Molecular Systems Biology (2006) doi:10.1038/msb4100069

www.molecularsystemsbiology.com
Article number: 2006.0026

molecular systems biology

Proteome analysis of yeast response to various nutrient limitations

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Received 8.8.05; accepted 5.4.06

We compared the response of *Saccharomyces cerevisiae* to carbon (glucose) and nitrogen (ammonia) limitation in chemostat cultivation at the proteome level. Protein levels were differentially quantified using unlabeled and ^{15}N metabolically labeled yeast cultures. A total of 928 proteins covering a wide range of isoelectric points, molecular weights and subcellular localizations were identified. Stringent statistical analysis identified 51 proteins upregulated in response to glucose limitation and 51 upregulated in response to ammonia limitation. Under glucose limitation, typical glucose-repressed genes encoding proteins involved in alternative carbon source utilization, fatty acids β-oxidation and oxidative phosphorylation displayed an increased protein level. Proteins upregulated in response to nitrogen limitation were mostly involved in scavenging of alternative nitrogen sources and protein degradation. Comparison of transcript and protein levels clearly showed that upregulation in response to glucose limitation was mainly transcriptionally controlled, whereas upregulation in response to nitrogen limitation was essentially controlled at the post-transcriptional level by increased translational efficiency and/or decreased protein degradation. These observations underline the need for multilevel analysis in yeast systems biology.
Molecular Systems Biology 16 May 2006; doi:10.1038/msb4100069
Subject Categories: proteomics
Keywords: nutrient limitation; proteomics; system adaptation; transcriptomics; yeast

Introduction

All living organisms rely on the uptake of nutrients from the environment to sustain energy, metabolism and growth. They have therefore evolved numerous alternative programs to adapt to their permanently changing environment. Such programs involve instantaneous responses (changes in intracellular metabolites, activation/inhibition of enzymes by effectors and of proteins through post-translational modifications) as well as slower processes that affect the levels of macromolecules (transcription, translation, mRNA and protein degradation). The availability of complete genome sequences and technologies that allow comprehensive analysis of global mRNA profiles has greatly expanded the ability to monitor the transcriptional reprogramming of cells in response to their environment. However, further studies (often conducted with yeast) indicate that transcripts are imperfect indicators of protein levels and of *in vivo* fluxes (Griffin *et al*, 2002; Washburn *et al*, 2003; Daran-Lapujade *et al*, 2004), and therefore bring limited understanding on whole biological systems.

Other 'omics' tools are not developed to the same degree as transcriptomics. Especially, quantitative analysis of the complete proteome still remains a major challenge. Conventional quantitative proteome analysis utilizes two-dimensional (2D) gel electrophoresis (O'Farrell, 1975) to separate complex protein mixtures followed by in-gel tryptic digestion and mass spectrometry for the identification of proteins. Although 2D gel electrophoresis allows the separation of hundreds of proteins simultaneously, it suffers many well-documented drawbacks such as the poor gel-to-gel reproducibility, the under-representation of low-abundant and hydrophobic proteins and the poor dynamic range of detection (Fey and Larsen, 2001; Rabilloud, 2002). To overcome some of the shortcomings of 2D gel electrophoresis, alternatives have been developed for quantitative proteomics. One of the most promising approaches relies on the labeling of proteins with stable isotopes (for reviews see Romijn *et al*, 2003; Julka and Regnier, 2004). The isotopic label can be incorporated into proteins via metabolic labeling of the living cells or into protein/peptides via chemical reaction after protein extraction. Mixed labeled and unlabeled protein extracts are then separated and

analyzed by mass spectrometry and the relative abundance of proteins can be determined by comparison of the integrated mass spectrometry peak areas of the labeled and unlabeled forms of the peptides. Metabolic labeling offers the earliest time point of stable isotope incorporation and is thus one of the most comprehensive labeling methods. Cells are grown on normal-abundance or stable-isotope-labeled media and the label is incorporated during protein synthesis. So far, different isotopic labels have been used, for example, ^{15}N fully labeled media (Oda *et al*, 1999; Krijgsveld *et al*, 2003), or ^{13}C- or ^{2}H- labeled amino acids (Ong *et al*, 2002; Blagoev *et al*, 2003), and metabolic labeling has been applied to a wide variety of organisms ranging from bacteria (Conrads *et al*, 2001), fruitflies (Krijgsveld *et al*, 2003) to rats (Wu *et al*, 2004a).

In quantitative approaches, not only the analytical techniques but also the experimental designs for cell cultivation have to be thoroughly devised. Laboratory-scale cultivation of microorganisms is predominantly performed in shake flasks. During the course of these batch fermentations, the physical and chemical environment constantly changes, which affects the specific growth rate and the regulation of many metabolic processes. As the time constants of transcription and translation are likely to differ, this dynamic nature of batch cultures complicates studies on correlation between mRNA and protein levels. The use of chemostat cultures enables the study of physiological adaptations to steady-state nutrient-limited growth. The medium that is continuously fed into a chemostat can be designed such that growth is limited by a single, defined nutrient, whereas all other nutrients remain present in excess. The culture broth is continuously replaced by fresh medium at a fixed and accurately determined dilution rate. This results in a constant dilution rate, which is equal to the growth rate. This offers the unique possibility to study metabolism and its regulation at a fixed and constant growth rate under tightly defined nutritional conditions, making chemostats excellent tools for quantitative transcriptome and proteome studies (Daran-Lapujade *et al*, 2004; Kolkman *et al*, 2005; Tai *et al*, 2005). Furthermore, the use of fermenters instead of shake flasks enables the tight control of critical culture parameters (e.g. pH, aeration, temperature).

