sensitive protein production was not a major target of Whi2-mediated TorC1 regulation either because: (i) of the extent to which Whi2 downregulated TorC1 or (ii) TorC1 control of its downstream targets was distinctly target-dependent and finely graded? Either interpretation would have been consistent with both earlier reported and present results.

As noted above, DAL80 is exquisitely activated by Gat1, more so than many other NCR-sensitive genes. What if that characteristic contributed to the preference of using the DAL80-GFP reporter both by Chen et al. (2018) and earlier experiments by Neklesa and Davis (2009)? In the Chen et al. experiment, GFP production was uniformly high in SC_{CSH} medium as well as three and six hours after wild type cells were transferred to SC_{ME} medium (see Supplemental Figure 3 in Chen et al., 2018). When the experiment was repeated in the \( \text{whi2}\Delta \), GFP production was uniformly lower (~2-3 fold) at 0, 3 and 6 hours after downshifting the cells to SC_{ME} medium.

Since the DAL80-GFP plasmid was not available, we assayed DAL80 expression directly (Figure 6B). In SC_{CSH} medium, DAL80 expression was initially almost undetectable in both wild type and \( \text{whi2}\Delta \) cells (Figure 6B, 0 or 1 hour in SC_{CSH} medium). After 6 hours in SC_{CSH} medium, there was a small (2-3 fold) increase in DAL80 mRNA in the wild type which was not convincingly lower in the \( \text{whi2}\Delta \) (Figure 6B one vs. six hrs SC_{CSH}). Note, however, that DAL80 expression behaved predictably, i.e., the longer the cells grew, depleting nitrogen as they did so, the more DAL80 expression increased.

When cells were transferred from the richer SC_{CSH} to the poorer SC_{ME} medium for one and then six hours, DAL80 expression in the wild type increased ~5 and 12-fold compared with ~2 and 5-fold in the \( \text{whi2}\Delta \); these comparisons were relative to the 0 hour SC_{CSH} control. However, the longer wild type and \( \text{whi2}\Delta \) cells were grown in the SC_{ME} medium the more DAL80 expression increased. It is important to note that the degree to which DAL80 expression was down-regulated in \( \text{whi2}\Delta \) cells was not dependent on the time that the cells spent in the poorer medium, as would normally be expected by the following reasoning. As the concentration of nitrogen decreased in the SC_{ME} medium from one
to six hours, so too would the level of TorC1 activation. In other words, TorC1 activation would be
lower at six hours than at one hour. Correspondingly, the degree to which Dal80-GFP increased due
to loss of Whi2 would also be expected to be greater at six than at one hour. Experimentally, the re-
duction in whi2Δ cells was about two-fold at both time points.

The above data were placed into proper perspective when we performed a standard in vivo assay
for NCR-sensitivity. Dal80 expression in derepressed proline-grown (Pro) cells was ~60 fold higher
than in the SC<sub>CSH</sub> medium (Figure 6B). Further, abolishing Whi2 only modestly decreased (<18%)
Dal80 expression in the proline-grown cells. In other words, NCR-sensitive regulation is a minor
target of Whi2. What remains unknown is whether Whi2 was acting directly or indirectly on TorC1.
The uncertainty derives from the fact that the major physiological response observed in the proteomic
data was a reorientation and up-regulation of amino acid biosynthesis that: (i) was independent of
Whi2 and (ii) was highly correlated with the amino acids whose concentrations differed between the
two SC media.

**Major protein targets altered only in wild type cells shifted from SC<sub>CSH</sub> to SC<sub>ME</sub> medium**

If NCR-sensitive gene expression was a minor target of Whi2-mediated regulation, what were Whi2’s
major targets? The earlier proteomic data we discussed (Figure 2) focused only on the proteins whose
levels changed in both wild type and whi2Δ strains cultured in SC<sub>CSH</sub> and SC<sub>ME</sub> media. We addition-
ally identified groups of proteins whose levels changed by absolute values of log<sub>2</sub> greater than 1 uniquely
in a wild type or whi2Δ strain after being transferred from SC<sub>CSH</sub> to SC<sub>ME</sub> medium. Indeed, unlike the
NCR-sensitive proteins, these changes were dramatic.

Twelve proteins increased by log<sub>2</sub> values greater than 5-6 in wild type cells after one hour in SC<sub>ME</sub>
relative to SC<sub>CSH</sub> medium, thereby yielding positive values for log<sub>2</sub> (1 Hr in SC<sub>ME</sub>/SC<sub>CSH</sub> medium) (Fig-
ure 8A, green bars and Table S-8). In contrast only two proteins decreased by a log<sub>2</sub> values less than -6
one hour in these conditions thereby yielding negative values for log<sub>2</sub> (1 Hr in SC<sub>ME</sub>/SC<sub>CSH</sub> medium)
(Figure 8A, red bars and Table S-8). Unfortunately, these sets of proteins could not be related to one
another with GO terms exhibiting a p-value equal to or below 0.01. There were, however, four regulatory
proteins whose levels changed dramatically: Rlo1, which is a serine kinase that participates in cell
cycle regulation and rDNA integrity; Snt2, which is a subunit of the Snt2 complex RING finger ubiqui-
tin ligase; Cdc36, which participates in regulating mRNA levels; and Cet1, an RNA 5’ triphosphatase
that participates in mRNA 5’ capping.

The levels of 18 proteins in wild type cells increased by a log<sub>2</sub> values greater than 5 (green bars)
and four decreased by a log<sub>2</sub> values smaller than -5 (red bars) six hours after being transferred to the
SC<sub>ME</sub> medium (Figure 8B and Table S-9). Four of these proteins were the same ones whose levels in-
creased after one hour of incubation in SC<sub>ME</sub> medium, Gim4, Snt2, Apa2 and YJL133C-A (asterisks in
Figures 8A and B). In contrast to expectation, the only NCR-sensitive protein was Ecm15 whose func-
tion is unknown. The most striking characteristic of the relatively large group of proteins in Figure 8B
was that they were not significantly associated with any GO process and the only significant GO fun-
ction was isocitrate dehydrogenase activity for the mitochondrial Idh1 and Idh2 proteins.

However, four of the 43 proteins in Figure 8B were loosely associated with carbohydrate metab-
olism/glycolysis: Gdb1, glycogen debranching enzyme required for glycogen degradation; Sol4, 6-
phosphogluconolactonase which increases in response to DNA replication stress; Gcy1, glycerol dehy-
drogenase which is involved in glycerol catabolism under microaerobic conditions; and Tdh2, glycer-
aldehyde-3-phosphate dehydrogenase that participates in glycolysis and gluconeogenesis. Also of po-
tential significance, Tip41, the Tap42 Interacting Protein, increased by a log<sub>2</sub> value greater than 7
(Figure 8B). Tip41 is a negative regulator of TorC1 that activates the PP2A-like phosphatase Sit4 by
comparing with its binding to Tap42 (Jacinto et al. 2001). It is interesting that even though there were 43 proteins whose levels changed by log_2 values of greater than 1 in Figure 8B, they did not appear to be functionally related in a GO process term analysis.

**Major protein targets altered only in the whi2Δ**

When a whi2Δ strain was transferred from SC_{CSH} to the poorer SC_{ME} medium for one hour, the levels of 10 proteins changed by absolute log_2 values greater than 1 (Figure 9A and Table S-10). Of these, two proteins increased by log_2 values greater than 5 (green bars), thereby yielding positive values for log_2 (1 Hr in SC_{ME}/SC_{CSH} medium). In contrast, seven proteins decreased by log_2 values less than -4 to -7x (red bars). Again, there were no GO process terms with p-values equal to or below 0.01 associated with this group of proteins.

Extending the time in the SC_{ME} medium to six hours resulted in a greater number of proteins being increased (14, green bars) or decreased (8, red bars) by an absolute log_2 value greater than 5 (Figure 9B and Table S-11). Again, however, they were not significantly associated with any GO process terms. Note that despite these large changes, only one of these proteins were represented among proteins whose levels changed both after one and six hours (Figures 9A and B). Overall, it was surprising that of the more than 90 wild type and whi2Δ proteins whose levels changed uniquely in only the wild type or the whi2Δ, we were unable to associate them with a GO process term.

**Proteins whose levels differed when comparing whi2Δ vs. wild type SC_{CSH}-grown cells**

To approach the proteomic data from a third vantage point, we identified proteins whose levels were markedly different when evaluated in wild type vs. whi2Δ cells. In amino acid rich SC_{CSH} medium, six proteins increased by log_2 values greater than 5 in a whi2Δ compared to wild type, thereby yielding log_2 (Wild Type/whi2Δ) that were negative (Figure 10A, green bars and Table S-12). They were: Tim13, a mitochondrial import translocase associated with inserting hydrophobic proteins into the mitochondrial inner membrane; Apa2, diadenosine tetraphosphate phosphorylase involved in the catabolism of nucleosidyl tetraphosphates; Yps3, aspartate yapsin-family protease associated with cell wall growth and maintenance; Pry2 a sterol binding protein associated with the export of fatty acids; Smc4, a subunit of chromosome condensin complex acting during mitosis and meiosis; and Gin 4, prefoldin subunit 2 which binds to cytosolic chaperonin and transfers target proteins to it.

Thirteen proteins decreased by log_2 values less than -1 with five decreasing by log_2 values less than -5 in a SC_{CSH}-grown whi2Δ compared to wild type, thereby yielding positive values for log_2 (Wild Type/whi2Δ) (Figure 10A, red bars and Table S-12). The five proteins most decreased in the whi2Δ were: Cet1, which participates in mRNA 5' capping; Cst6, a basic leucine zipper transcription factor that participates in the stress response regulatory network; and Pom33 a nucleoporin; Xks1, xylulose kinase; and Yta7, a chromatin-binding ATPase regulating histone gene expression. Although these five proteins were insufficiently related to generate a positive GO correlation, Cet1, Cst6, Pom33 and Yta7 are loosely related to RNA metabolism. Interestingly, the fifth member of this group encodes xylulose kinase the rate limiting step in xylulose metabolism.

Four of the 13 proteins identified were highly enriched for the gene ontology (GO) process, carbohydrate catabolism -log(*corrected p-value) = 2.16; p-value = 0.007: Xks1 (xylulokinase), Gph1 (glycogen phosphorylase), Hxk1 (Hexokinase 1), and Pgm2 (phosphoglucomutase) (Table S-13). On the other hand, only two of the 20 proteins whose levels increased or decreased in the whi2Δ relative to wild type were associated with amino acid metabolism, Gad1 catalyzing the decarboxylation of glutamate that participates in a response to oxidative stress and Arg3 required for the biosynthesis of cit-
rulline and arginine. Changes in the levels of these proteins were moderate (absolute log₂ values of 1-2) relative to the eleven proteins whose levels changed the most (Figure 10A, red and green bars).

* A corrected p-value is the smallest familywise significance level at which a particular comparison will be declared statistically significant as part of the multiple comparison testing.

Proteins whose levels differed when comparing a whi2Δ to wild type cells were transferred from SC<sub>CSH</sub> to SC<sub>ME</sub> medium for one hour

The lack of Whi2 had a great effect on 21 proteins relative to wild type when cells were transferred from SC<sub>CSH</sub> to poorer SC<sub>ME</sub> medium for one hour (Figure 10B, Table S-14). Of these 21 proteins, five increased by log₂ values greater than 6 in the whi2Δ, thereby yielding negative values for log₂ (Wild Type/whi2Δ) (green bars). They were: Far8, which acts in the cell cycle arrest recovery process; Mnn10, a subunit of the Golgi mannosyltransferase complex; Sgv1, a cyclin (Bur2p)-dependent protein kinase functioning in transcription; Tcd1, tRNA threonylcarbamoyladenosine dehydratase required for tRNA base modification and Kss1, a MAPK kinase involved in signal transduction pathways that control filamentous growth and pheromone response.

Twelve proteins decreased by log₂ values less than -5 to -7 and four by log₂ values of less than -1 to -2 when the whi2Δ was grown in SC<sub>ME</sub> for one hour compared to wild type, thereby yielding positive values for log₂ (Wild Type/whi2Δ) (Figure 10B, red and black bars, respectively; Table S-14). Three of these proteins exhibiting decreased levels in this condition were highly enriched [-log(*corrected p-value) = 2.24] for the GO processes associated with glucose-6-phosphate metabolism, and more specifically the pentose phosphate pathway (Table S-15). They were: Sol4 (the gene encoding Sol4 was isolated as a suppressor of the los1-1 mutation), 6-phosphogluconolactonase 4, converting 6-phosphogluconolactone to 6-phosphogluconic acid required for the oxidative phase of the pentose pathway, which decreased by nearly 6X; Hxk1, hexokinase 1, catalyzing phosphorylation of glucose to yield glucose-6-phosphate, which is highly derepressed when cells are provided with non-glucose carbon sources and is the first step in the conversion of glucose to pentoses; and Pgm2, phosphoglucomutase, catalyzing the interconversion of glucose-6-phosphate and glucose-1-phosphate G-1-P). It is pertinent that G-1-P is the first unique step in the pentose phosphate pathway that also participates in glycogen and trehalose metabolism. Hxk1 and Pgm2 were decreased only modestly by a log₂ values between about -1 to -1.4, in the whi2Δ after one hour in SC<sub>ME</sub> medium. Together, these data suggested that the pentose phosphate pathway was significantly down-regulated in a whi2Δ.