In their natural environments, as well as in industrial processes, growth of microorganisms is often limited by a single nutrient. For instance, during baker's yeast production, yeast is grown aerobically under sugar limitation to achieve high biomass yields. Conversely, during processes such as beer fermentation and dough fermentation, high concentrations of fermentable sugars are present under anaerobic conditions, and growth is limited by other nutrients (e.g. oxygen and/or nitrogen). In recent large-scale transcriptome analyses of *Saccharomyces cerevisiae* grown under various nutrient limitations (Boer *et al*, 2003; Daran-Lapujade *et al*, 2004; Saldanha *et al*, 2004; Wu *et al*, 2004b; Tai *et al*, 2005), specific transcriptional responses to the limiting nutrient were identified. However, it is not known to what extent these major transcriptional responses are actually translated into quantitatively identical responses at the protein level.

The aim of this study was to investigate the proteomic response of *S. cerevisiae* grown under different nutrient limitation regimes (carbon and nitrogen limitation) and to assess to what extent changes at the transcriptional level are reflected in changes at the protein level. For this purpose, we performed a quantitative proteome analysis of chemostat-cultivated *S. cerevisiae* limited for glucose or ammonia. The proteins were labeled *in vivo* by metabolic stable isotopic labeling with ^{15}N and quantified by mass spectrometry. The protein data set was less biased compared to standard 2D gel-based analysis, as proteins originating from different subcellular compartments, including membrane proteins, and proteins with extreme isoelectric points and molecular weights were identified. In order to reduce error and noise in the data, we used rather stringent criteria to filter the protein expression levels, resulting in a data set of 102 proteins that were considered as significantly changed. The functional annotation of these 102 proteins provided insight into how the yeast cell copes with nitrogen and carbon limitation at the protein level. Moreover, by comparing the proteome data with corresponding transcriptome data, it was found that transcriptional control mechanisms play a significant role in gene expression regulation under glucose limitation, whereas under ammonia limitation protein expression was mainly regulated post-transcriptionally. These observations clearly underline the need for multilevel analysis in yeast systems biology approaches.

Results

Data quantity and quality

S. cerevisiae was cultivated in chemostat cultures limited for either the carbon (i.e. glucose) or the nitrogen source (i.e. ammonia). Metabolic labeling with ^{15}N as stable isotope was used to uniformly label the yeast proteins in the nitrogen-limited chemostats (Figure 1). Mass spectrometric analysis showed that the ^{15}N stable isotope label was fully incorporated in the proteins after growth on the ^{15}N-labeled media (data not shown). Equal amounts of protein extracts of the carbon-limited and nitrogen-limited yeast cultures were mixed and subsequently separated by 1D SDS–PAGE. The gel lane was cut into 40 slices and each slice was digested in-gel with trypsin. The resulting complex tryptic digests were analyzed by nanoflow-LC-MS/MS. Proteins were identified using MS/MS and relative protein expression levels were obtained by comparing the extracted ion chromatograms of the ^{14}N- and ^{15}N-labeled peptide.

It is well documented that replicate nanoflow-LC-MS/MS measurements of the same complex peptide mixture are beneficial as they lead to increasing number of peptide identifications (Liu *et al*, 2004; Taoka *et al*, 2004). Indeed, despite separation of the peptide mixtures with high-resolution liquid chromatography before MS analysis, still many peptides coeluted. The LCQ mass spectrometer is then unable to collect tandem mass spectra from all these coeluting peptides via data-dependent acquisition. Therefore, the 40 tryptic digests were analyzed twice with nanoflow-LC-MS/MS, in order to increase the number and the reliability of identified and relatively quantified proteins. This replicate analysis led to the identification of 928 proteins (Table I and Supplementary Table SI). Among these identified proteins, 581 were found in both analysis sets, whereas 172 were exclusively detected in the first analysis set, and 176 solely in the second analysis.

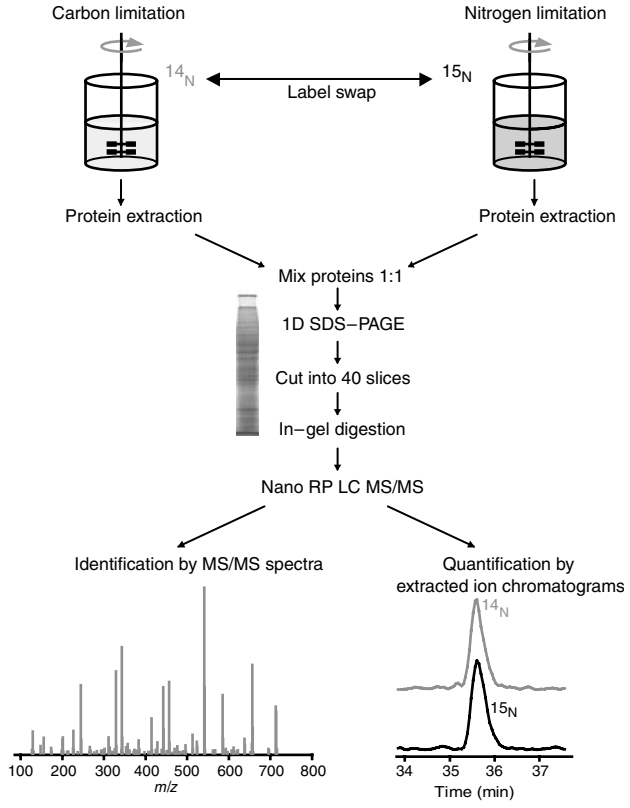

Figure 1 Schematic overview of quantitative proteomics approach used to study the effect of nutrient limitation on the yeast proteome.