Seven proteins, whose levels increased or decreased (by an absolute log₂ value equal to or greater than 1) relative to wild type after the whi2Δ was grown for one hour SC<sub>ME</sub> medium, were highly enriched [-log(*corrected p-value) = 2.76] for the GO function, transfer of phosphorus groups (Figure 10B and Tables S-14 and S-16): Hxk1, Hexokinase 1; Pol2, the catalytic subunit of DNA polymerase ε; Kss1, the MAPK that controls filamentous growth; Cdc8, a nucleoside monophosphate – nucleoside diphosphate kinase; Psk1, a serine/threonine protein kinase that coordinates the regulation of sugar flux and translation; Sgv1, the cyclin-dependent protein kinase component of the BUR complex, phosphorylates the C-terminal domains of RNA polymerase II and elongation factor Spt5-Sgv1; Uba4, an E1-like protein that acts in the thiolation of the wobble base of tRNAs and Sro7, whose loss prevented filamentation and invasive growth in Σ-1278b strains. All but Hxk1 increased or decreased by an absolute log₂ values greater than 5.

Proteins whose levels differed between a whi2Δ and wild type down-shifted from SC<sub>CSH</sub> to SC<sub>ME</sub> medium for 6 hours

After 6 hours of growth in the poorer SC<sub>ME</sub> medium, 20 of the 47 proteins decreased by log₂ values less than 3 in the whi2Δ, thereby yielding positive values for log₂(Wild Type/whi2Δ) (Figure 10C, red
bars and Table S-17). Eleven of these proteins were highly enriched for the gene ontology (GO) process, carbohydrate and energy metabolism (Figure 10D and Table S-18). The four proteins most affected, decreased by log₂ values between -3 and -8: Gph1, a glycogen phosphorylase; Psk2 serine/threonine kinase; Gdb1, a glycogen debranching enzyme; and Sol4, the phosphogluconolactonase required for the oxidative branch of the pentose pathway. The others decreased by only log₂ values of less than -1 to -2 (Figure 10C, black bars up to Gsy2).

There were also nine proteins whose levels increased by log₂ values greater than 4-7 in the whi2Δ thereby yielding negative values for log₂ (Wild Type/whi2Δ) (Figure 10C green bars and Table S-17). However, their functions were not easily related to one another. They were Bud20, a zinc finger protein required for ribosome assembly; Utp30, a subunit of U3-containing 90S preribosome complex; YCR102C, a putative quinone oxidoreductase associated with acid stress resistance; Elp6, a RcA-like ATPase Elp456 Elongator subcomplex required for modification of tRNA; Nst1, a protein involved in signal transduction pathways mediating responses through cell wall integrity, high-osmolarity glycerol and pheromone pathways; Flc2, a putative calcium channel involved in calcium release under hypotonic stress, required for uptake of FAD into endoplasmic reticulum and involved in cell wall maintenance; Irc22, a protein of unknown function that may localize to the ER; Ecm15, a protein that may be associated with cell wall biogenesis and Bud32, a Protein kinase that is a component of the EKC/KEOPS complex which is required for tRNA modification and telomeric recombination.

Only four of the 47 proteins in Figure 10C were related to NCR-sensitive regulation (green text). One, YMR125W, an uncharacterized vacuolar membrane protein decreased by a log₂ value of nearly -5 in a whi2Δ. Also decreased, but only by log₂ values of -1 to -3, were the putatively NCR-sensitive proteins, Hxk1 and YML196W. In contrast, the uncharacterized vacuolar membrane protein, Emc15, increased in the whi2Δ by a log₂ value greater than 7.

**DISCUSSION**

**Explanation of DAL80 expression vs. Dal80-GFP production in Synthetic Complete medium**

Present experiments were initiated in response to a paradoxical question, how to explain highly NCR-sensitive DAL80 expression in nitrogen replete Synthetic Complete medium containing 0.5% ammonium sulfate plus 0.18% (SCSH) or 0.12% (SCME) total amino acids? The major NCR-sensitive, TorC1-responsive transcription activator, Gln3, was not demonstrably responsible for the DAL80 expression. It remained staunchly sequestered in the cytoplasm in these excess nitrogen conditions in both wild type and a whi2Δ. In contrast, Gat1 - being more resistant to NCR (Georis et al. 2008), autogenously regulated (Coffman et al. 1996) and a significant contributor to DAL80 expression (Cunningham et al. 2000) - did modestly enter the nuclei of wild type and whi2Δ cells. Therefore, we suggest that Gat1 likely accounted for the small amount of DAL80 expression we could demonstrate using qPCR assays as well as the modest effect of a whi2Δ on that expression: a decrease of 50% after 6 hours in SCME, but only 18% when DAL80 expression was more fully derepressed in proline medium.

**Whi2 is only a minor regulator of NCR-sensitive protein production**

The modest NCR-sensitive DAL80 expression argued that Whi2 was only a minor regulator of NCR-sensitive protein production. This conclusion was supported by our proteomic data. Only 16 of 125 known or potential NCR-sensitive proteins, or proteins emanating from genes with GATA elements in their promoters exhibited significant changes in our combined analyses. Further, only three of these 16 were associated with catabolic activity and those three participated in amino acid interconversions. In contrast, 32 of the 125 proteins were present in the proteomic data but did not change. By inductive reasoning, if one accepts that the effects of the whi2Δ on DAL80 expression likely derived from the
down regulation of TorC1, then one must also conclude that Whi2 only modestly regulates TorC1 after six hours of growth in SCME medium. This is consistent with the earlier observation that a low concentration of rapamycin (2.5 ng/ml) was sufficient to suppress overgrowth of whi2 mutants relative to wild type (Chen et al. 2018).

One of the NCR-sensitive proteins that failed to appear in our proteomic data was Mep2, ammonia permease. That may have occurred for purely technical reasons. However, it prompted us to look for evidence of Whi2 controlling MEP gene expression. In doing so, we realized a curious set of observations. Mep2 is activated via its phosphorylation by Npr1, which is up-regulated when TorC1 is down-regulated (Vandenbol et al. 1990; Schmidt et al. 1998; Feller et al. 2006; Tate et al. 2006; Boeckstaens et al. 2014). Since Whi2 down-regulates TorC1, one would a priori expect it to up-regulate Npr1 and Mep2 activities. However, Boeckstaens et al. (2014) demonstrated Whi2’s binding partners, Psr1/Psr2 dephosphorylate and down regulate Mep2 activity.

Extended growth in SCME medium elicits large scale reorganization of amino acid metabolism

Despite the quite modest Whi2-dependent regulation of NCR-sensitive transcription, dramatic changes were observed to be independent of Whi2 when cells were transferred from SCcsh to SCME medium. Amino acid biosynthesis dramatically increased. Eighty-one proteins significantly increased and did so more or less equivalently in wild type and whi2Δ cells (Figure 2). In contrast, only ten proteins decreased. This massive reorganization of amino acid metabolism did not, however, involve all amino acids. The predominant increases occurred for basic arginine, lysine and histidine, aliphatic leucine, isoleucine and methionine, aromatic phenylalanine, tryptophan and tyrosine, and serine related asparagine and glycine. On the other hand, proteins required to synthesize multiple other amino acids that were omitted completely in the SCME medium, i.e., cystine, glutamine, and proline, were largely unaffected, which coincides with earlier observations made in a gcn2Δ at the level of tRNA charging (Zaborske et al. 2010). Together, these observations argued that Whi2 was not a significant regulator of amino acid biosynthesis.