Table I Summary of proteins identified and/or quantified in this study

	Number of proteins
Identified in both first and second LC-MS/MS run	581
Identified only in first LC-MS/MS run	172
Identified only in second LC-MS/MS run	176
Total no. of identified proteins	928
Quantified proteins	645
'On/off' proteins	114
Nonquantified	165
Total no. of quantified proteins	759

These results clearly illustrate the limits of the used nanoflow-LC-MS/MS approach.

To obtain a global view of the 928 identified proteins and to estimate how our data set compares to standard 2D gel techniques, a virtual 2D gel of the identified proteins and of the total *S. cerevisiae* proteome was constructed (Figure 2A). The virtual 2D gel of the identified proteins largely overlaps the 2D gel of the whole yeast proteome, 29% of the identified proteins falling outside the p*I* and Mw range of a typical 2D gel (p*I* 4–9, Mw 15–150 kDa). Proteins of all p*I* were identified, clearly showing that our data set is not biased by this biochemical property. However, our data set apparently excluded low molecular weight proteins (e.g. proteins with a molecular weight smaller than 10 kDa). This bias could be reasonably explained by the use of a 12% SDS–PAGE gel, on which only proteins with molecular weights above 15 kDa can be well resolved.

Another well-known drawback of classical 2D gels is the restriction to proteins with high abundance in the cells. In order to investigate if our data set was also biased by protein abundance, the codon adaptation index (CAI) distribution of the identified proteins was plotted and compared to the distribution of the complete yeast proteome (Figure 2B). Clearly, proteins with the complete range of CAI were identified (from 0 to 1); however low expressed proteins with a CAI smaller than 0.2 were under-represented compared to the yeast proteome. Conversely, highly abundant proteins with a CAI greater than 0.2 were over-represented in our data set compared to the complete yeast proteome. These results, in good agreement with previous studies (Liu *et al*, 2004), reveal a limitation of the so-called 'shotgun proteomics' approaches in which the high complexity of the peptide mixtures results in the preferential identification of high-abundance proteins by the sampling process.

The subcellular localization of the 928 identified proteins was compared to that of the total *S. cerevisiae* proteome using the *Saccharomyces* Genome Database for classification (Figure 2C). The largest group of identified proteins originated from the cytoplasm (34%) and, like the group of ribosomal proteins, was over-represented compared to the complete yeast proteome. This enrichment was probably owing to the extraction method applied and to the fact that these proteins are relatively abundant in the cell. The other subcellular compartments were rather well represented in our analysis. It is noteworthy that without any specific enrichment or isolation methods for membrane proteins, we were able to detect 95 membrane proteins of which 49, 19 and 9% originated from mitochondrial, plasma and vacuolar membranes, respectively.

Differential expression

Relative protein expression levels (carbon limitation versus nitrogen limitation) were obtained by using the quantitative proteomics algorithm RelEx (MacCoss *et al*, 2003). This program compares the extracted ion chromatograms of the unlabeled (^{14}N) with the corresponding labeled (^{15}N) peptides.

Several proteins could be detected by a set of unique peptides under one growth-limiting condition, with their counterparts from the other condition being below the detection level. Examples of these 'on/off' peptides are depicted in Figure 3A. The panel on the left shows the extracted ion chromatograms of the ^{14}N and ^{15}N version of the DIDIEYHQNK-peptide of heat shock protein 26 (Hsp26p). Only the ^{14}N version of the peptide, which originated from the carbon-limited chemostat culture, could be detected. On the right side, the extracted ion chromatograms of the ^{14}N and ^{15}N version of the GTMITLNDR-peptide from the yeast protein L-asparaginase II (Asp3p) are shown. It is clear that only the ^{15}N-labeled version of this peptide, which originated from the nitrogen-limited chemostat culture, could be detected. All detected 38 peptides of Hsp26p showed this extreme upregulation in the carbon-limited chemostat culture and also all 16