It is worthy of emphasis that the nine amino acids whose biosynthesis increased in this study are the same ones whose concentrations were decreased in SCME relative to SCcsh medium (Table S-1). This likely contributes to explaining why multiple amino acids, in addition to leucine, were needed to overcome the effect of a whi2Δ (Teng et al. 2018). The importance of the correlations we report is that cellular events differed significantly in the different SC formulations and did so over the time of incubation in them. Hence the extent to which these differences are important to future investigations, different formulations of SC media cannot be prudently used interchangeably.

Gcn2, Whi2 and regulation of TorC1 and amino acid biosynthesis in SCME medium

The highly increased production of amino acid biosynthetic pathway proteins, we observed in Figure 2 and Table S-4 prompts us to query the regulatory systems involved. Here, the General Amino Acid Control (GAAC) pathway immediately comes to mind (Hinnebusch 1988; 1993; 2014). In general, amino acid limitation results in decreased charged tRNA levels which in turn activate Gcn2 kinase that inhibits overall protein synthesis and TorC1 activity (Staschke, et al., 2010; Zaborske et al. 2009, 2010; Yuan, et al. 2017). The inhibition of TorC1 by both Whi2 and Gcn2 raises an important, unanswered question. Do Gcn2 and Whi2 function in parallel to inhibit TorC1 or alternatively in tandem?

Gcn2 activity also stimulates translation of a select group of mRNAs, including that of GCN4, and is required for nuclear Gln3 localization. Gcn4 is a central participant in the activation of many amino acid biosynthetic, nutrient reutilization and some stress-related genes (Hinnebusch and Natarajan 2002; Hinnebusch 1994). This could straightforwardly lead to the conclusion that GAAC control ex-
explains the increased amino acid pathway proteins and DAL80 expression we observed. Explanations of our data, however, are more complicated.

Zaborske, et al. (2009; 2010) measured the growth and charging profiles of all tRNAs in wild type and gcnaΔ cells cultured in SCME medium from which each of the 20 amino acids were individually omitted. In wild type cells, none of the omissions had a significant effect on tRNA charging levels or growth. Remarkably in the gcnaΔ, the omission of only tryptophan or arginine gradually affected growth and tRNA charging levels. In the case of tryptophan deficiency, the tRNA charging levels were restored if tyrosine and phenylalanine were omitted along with tryptophan or all three amino acids were present. This result argued that both GAAC and release from metabolite feedback inhibition of aromatic amino acid biosynthesis were necessary to maintain a wild type response. In the case of arginine, analogous analyses argued that both GAAC and arginine related metabolites, ornithine and citrulline influence the capacity for arginine biosynthesis.

Our findings of increased methionine and arginine biosynthetic pathway proteins one hour after transferring cells to SCME medium, correlates well with the findings of Zaborske et al. (2009; 2010). In the case of methionine, Zaborske et al. (2010) observed that omission (or limiting concentrations) of tryptophan, one of the amino acids whose concentration is reduced in SCME medium, also decreased charged tRNA^{MET}. However, by this reasoning, why did we not see the aromatic amino acid biosynthetic pathway proteins increased after 60 minutes in SCME medium? We suggest this result derives from the fact that tryptophan, phenylalanine and tyrosine were all present in the SCME medium, and that there was still sufficient tryptophan present at one hour in SCME medium to mitigate the presence of phenylalanine and tyrosine, the feedback inhibitors of the pathway. However, by six hours in SCME medium this was no longer the case and one observes significant increases in aromatic amino acid biosynthetic proteins.

We also observed increased levels of aliphatic branched chain amino acid biosynthetic proteins one hour after transfer to SCME medium. Zaborske et al. (2010), however, did not see a change in tRNA profiles when these amino acids were omitted. Here we suggest that the leucine auxotrophy of our strains abrogated or reduced the capacity for aliphatic branched-chain amino acid synthesis. That coupled with transferring cells to SCME medium, further reducing leucine availability to the growing cells, triggered the results we observed. All of these effects were amplified six hours after transfer of the cells to SCME medium as all of the amino acids were being depleted by the increased number of cells assimilating them.

Finally, why did Gcn2 activation, as signaled by increased amino acid biosynthesis, fail to elicit greater nuclear Gln3 localization and increased expression of NCR-sensitive genes? This is likely because the regulation of intracellular Gln3 localization is multi-faceted: (i) Nuclear Gln3 localization is inhibited by up-regulation of TorC1 and down regulation of Gcn2 that occurs in nitrogen replete medium (Tate et al. 2017). It is also important to recall that TorC1 and Gcn2 reciprocally regulate one another’s activities (Cherkasova and Hinnebusch 2003; Yuan 2017). As amino acids are lower and depleted by growth in the SCME medium, Gcn2 activity increases, but overall nitrogen availability both from remaining amino acids and de novo assimilation of ammonia remains high. Our Gln3 localization and NCR-sensitive protein production data suggest that TorC1’s negative regulation of Gln3 is stronger than is Gcn2’s positive regulation. Further, wild type Whi2 activity along with Gcn2 activation after six hours in SCME medium still remains insufficient to overcome the negative regulation of Gln3 localization. Hence Gln3 localization remains almost completely cytoplasmic and NCR-sensitive gene expression minimal. (ii) Additionally, intracellular glutamine concentration, which would remain high in ammonia assimilation, elicits rapid Gln3 exit from the nucleus before it can activate NCR-sensitive transcription (Rai et al., 2015). These explanations must remain tentative, however,
because they do not adequately address the possibility that the downstream effects of TorC1, Gcn2
and Whi2 are likely individually and/or collectively graded, and if so, to what extent(s). That Whi2 so
minimally affects NCR-sensitive gene expression in the face of much greater control of Rps6 phos-
phorylation argues strongly in favor of such graded downstream responses by these global regulators
(present work and Chen et al. 2018).

Our data also suggest that the relative NCR-insensitivity and autogenous regulation of GAT1 ex-
pression and Gat1 activity likely accounts for the little nitrogen-responsive DAL80 expression we ob-
erved (Georis et al. 2008). The evidence, DAL80 expression increases after six hour’s relative to one
hour’s growth in SCME medium. Present data do not, however, answer the question, does the increase
derive from increased Gcn2 or decreased TorC1 activities or both? Whi2 modestly influences that ex-
pression in SCME but not SCCSH medium. What is clear is that the increase is small relative to normal
NCR-sensitive derepression. The extent of Whi2’s influence on DAL80 expression appears to be in-
dependent of the growth-time and hence amino acid concentration in SCME medium because DAL80 ex-
pression was similarly lowered (~50%) at both growth times in whi2Δ cells. This may account for the
similar amounts of Dal80-GFP observed in the work of Chen et al. 2018.

The major targets of Whi2 were not demonstrably related
The levels of many proteins drastically changed in a strain specific manner after one and six hours in
SCME medium; 57 in the case of wild type cells and 35 in the whi2Δ (Figures 8B and 9B). There were
three outstanding characteristics of the changes we observed during our experiments: (i) A large ma-
jority of the changes were dramatic, by absolute \( \log_2 \) values greater than 3 and could be speculated to
be binary on or off in terms of a protein’s presence. (ii) Whi2 affects processes far more diverse than
those expected if its primary function is to negatively regulate TorC1 activity. Further, in most cases it
was not possible to obtain a GO analysis result with a p-value of <0.01. In the single case where GO
data were obtained, i.e., comparing wild type to whi2Δ protein levels after six hours in SCME medium,
the protein relationships center on carbohydrate metabolism, energy generation and stress responses.
(iii) In very few cases did the changed proteins behave coordinately in the one and six-hour samples;
four in wild type and only one in the whi2Δ. These data again emphasize the need for caution in the
interpretation of data collected in different, even seemingly highly related media, and different condi-
tions of growth and experimental perturbation. The remarkable dynamics exhibited by strains, as they
grow, should be no surprise though at times overlooked.

A further characteristic that clearly distinguished the wild type and whi2Δ strain protein composi-
tions was a difference in the number of regulatory proteins whose levels changed, seven in the case of
wild type compared to only three in the whi2Δ (Figures 8 and 9; Tables S-8 – S-11). In wild type cells
grown one hour in SCME medium, three control proteins increased by \( \log_2 \) values greater than 6: Rio1,
a serine kinase involved in cell cycle regulation and rDNA integrity; Snt2, a ring-finger ubiquitin lig-
ase (E3) that binds with other proteins to the promoters of some stress response genes; and Cdc36
that participates in the transcription and destabilization of mRNAs (Table S-8). At six hours in SCME
medium, five regulatory proteins increased by \( \log_2 \) values between 4 and 7 in the wild type: Tip41, a
Tap42 interacting protein that negatively regulates TorC1 and activates Sit4 phosphatase; Rtk1, a put-
tative protein kinase that is phosphorylated by Cdc28 and increases during DNA replication stress;
Ume1, a component of histone deacetylase complexes and negative regulator of meiosis; Atg1, a ser-
ine/threonine protein kinase that participates in autophagic vesicle formation; and Snt2 (Table S-9).
Note that only one protein, Snt2, increased at both time points.

In contrast, we identified only two regulatory proteins whose levels change in the whi2Δ after one
hour in SCME medium (Figure 9): Sgv1, a cyclin dependent protein kinase that participates in tran-
scriptional regulation increased by a \( \log_2 \) value greater than 6, whereas Skn7, a transcription factor
required for induction of heat shock genes responding to oxidative stress, decreased by a log₂ value less than -7 (Table S-10). At six hours in SC_{ME} medium, only one regulatory protein increased, Ych1, a Cdc25 family tyrosine phosphatase, by a log₂ value greater than 2 (Table S-11). Two additional regulatory proteins decreased by log values greater than 7: Slm1, a phosphoinositide PI4,5P binding protein and TorC1 target that increases response to DNA replication stress; and Yip4, which interacts with Rab GTPases at late Golgi vesicles.

When wild type and whi2Δ proteomes are compared directly (Figure 10), the levels of two serine/threonine protein kinases changed by log₂ values greater than 5 after one hour in SC_{ME} medium: Psk2, that regulates sugar flux decreases in the whi2Δ whereas, Sgv1, a cyclin-dependent kinase whose loss results in myo-inositol auxotrophy increases to a similar degree (Table S-14). After six hours in SC_{ME} medium, four regulatory proteins decreased by log₂ values less than -5 in the whi2Δ relative to wild type: Yap1, a transcription factor required for stress tolerance, Ypi1, the regulatory subunit of a Type I protein phosphatase and regulates glycogen metabolism and mitosis. As occurred after one hour in SC_{ME} medium, Slm1 and Psk2 were also down regulated in a whi2Δ (Table S-17).

**Differences in the wild type and whi2Δ proteomes of liquid cultures compared to colonies on plates**

It also was striking how different the effects of a whi2Δ were in colonies previously grown in complex respiratory medium on plates compared to the SC liquid cultures described here. 65 proteins increased in whi2 and psr1/psr2 colonies relative to wild type (37 by log₂ values greater than 2.4 and 28 by log₂ values greater than 0.85) (Marsikova et al. 2020, Figures S3A and B). The most significant GO categories observed among these proteins (with log₂ values greater than 2.4) were those associated with transporter activity or proteins localized to the cell periphery. A smaller difference (log₂ values greater than 0.85) was observed for metabolic proteins related to alcohol metabolism and polyol synthesis (Marsikova et al. 2020, Figures S3 A and B). Only one of these 65 proteins identified in colonies was also identified in the current analyses.

In colonies, an additional 31 proteins were identified that were significantly decreased in whi2 and psr1/psr2 relative to wild type (Marsikova et al. 2020, Figure S4). Nine of these proteins decreased by log₂ values less than -2.6 to -8.0 and 22 by log₂ values less than -0.8 to -1.9. Significant GO categories (p-value log<0.01) included proteins associated with the cell periphery, extracellular proteins, and proteins involved in cofactor and coenzyme metabolic processes.

Five of the 31 proteins whose levels changed significantly in colonies were also identified in the liquid culture analyses. All five proteins increased in wild type relative to the whi2Δ either: (i) after culture in SC_{ME} medium for six hours (Ygp1, Sag1, Ssa4), (ii) after one or six hours in SC_{ME}, or for one hour in SC_{CSH} (Gad1) or (iii) after six hours in SC_{ME} and one hour in SC_{CSH} (Gph1). Two of these proteins are related to the cell response to starvation and stress: cell wall glycoprotein Ygp1, heat shock protein Ssa4 and glutamate decarboxylase Gad1. Three are metabolic proteins: glycogen phosphorylase Gph1 and Gad1, and a third is alpha-agglutinin Sag1p. Expression of the genes for all these proteins is induced with varying intensity during the transition of cells growing on YPD medium to stationary phase (Gasch et al. 2000).

It has been previously reported that the Whi2p-Psr1p/Psr2p complex plays a role in the general stress response, with cells defective in this complex being more sensitive to stress (Kaida et al. 2002). This stress response is related to the function of the transcriptional regulator Msn2p/Msn4p, which appears to be involved in the regulation of three of the above genes identified in both liquid cultures and colonies on plates (Ygp1p, Ssa4p, and Gph1p).
Overall, a comparison of the proteomic differences between wild type and \textit{whi}2 identified in liquid cultures with those identified in colonies cultured on plates showed very little commonality. This result is not surprising since the cultivation conditions were significantly different in both types of experiments - colonies versus liquid cultures and complex respiratory medium versus fermentative glucose medium with different amino acid additions. Moreover, for colonies, only proteins significantly different from wild type in both \textit{whi}2 and \textit{psr}1/\textit{psr}2 strains were considered. On the other hand, it is not surprising that there was some agreement between changes observed between colonies and liquid cultures after six-hours of culture in SC_{ME}, where a portion of the initial nutrients (including glucose) had been consumed, conditions more similar to those in colonies.