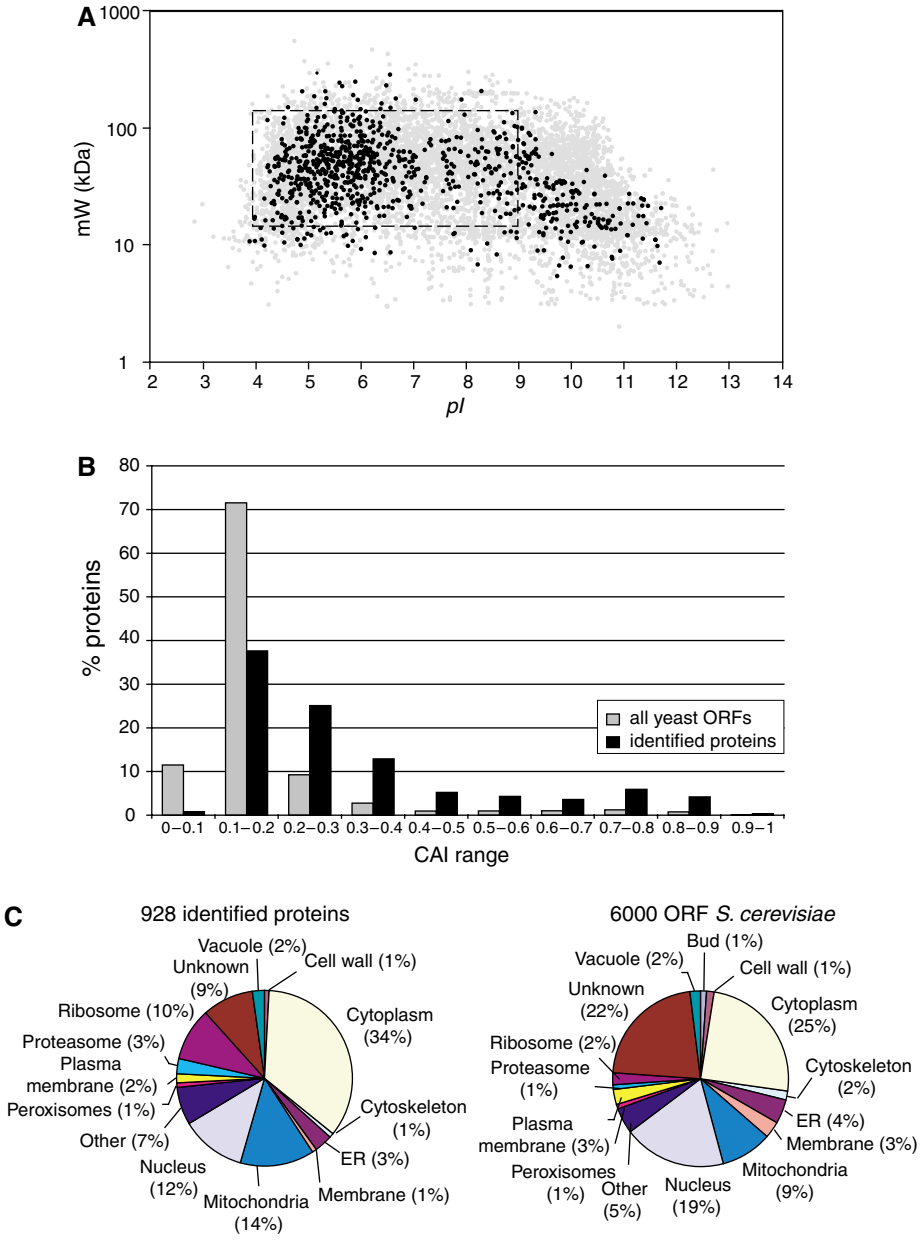

Figure 2 Comparison of the properties of the 928 identified proteins and the total *S. cerevisiae* proteome. (**A**) Virtual 2D gel of the identified proteins (black dots) and of all ORFs of *S. cerevisiae* (gray dots). The area covered by a typical 2D gel (p*I* 4–9, Mw 15–150 kDa) is indicated with a dashed rectangle. (**B**) Distribution of the CAI of the proteins identified in this study and of the complete yeast proteome. (**C**) Subcellular localization distribution of the 928 identified proteins (left) and of all ORFs of *S. cerevisiae* (right) as defined by the *Saccharomyces* Genome Database. The percentage of proteins present in each subcellular localization is given in parentheses.

peptides of Asp3p were detected only in the nitrogen-limited chemostat culture. These 'on/off' peptide ion ratios showed poor correlation using the RelEx algorithm and would therefore be automatically filtered out of the data set if the correlation filter option in the software is used. Therefore, all relative protein expression levels obtained using the RelEx software were also inspected manually to detect the 'on/off' proteins. Next, the remaining data set was filtered using the signal to noise and the regression filter within the RelEx software. The remaining peptide ratios were used for determination of protein expression ratios and their respective standard deviation. In total, 759 of the 928 identified proteins were relatively quantified. The list of quantified proteins can be found in Supplementary Table II.

Reciprocal labeling control

In order to evaluate the influence of sample processing and biological variation on the relative quantification of proteins, a complete parallel analysis using independent yeast cultivations was performed. Therefore, two new chemostats were run in which we inverted the ^{15}N label, that is, the nitrogen-limited culture was now grown on normal ^{14}N media, whereas the carbon-limited culture was grown on ^{15}N-labeled media.

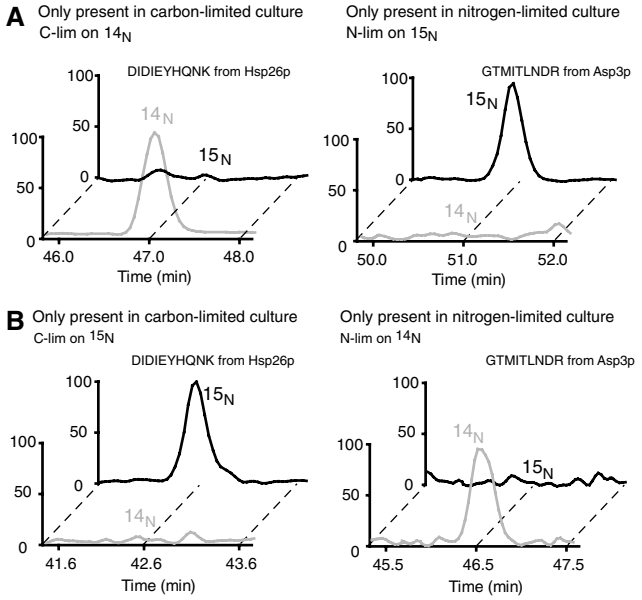

Figure 3 Typical examples of extremely regulated proteins. (**A**) The panel on the left side shows the extracted ion chromatograms of the ^{14}N- and ^{15}N-labeled version of the DIDIEYHQNK peptide from Hsp26p, which could only be detected in the ion chromatogram of the carbon-limited culture (^{14}N version). The panel on the right shows the extracted ion chromatograms of the ^{14}N- and ^{15}N-labeled GTMITLNDR peptide from Asp3p. This peptide is only present in the nitrogen-limited yeast culture (^{15}N version). (**B**) Extracted ion chromatograms from the same peptides as in panel A are shown for the reverse labeling experiment. In this experiment, the nitrogen-limited culture was grown on ^{14}N media and the carbon-limited culture on ^{15}N-labeled media.