**CONCLUSIONS**

This work initially investigated unexpected NCR-sensitive \textit{DAL80-GFP} gene expression (Dal80-GFP production) in two nitrogen replete Synthetic Complete media (SC_{CSH} and SC_{ME}) that are routinely used interchangeably as reference conditions for yeast physiology investigations. GATA activation factor localization and proteomic data obtained with these media demonstrated: (i) Gln3 is staunchly cytoplasmic irrespective of the SC formulation used and the time wild type and \textit{whi}2\textDelta cells are incubated in them, one or six hours. (ii) Gat1, being autogenously regulated and more insensitive to NCR than Gln3, partially localizes to the nucleus accounting for the modest \textit{DAL80} expression observed. (iii) There is massive and equivalent reorientation of amino acid biosynthetic protein production in both wild type and \textit{whi}2\textDelta cells transferred from SC_{CSH} to SC_{ME} medium. Whi2 does not play a demonstrable role in regulating this reorientation. However, the amino acid biosyntheses most affected by the transfer are those whose concentrations are diminished in SC_{ME} medium. These observations correlate well with those expected from earlier studies of Gcn2 (GAAC) regulation of amino acid biosynthesis. These results may also contribute to explaining the earlier conclusions that other amino acids in addition to the absolute level of leucine were sensed by wild type and ignored by \textit{whi}2\textDelta cells. As a result, some or as many as 13 amino acids in addition to low leucine are required to suppress wild type growth relative to that of \textit{whi}2\textDelta cells in SC_{ME} medium. (iv) Although loss of Whi2 modestly diminishes \textit{DAL80} expression (two-fold), it does not demonstrably regulate overall NCR-sensitive or TorC1-regulated protein production. Loss of Whi2, on the other hand, drastically effects the production of 58 proteins which, with two exceptions - carbohydrate metabolism and oxidative stress, are not related to one another in GO analyses. We suggest that SC_{CSH} and SC_{ME} media cannot be prudently used interchangeably. Further, data from this and earlier works argue that control of TorC1 downstream targets is highly specific and graded. This work also prompts the important question of whether Gcn2 and Whi2 regulate TorC1 in parallel or in tandem. Our data are speculatively consistent with these regulators functioning in tandem.

**Data availability**

Following publication, strains and plasmids will be provided upon request, but only for non-commercial purposes. Commercial and commercial-development uses are prohibited. Materials provided may not be transferred to a third party without written consent. This will be done in accordance with NIH guidelines. Publicly available datasets were analyzed in this study. This proteomic data set has accession number PXD028004 and can be found at [http://www.ebi.ac.uk/pride/archive/projects/PXD028004](http://www.ebi.ac.uk/pride/archive/projects/PXD028004).

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FIGURE LEGENDS

Figure 1. Diagram of the TorC1 regulatory pathway as it is generally viewed and Whi2 participation as reported (Chen et al. 2018). It is important to emphasize that “active” or “inactive”, as used in the figure, indicates overall activity. The designations may not apply to other functions or conditions that the depicted regulatory proteins may also be executing. For example, Sit4 functions both when TorC1 is highly active as well as inactive, i.e., when complexed with Tap42 or not associated with Tap42; Sit4 and PP2A are both required along with Ure2 to maintain Gln3 in the cytoplasm in nitrogen-rich me-
Cultures were pre-grown overnight in SC medium (contains 367 mg/L leucine) to a cell density of $A_{600 \text{ nm}} = 0.5$. The cultures were then split with one half transferred back into fresh SC medium and the other half transferred to fresh SCS medium (contains 30 mg/L leucine) for 0-6 hours; both cultures to a cell density of $A_{600 \text{ nm}} = 0.5$. (Panels C and D). Cultures were pre-grown overnight to saturation in SCS medium (ON) and then transferred to fresh SC medium for 1 hour (FrSC). Each of the cultures was then split. One half of the cultures were resuspended to a cell density of $A_{600 \text{ nm}} = 0.1$ (the histogram) or $A_{600 \text{ nm}} = 0.5$ (filled histogram). Sensitive genes in the proteomic data set but their levels did not significantly change in any of the conditions we assayed. Genes in black text were not represented in the proteomic data set. Genes in red text (labelled PNC, Present in proteomic data but No Change) were represented in the proteomic data set but their levels did not significantly change in any of the conditions we assayed during the course of all experiments we performed. Genes in green text (SC, Significant Change) underwent a significant change in one or more of the conditions we assayed during the course of experiments presented in this work. (Panel B) GO process analysis output using the genes in Panel A as the query. The GO list is incomplete. It is presented only to a $-\log^2(p$-value) between 5-6 and 12.
circles) in \( SC_{CSH} \) or \( SC_{ME} \) medium for 0-6 hours. The experiments in panels A and B represent data from different cultures performed on different days than those in panels C and D. We did not repeat these experiments because all four panels are variations of the same experiment and all gave identical results.

Figure 6. (Panel A) Responses of Gat1-Myc\textsubscript{13} intracellular localization in wild type (P1) and \( \text{whi2}\Delta \) (P1-whi2) cells provided with YNB-glutamine, ammonia or proline as nitrogen source and with ramicin added to glutamine (Gln + Rap) medium as described in Materials and Methods (our standard assay conditions). Data presentations are as described in Figure 3. (Panel B) qPCR measurements of \( DAL80 \) expression in wild type (P1, green bars) and \( \text{whi2}\Delta \) (P1-whi2, magenta bars) in cells grown for 0, 1 and 6 hours in either \( SC_{CSH} \) or \( SC_{ME} \) medium. Cells were also cultured in \( SC_{CSH} \) and YNB-proline (Pro) to demonstrate \( DAL80 \) expression in response to nitrogen catabolite repression.

Figure 7. Gat1-Myc\textsubscript{13} localization in wild type (P1) or \( \text{whi2}\Delta \) (P1-whi2) cells grown according to protocols reported by Chen et al. (2018). (Panels A and B) Cultures were pre-grown overnight in \( SC_{CSH} \) medium (contains 367 mg/L leucine) to a cell density of \( A_{600 \text{ nm}} = 0.5 \). The cultures were then split with one half transferred back into fresh \( SC_{CSH} \) medium and the other half transferred to fresh \( SC_{ME} \) medium (contains 30 mg/L leucine) for 0-6 hours; both cultures to a cell density of \( A_{600 \text{ nm}} = 0.5 \). (Panels C and D). Cultures were pre-grown overnight to saturation in \( SC_{CSH} \) medium (ON) and then transferred to fresh \( SC_{CSH} \) medium for 1 hour (FrSC). The cultures were then split and then transferred to cells to fresh \( SC_{CSH} \) and \( SC_{ME} \) media at a starting \( A_{600 \text{ nm}} = 0.4 \) for 30 minutes, 1, 3 and 6 hours. Experiments in panels A and B represent data from different cultures (biological replicates, N=2) analyzed on different days than those in panels C and D (N=1, because data are overall consistent with those obtained in Panels A and B).

Figure 8. Proteins whose levels changed by absolute log\textsubscript{2} values equal to or greater than 1 (i.e., equal to or greater than 2-fold) in wild type (P1) cells but not in \( \text{whi2}\Delta \) (P1-whi2) cells. Cells were cultured for 1 (Panel A) or 6 (Panel B) hours in \( SC_{ME} \) medium and the results compared to those obtained after cells were cultured for 1 hour in \( SC_{CSH} \) medium. Prior to the beginning of the experiment, cells were pre-grown over night in \( SC_{CSH} \) medium to an \( A_{600 \text{ nm}} = 0.35 \). Genes whose proteins changed at both 1 and 6 hours are marked with an asterisk. NCR-sensitive genes appear in green text. SGD GO process analysis of the genes in Panel A or B did not yield any significant results with p-value equal to or <0.01.

Figure 9. Proteins whose levels changed by absolute log\textsubscript{2} values equal to or greater than 1 (i.e., equal to or greater than 2-fold) in \( \text{whi2}\Delta \) (P1-whi2) but not wild type (P1) cells. Cells were cultured for 1 (Panel A) or 6 (Panel B) hours in \( SC_{ME} \) medium and the results compared to those obtained after cells were cultured for 1 hour in \( SC_{CSH} \) medium. Prior to the beginning of the experiment, cells were pre-grown over night in \( SC_{CSH} \) medium to an \( A_{600 \text{ nm}} = 0.35 \). SGD GO process analyses of the genes in Panels A and B did not yield any significant results with p-value <0.01.

Figure 10. Proteins whose levels differed by absolute log\textsubscript{2} values equal to or greater than 1, (2-fold), in a \( \text{whi2}\Delta \) relative to wild type cells grown under the same condition. All cultures were pre-grown over night in \( SC_{CSH} \) medium to an \( A_{600 \text{ nm}} = 0.35 \). (Panel A) Cells were cultured in \( SC_{CSH} \) medium. (Panel B) Cells were transferred from \( SC_{CSH} \) to \( SC_{ME} \) medium and grown for 1 hour before being assayed. (Panel C) Cells were transferred from \( SC_{CSH} \) to \( SC_{ME} \) medium and grown for 6 hours prior to assay. Known or potentially NCR-sensitive genes are in green text. (Panel D) GO process analysis for the genes identi-
fied in Panel C. GO process analyses for genes in panels A and B were negative when a p-value of 0.01 was used.

**Table 1**

**Strains, Plasmids and Primers Used in This Work**

<table>
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<th>Strain/Plasmid</th>
<th>Genotype</th>
<th>Reference</th>
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<td>(SM, smooth colony morphology)</td>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>TBP1</td>
<td>5′-TATAACCCAAGCCTTGTGC-3′</td>
<td>Georis et al. 2009</td>
</tr>
<tr>
<td></td>
<td>5′-GCCAGCTTTGAGTCATCCTC-3′</td>
<td></td>
</tr>
</tbody>
</table>

¹BY4742 clone ²plasmids contain URA3 as the selectable marker.
Figure 1

SC<sub>CCH</sub> Medium
Excess Amino Acids
Significantly Met, Leu, Gln,

- High Leucine
  - LeuRS (Leucine)
    - Active
  - Gtr2 [Gtr1]
    - GDP
  - GTP
    - Inactive
- Ego1/3
  - Active
  - Tap42
  - PP2A
    - Active
  - Sit4
  - Inactive
  - Lst4-7 (GAP)
  - Inactive
  - Ure2
  - Gln3
  - Excluded from Nucleus

NCR-sensitive & ATG14
Transcription Repressed
Nucleus

PP2A (CH<sub>3</sub>)

N-Stravation, Poor or
Limiting Amino Acids
Significantly Met, Leu, Gln,

- Low Leucine
  - LeuRS (Leucine)
    - Inactive
  - Gtr2 [Gtr1]
    - GDP
  - GTP
    - Inactive
- Ego1/3
  - Active
  - Tap42
  - PP2A
    - Active
  - Sit4
  - Inactive
  - Lst4-7 (GAP)
  - Inactive
  - Ure2
  - Gln3
  - Inactive

NCR-sensitive & ATG14
Transcription Activated
Nucleus
Wild Type and/or whi2Δ proteins whose levels change by a Log₂ value greater than 1 after one Hr in ME medium compared to those after one Hr in CSH medium

(A)  
```
<table>
<thead>
<tr>
<th></th>
<th>Arg1</th>
<th>Arg3</th>
<th>Che1</th>
<th>Leu1</th>
<th>Met17</th>
<th>Trmt1</th>
<th>YHR2068W</th>
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</thead>
<tbody>
<tr>
<td>WT</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
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<tr>
<td>ΔWT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
```

(B) GO Process
- Cellular amino acid biosynthetic
- Branched amino acid biosynthetic
- Leucine biosynthetic
- Aspartate family aa metabolic
- Arginine biosynthetic
- Isoleucine biosynthetic
- L-methionine biosynthetic

(C) Wild Type and/or whi2Δ proteins whose levels change by a Log₂ value greater than 1 after six Hrs in ME medium compared to those after one Hr in CSH medium

(D) GO Process
- Cellular amino acid biosynthesis
- Carboxylic acid metabolic
- Aspartate family aa biosynthetic
- Branched-chain aa biosynthetic
- Arginine biosynthetic
- Serine family aa metabolic
- Glutamine family aa biosynthetic
- Isoleucine biosynthetic
- Dicarboxylic acid metabolic
- Sulfur aa biosynthetic
- Drug metabolic

- Log (Corrected p-value)
### Figure 3

#### (A) NCR Sensitive

<table>
<thead>
<tr>
<th>AGP1</th>
<th>DAL7</th>
<th>MEP3</th>
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<tbody>
<tr>
<td>ASP3-1</td>
<td>DAL80</td>
<td>PEP4-PNC</td>
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<td>ASP3-2</td>
<td>DCG1</td>
<td>PRB1-PNC</td>
</tr>
<tr>
<td>ASP3-3</td>
<td>DUR1.2-PNC</td>
<td>PUT1</td>
</tr>
<tr>
<td>ASP3-4</td>
<td>DUR3</td>
<td>PUT2-PNC</td>
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<tr>
<td>ATG14</td>
<td>ECM38</td>
<td>PUT4</td>
</tr>
<tr>
<td>BAT2-SC</td>
<td>GAP1</td>
<td>UGA1-PNC</td>
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<tr>
<td>CAN1</td>
<td>GAT1</td>
<td>UGA4</td>
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<tr>
<td>CAR1-PNC</td>
<td>GDH2-SC</td>
<td>VID30</td>
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<td>GDH3</td>
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<td>LAP4</td>
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<tr>
<td>DAL4</td>
<td>MEP1</td>
<td></td>
</tr>
<tr>
<td>DAL5</td>
<td>MEP2</td>
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</tbody>
</table>