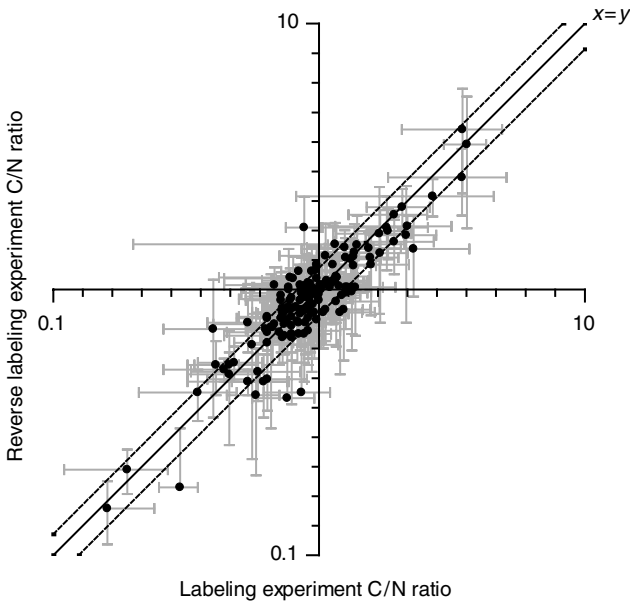

Figure 4 Scatter plot of labeling and reverse labeling experiments. Only proteins that were quantified on the basis of at least three peptide peak pairs in both experiments were considered in this comparison (n=156). The solid line in the graph indicates a perfect correlation between the two experiments. The dashed lines indicate a 20% deviation in protein ratios.

The same procedure as described in Figure 1 was followed for this second experiment, except that the 1D gel was cut into 10 pieces instead of 40 and that one nanoflow-LC-MS/MS was run per gel slice instead of two. Consequently, the protein expression data set of the reverse experiment was smaller. Nevertheless, we were able to identify 171 proteins in both experiments that could be quantified on the basis of at least three peptide peak pairs in each of the two experiments. From these 171 proteins, a subset of 15 so-called 'on/off' regulated proteins was detected in both data sets and the regulation was consistent between the inverse labeling experiments, for example, a protein was found solely in the nitrogen-limited culture in both experiments (Figure 3A and B). Additionally, plotting the expression ratios of the other 156 proteins obtained from the labeling and reverse labeling experiments versus each other (Figure 4) showed a good correlation between the two experiments. Indeed, three-quarter of the proteins showed less than 20% deviation in the forwarded and reverse labeling ratios, reflecting good experimental and biological reproducibility. Furthermore, by performing the reversed labeling experiment, we also showed that the ^{15}N mass label did not influence protein expression patterns in chemostat-cultivated yeast. As the experimental design of the reverse labeling was slightly different from the design used for the standard labeling experiment, it was not further used for data analysis.

Additionally, to validate the quantitative proteome data set, we performed Western blots of a subset of proteins. These results are shown in Supplementary Figure S1 and revealed a good agreement between the Western blot and mass spectrometry-based quantification.

Proteome responses to carbon and nitrogen limitation

To investigate the response of *S. cerevisiae* to carbon and nitrogen limitation, we focused on the analysis of the 759 quantified proteins identified from the labeling experiment (Supplementary Table SII). An important parameter to assess the accuracy and reliability of the proteome analysis for the quantification of protein expression is the number of peptides identified per protein. In our data set, as many as 68% of the proteins were quantified based on two peptides or more, and 40% with five peptides or more (Figure 5A). Thus, for most proteins, multiple peptide ratios were used to calculate the relative expression levels of the protein leading to meaningful standard deviations for quantification. The average coefficient of variation for all quantified proteins was 30%. The distribution of the expression ratios of these proteins is depicted in Figure 5B.

We used rather stringent data mining criteria (see Materials and methods) to filter our data to obtain a high-quality data set of proteins that are differentially expressed. In this high-quality data set, 51 proteins were specifically upregulated in response to glucose limitation (Table IIA), whereas 51 proteins were downregulated under ammonium limitation (Table IIB). This set of 102 proteins was further evaluated to determine if proteins that belong to certain functional categories were specifically regulated in response to carbon or nitrogen limitation (Table III). Both limitations resulted in the over-representation of proteins in the category 'metabolism'