#### Putatively NCR-Sensitive

<table>
<thead>
<tr>
<th>AAH1-PNC</th>
<th>LEE1</th>
<th>SLX9-PNC</th>
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</thead>
<tbody>
<tr>
<td>ALD4-SC</td>
<td>MIG2</td>
<td>UGA3</td>
</tr>
<tr>
<td>ARG4-SC</td>
<td>MOH1</td>
<td>UGK2</td>
</tr>
<tr>
<td>AVT1</td>
<td>NIT1</td>
<td>URK1</td>
</tr>
<tr>
<td>AVT4</td>
<td>NPR2</td>
<td>VBA1</td>
</tr>
<tr>
<td>AVT7</td>
<td>NRK1</td>
<td>YDL237W-PNC</td>
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<tr>
<td>CHA1-SC</td>
<td>OPT1</td>
<td>YDR90C</td>
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<tr>
<td>DIP5</td>
<td>OPT2</td>
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<td>ECM37</td>
<td>PMP1</td>
<td>YGL196W-PNC</td>
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<td>GCN4</td>
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<td>GDH1-PNC</td>
<td>RNY1-SC</td>
<td>YJR011C</td>
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<td>GGC1-SC</td>
<td>RPS08-PNC</td>
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<tr>
<td>GLT1-PNC</td>
<td>RSM10</td>
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<td>GUD1</td>
<td>RTS3-SC</td>
<td>YOR052C</td>
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<tr>
<td>HXK1-SC</td>
<td>SDL1</td>
<td></td>
</tr>
</tbody>
</table>

#### Promoter Contains GATA Elements

| ACE2 | PRC1-PNC | YKL137W |
| ALT1-SC | PST2-PNC | YKR033C |
| APE2-PNC | QDR2-SC | YLR149C |
| COX12 | RAS2-PNC | YLR164W |
| ECM15-SC | RPN6-PNC | YMR181C |
| FMP16 | RPT2-PNC | YMR196W-SC |
| GPG1 | SNA2 | YMR226C-PNC |
| GRX1-PNC | YBR085C-A-PNC | YOL019W |
| HOR7 | YBR139W-PNC | YPR098C |
| ICY1 | YER030W-PNC | ZSP |
| INO4 | YET1-PNC |
| LAP3-PNC | YFR055W |
| PBI2-PNC | YIL087C |
| PHM8-SC | YIL165C |
| PMP3 | YJR096W-PNC |

---

**Not represented in our data set**

**Protein present in our data set but no significant change**

**Present in our data set with significant change**

#### (B) GO Process

**GO Process**

- cellular amino acid catabolic
- organonitrogen compound catabolic
- nitrogen utilization
- small molecule metabolic
- glutamine family amino acid metabolic
- cellular amino acid metabolic
catabolic
- amino acid transport
- allantoin catabolic
- organic acid transport
- cellular response to nitrogen starvation
- proline transport
- purine nucleobases metabolic

**GO Process**

- glutamate metabolic
- regulation of nitrogen utilization
- nucleobase metabolic
- glutamate biosynthetic
- glutamine family amino acid biosynthetic
- aromatic amino acid transport
- ion transport
- aspartate family amino acid catabolic
- asparagine catabolic
- purine nucleobase catabolic
- alpha amino acid biosynthetic
- oligopeptide transmembrane transport
- proline membrane transport

---

**-Log (Corrected p-value)**

- 0.5
- 1.0
- 1.5
- 2.0
- 2.5
- 3.0
- 3.5
- 4.0
- 4.5
Figure 6

(A) Gat1-Myc\textsubscript{13} Localization

- W.T. whi2\(\Delta\)
- W.T. whi2\(\Delta\) + Gln + Rap
- W.T. whi2\(\Delta\) Ammonia
- W.T. whi2\(\Delta\) Proline

Percentage of Cells

(B) DAL80 Expression

- Wild Type (P1)
- whi2\(\Delta\) (P1-whi2)

Fold Increase Relative to the Wild Type 0 Hr CSH Value

Culture Conditions:
- 0 Hr CSH
- 1 Hr CSH
- 6 Hrs CSH
- 1 Hr ME
- 6 Hrs ME
- CSH
- Pro
Figure 7  Gat1-Myc\textsubscript{13} Localization

(A) SC\textsubscript{CSH} Medium  
- Cytoplasmic  
- Nucl.-Cyto.  
- Nuclear

Percentage of Cells

0 1 3 6 0 1 3 6

Hrs after shift to SC\textsubscript{CSH} medium  
Wild Type  
whi2\Delta

SC\textsubscript{CSH} OD = 0.5  (367 mg/L Leu)

SC\textsubscript{ME} OD = 0.5  (30 mg/L Leu)

SC\textsubscript{CSH} Over Night to Saturation  
Fresh SC\textsubscript{CSH} 1 Hr.

(B) SC\textsubscript{ME} Medium  
- Cytoplasmic  
- Nucl.-Cyto.  
- Nuclear

Percentage of Cells

0 1 3 6 0 1 3 6

Hrs after shift to SC\textsubscript{ME} medium  
Wild Type  
whi2\Delta

(C) SC\textsubscript{CSH} Medium  
- Cytoplasmic  
- Nucl.-Cyto.  
- Nuclear

Percentage of Cells

ON FrSc 0.5 1 3 6 ON FrSc 0.5 1 3 6

Hrs after shift to SC\textsubscript{CSH} medium  
Wild Type  
whi2\Delta

SC\textsubscript{CSH} OD = 0.4  (367 mg/L Leu)

SC\textsubscript{ME} OD = 0.4  (30 mg/L Leu)

(D) SC\textsubscript{ME} Medium  
- Cytoplasmic  
- Nucl.-Cyto.  
- Nuclear

Percentage of Cells

ON FrSc 0.5 1 3 6 ON FrSc 0.5 1 3 6

Hrs after shift to SC\textsubscript{ME} medium  
Wild Type  
whi2\Delta
**Figure 8**

(A) **Wild Type (P1) proteins whose levels change by an absolute Log₂ value greater than 1, one Hr after shift to ME medium relative to CSH medium**

(B) **Wild Type (P1) proteins whose levels change by an absolute Log₂ value greater than 1, six Hrs after shift to ME medium relative to CSH medium**
**Figure 9**

**A**

*whi2Δ (P1-whi2)* proteins whose levels change by an absolute log2 value equal to or greater than 1, one hr after shift to ME medium relative to one hr in CSH medium.

**B**

*whi2Δ (P1-whi2Δ)* proteins whose levels change by an absolute log2 value equal to or greater than 1, six hrs after shift to ME medium relative to one hr CSH medium.
Proteins whose levels change by an absolute Log$_2$ value equal to or greater than 1 when CSH-grown (one Hr) Wild Type and whi2Δ cells are compared.

Proteins whose levels change by an absolute Log$_2$ value equal to or greater than 1 when ME-grown (one Hr) Wild Type and whi2Δ cells are compared.

Proteins whose levels change by an absolute Log$_2$ value of equal or greater than 1 when Wild Type (P1) and whi2Δ cells (P1-Whi2), grown for six Hrs in ME medium, are compared.

(D) GO Process
- glycogen metabolic
- energy reserve metabolic
- carbohydrate metabolic
- precursor and energy generation
- cellular oxidative stress
- carbohydrate catabolic

-Log (Corrected p-value)