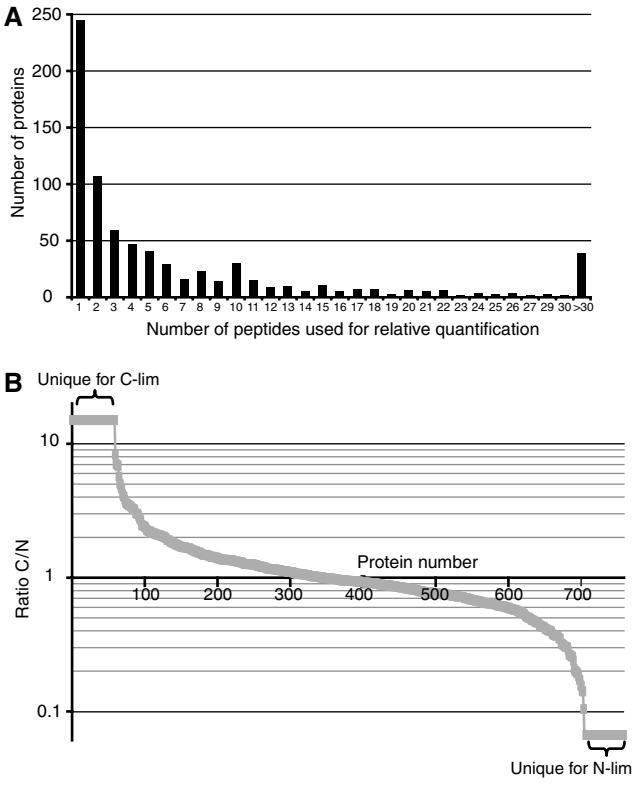

Figure 5 Protein ratio distribution and the number of peptide pairs used for relative quantification. (**A**) Distribution of the 759 proteins as a function of the number of peptide pairs used for quantification. (**B**) Plot of relative protein expression levels from the carbon-limited versus the nitrogen-limited chemostat culture of all 759 relatively quantified proteins.

(42 proteins), which reflected a major metabolic rearrangement in yeast in order to adapt to the altered nutrient availability. As anticipated, under carbon limitation, the metabolic changes mainly concerned carbon metabolism-related proteins (18 proteins). Interestingly, proteins localized in mitochondria (12 proteins) and peroxisomes (nine proteins) were particularly overexpressed under carbon limitation. An in-depth analysis of the differentially expressed proteins revealed that at least 31 of the 102 differentially expressed proteins were consistent with the anticipated yeast response to carbon and nitrogen limitation (Figure 6).

When comparing ammonia-limited to glucose-limited cultures, yeast has to face two major changes in the environment: an excess of glucose and a shortage of ammonia. The effect of these changes on yeast physiology has already been described (Boer *et al*, 2003) and it will not be fully repeated here. Important for this study, glucose excess is known to trigger carbon catabolite repression via several signaling pathways, which has many consequences on gene and protein expression (for review see Gancedo, 1998). First of all, the presence of glucose, which is the preferred carbon source of *S. cerevisiae*, downregulates the expression of proteins involved in the utilization of alternative carbon sources. Typical genes subjected to glucose repression and/or inactivation, such as *ADH2*, *ACS1* or *MLS1* involved in C2-compounds (ethanol, acetate) utilization, *SUC2* and *CYB2* for sucrose and lactate degradation respectively, consistently displayed a decreased protein expression under nitrogen limitation, that is, glucose excess (Figure 6). Furthermore, while yeast cells grow with a full respiratory metabolism when glucose is the limiting nutrient, the excess of glucose triggers a mixed respiro-fermentative metabolism (so-called long-term Crabtree effect). Therefore, under glucose excess, the expression of proteins involved in respiratory metabolism is repressed, as confirmed by the downregulation of the tricarboxylic acids cycle (Mdh3p, Idp2p) and oxidative phosphorylation (Cyc1p, Nde1p) proteins (Figure 6). Most of these proteins are localized in the mitochondria, which corroborates the over-representation of mitochondrial proteins among the differentially expressed proteins under carbon limitation in our data set. Furthermore, glucose excess represses the expression of genes involved in fatty acid β-oxidation via Adr1p, the glucose repressible activator of the two major transcription factors involved in β-oxidation and peroxisome biogenesis, Oaf1p and Pip2p (Gurvitz *et al*, 2001). This is consistent with the down-regulation under glucose excess of Pox1p, Faa2p, Fox2p and Pot1p, all targets of Oaf1p and Pip2p that catalyze critical steps in β-oxidation (Figure 6). These metabolic proteins are localized in peroxisomes, which partly explained the over-representation of peroxisomal proteins among the glucose-responsive proteins (Table III). Several proteins specialized in the utilization of glucose when present at low concentration (hexose transporter Hxt6p and hexokinases Glk1p and Hxk1p) were also downregulated when glucose was in excess. Conversely, several proteins involved in substrate level phosphorylation (the main pathway for energy generation under fermentative metabolism) were upregulated when nitrogen was the limiting nutrient (Tpi1p, Tdh2p, Gpm1p, Pdc1p, Pdc5p).

The other expected cellular response concerns nitrogen availability. Indeed nitrogen availability, as well as the type of nitrogen source (ammonia is the preferred nitrogen source of *S. cerevisiae*), triggers a complex and not yet fully understood metabolic rearrangement (for review see ter Schure *et al*, 2000; Wek *et al*, 2004). In literature, nitrogen starvation rather than nitrogen limitation has been the subject of intensive study and little is known about the differences between these two growth conditions. Recently, a yeast transcriptome analysis revealed that leucine limitation in chemostat and leucine starvation in batch resulted in comparable gene expression profiles (Saldanha *et al*, 2004). Similarly, in our data set, we can see two major responses that have also been previously described under nitrogen depletion, that is, scavenging of alternative nitrogen sources (Magasanik and Kaiser, 2002) and efficient protein turnover, also known as macroautophagy (Takeshige *et al*, 1992). Indeed, in response to low ammonia supply, yeast cells induced the expression of proteins involved in the transport (Gap1p) and the degradation (Dal7p, Put2p, Asp3-1p) of alternative nitrogen sources. Another way of optimizing nitrogen utilization in the cell is to recover the amino acids by protein turnover. This response is reflected by the upregulation of four ubiquitin-dependent and -independent proteases (Figure 6).

It is noteworthy that several pathways involved in carbon and nitrogen metabolism, relevant for this study, were almost completely identified. This is particularly true for glycolysis (24 proteins identified out of the 27 proteins in this pathway)

Table II Proteins significantly upregulated in response to (A) glucose and (B) nitrogen limitation

Systematic name	Gene name	Functional category	Protein				Transcript (Boer *et al*, 2003)		
			No. of peptides[a]	Avg C/N[b]	s.d.	CV[c] (%)	Avg C/N[d]	s.d.	*P*-value[e]
(A) Upregulation under carbon limitation									
YBL015W	*ACH1*	Carbohydrate metabolism	10	3.4	0.9	26	*3.1*	*1.4*	*0.01*
YAL054C	*ACS1*	Carbohydrate metabolism	35	C			**21.3**	**3.8**	**0.01**
YDL124W	YDL124W	Carbohydrate metabolism	5	1.9	0.4	20	1.4	0.3	0.09
YDR516C	YDR516C	Carbohydrate metabolism	3	4.1	1.5	38	1.4	0.3	0.07
YNL134C	YNL134C	Carbohydrate metabolism	2	C			**0.36**	**0.09**	**0**
YML128C	*MSC1*	Cell cycle and DNA processing	4	5.1	2.1	42	**3.8**	**1.2**	**0.05**
YLR286C	*CTS1*	Cell wall maintenance	2	C			0.7	0.1	0
YNL079C	*TPM1*	Cytoskeleton	2	C			1	0.2	0.87
YPL276W	*FDH2*	Energy	10	C			**86.8**	**22.2**	**0.03**
YMR145C	*NDE1*	Energy	5	2.2	0.6	26	**2**	**0.3**	**0**
YOR374W	*ALD4*	Ethanol utilization	107	C			**3.8**	**0.9**	**0**
YGL205W	*POX1*	Fatty acid metabolism	8	C			**18.2**	**3**	**0**
YER015W	*FAA2*	Fatty acid metabolism	3	C			**8.5**	**1.8**	**0.02**
YKR009C	*FOX2*	Fatty acid metabolism	2	7.1	1.1	15	**8.9**	**3**	**0.03**
YIL160C	*POT1*	Fatty acid metabolism	2	C			**16.7**	**5**	**0.04**
YIL155C	*GUT2*	Glycerol metabolism	41	C			**3**	**0.9**	**0.02**
YMR303C	*ADH2*	Glycolysis	26	C			**147.5**	**54.1**	**0.05**
YBR145W	*ADH5*	Glycolysis	2	C			1.3	0.1	0.01
YCL040W	*GLK1*	Glycolysis	13	4.2	0.8	20	2.1	0.8	0.13
YFR053C	*HXK1*	Glycolysis	44	C			**3.5**	**1.2**	**0.01**
YKL127W	*PGM1*	Glycolysis	2	3	0.2	5	0.8	0.2	0.21
YMR125W	*STO1*	Glycolysis	2	1.7	0.1	3	1	0.1	0.55
YNL117W	*MLS1*	Glyoxylate cycle	2	1.5	0.3	20	*0.4*	*0.1*	*0.01*
YJL153C	*INO1*	Inositol metabolism	27	3.4	1.6	47	**2.8**	**0.6**	**0.03**
YDL120W	*YFH1*	Iron homeostasis	2	C			**1.5**	**0.2**	**0.04**
YML054C	*CYB2*	Lactate utilization	12	C			**3.6**	**0.7**	**0**
YNL104C	*LEU4*	Leucine biosynthesis	4	2.4	0.5	19	**1.8**	**0.2**	**0.03**
YDR234W	*LYS4*	Lysine biosynthesis	3	2	0.6	27	1.5	0.3	0.12
YIL136W	*OM45*	Mitochondrial organization	3	4.6	1.4	30	**4.8**	**1.4**	**0.04**
YMR294W	*JNM1*	Mitosis, nuclear migration	2	C			*0.61*	*0.14*	*0.04*
YBR237W	*PRP5*	mRNA splicing	2	C			0.76	0.14	0.07
YGR159C	*NSR1*	Nuclear protein targeting	9	3.6	0.6	18	**4.5**	**2.1**	**0.02**
YJR048W	*CYC1*	Oxidative phosphorylation	5	1.7	0.1	6	**1.7**	**0.3**	**0.02**
YFR033C	*QCR6*	Oxidative phosphorylation	2	1.5	0.1	8	1.3	0.1	0.07
YDR256C	*CTA1*	Oxidative stress response	13	C			**102.2**	**17.3**	**0.01**
YOL147C	*PEX11*	Peroxisome biogenesis	4	3.5	1.4	39	**2.2**	**0.4**	**0.02**
YJR110W	YJR110W	Phosphate metabolism	2	C			0.85	0.17	0.24
YNL015W	*PBI2*	Protein fate	3	1.7	0.2	9	1.4	0.2	0.04
YOR020C	*HSP10*	Protein folding	9	1.7	0.3	17	**1.6**	**0.2**	**0.01**
YOR027W	*STI1*	Protein folding	20	2.1	0.5	26	**1.8**	**0.3**	**0.01**
YPL160W	*CDC60*	Protein synthesis	4	2.1	0.6	29	0.79	0.14	0.14
YEL034W	*HYP2*	Protein synthesis	8	2.1	0.6	30	0.84	0.29	0.49
YBR072W	*HSP26*	Stress	38	C			**8**	**1.6**	**0.01**
YIL162W	*SUC2*	Sucrose utilization	11	C			**5.8**	**1**	**0.02**
YLR174W	*IDP2*	TCA cycle	10	C			**13.2**	**2.4**	**0.01**
YDL078C	*MDH3*	TCA cycle	3	2.9	0.3	12	**1.9**	**0.2**	**0.02**
YDR343C	*HXT6*	Transport	7	C			1.2	0.3	0.41
YCR010C	*ADY2*	Transport	5	C			**86**	**10.8**	**0.01**
YML091C	*RPM2*	tRNA processing, mitochondrial	2	C			1.1	0.2	0.43
YPL186C	*UIP4*	Unknown	2	3.4	0.1	3	**2.2**	**0.5**	**0.05**
YOR285W	YOR285W	Unknown	10	3.5	1.4	41	**1.5**	**0.1**	**0.02**
(B) Upregulation under nitrogen limitation									
YIR031C	*DAL7*	Allantoin utilization	4	N			**0.08**	**0.02**	**0**
YHR018C	*ARG4*	Arginine metabolism	5	0.48	0.09	18	0.85	0.2	0.32
YLR155C	*ASP3-1*	Asparagine metabolism	16	N			**0.62**	**0.11**	**0.01**
YGL252C	*RTG2*	Carbohydrate metabolism	2	N			0.67	0.1	0.05
YMR215W	*GAS3*	Carbohydrate metabolism	8	N			**0.61**	**0.1**	**0.01**
YKR043C	YKR043C	Carbohydrate metabolism	2	0.5	0.12	24	0.87	0.08	0.08
YDR033W	*MRH1*	Cell rescue, defense and virulence	2	N			**0.4**	**0.04**	**0**
YNL030W	*HHF2*	Chromatin structure	12	0.46	0.12	26	0.93	0.13	0.5
YNL031C	*HHT2*	Chromatin structure	2	0.42	0.06	15	0.94	0.04	0.09
YDR174W	*HMO1*	Chromatin structure	4	0.43	0.11	25	0.86	0.13	0.19
YDR225W	*HTA1*	Chromatin structure	12	0.46	0.14	30	1.3	0.2	0.12
YOR144C	*ELG1*	DNA replication	2	N			0.71	0.11	0.05
YKR046C	*PET10*	Energy	11	0.3	0.09	30	0.95	0.13	0.56
YPL117C	*IDI1*	Fatty acid metabolism	3	0.56	0.02	3	0.95	0.1	0.46
YLR229C	*CDC42*	Filamentous or polarized growth	2	0.6	0.02	3	0.96	0.11	0.57
YOR375C	*GDH1*	Glutamate metabolism	62	0.41	0.09	21	1	0.03	0.11
YPR035W	*GLN1*	Glutamin metabolism	8	0.31	0.08	26	0.84	0.11	0.13

Table II Continued

Systematic name	Gene name	Functional category	Protein				Transcript (Boer *et al*, 2003)		
			No. of peptides[a]	Avg C/N[b]	s.d.	CV[c] (%)	Avg C/N[d]	s.d.	*P*-value[e]
YER133W	*GLC7*	Glycogen metabolism	2	0.62	0	0	0.71	0.18	0.07
YMR083W	*ADH3*	Glycolysis	4	0.61	0.11	18	0.73	0.08	0.02
YKL152C	*GPM1*	Glycolysis	63	0.63	0.1	16	0.96	0.16	0.72
YLR044C	*PDC1*	Glycolysis	78	0.16	0.08	50	0.81	0.17	0.2
YLR134W	*PDC5*	Glycolysis	7	0.1	0.04	36	**0.06**	**0**	**0**
YJR009C	*TDH2*	Glycolysis	47	0.67	0.11	17	1.1	0.07	0.09
YDR050C	*TPI1*	Glycolysis	32	0.48	0.13	27	0.8	0.14	0.16
YGR155W	*CYS4*	Methionine biosynthesis	10	0.5	0.11	22	**0.65**	**0.09**	**0.04**
YJL203W	*PRP21*	mRNA splicing	2	N			1.2	0.23	0.32
YER009W	*NTF2*	Nuclear protein targeting	3	0.64	0.06	10	0.88	0.08	0.12
YBR106W	*PHO88*	Phosphate transport	5	0.6	0.11	18	0.95	0.09	0.43
YHR037W	*PUT2*	Proline metabolism	10	0.3	0.13	43	**0.35**	**0.04**	**0**
YBR286W	*APE3*	Protein degradation	5	0.24	0.11	48	1.1	0.33	0.66
YJL172W	*CPS1*	Protein degradation	20	N			0.12	0.08	0.07
YKL103C	*LAP4*	Protein degradation	5	0.26	0.13	50	**0.33**	**0.06**	**0.03**
YMR314W	*PRE5*	Protein degradation	2	0.64	0	0	0.98	0.17	0.8
YDR533C	*HSP31*	Protein fate	5	0.21	0.05	22	**0.42**	**0.06**	**0.01**
YOR370C	*MRS6*	Protein processing	2	0.65	0.13	19	0.77	0.08	0.02
YGL245W	YGL245W	Protein synthesis	29	0.66	0.09	14	0.8	0.09	0.05
YLL024C	*SSA2*	Protein translocation	9	0.47	0.09	19	1.1	0.19	0.63
YBL047C	*EDE1*	Protein translocation	2	0.6	0.12	20	0.74	0.14	0.14
YDR086C	*SSS1*	Secretion	2	0.51	0.06	11	0.9	0.14	0.35
YMR226C	YMR226C	Serine metabolism	13	0.3	0.05	17	0.86	0.09	0.13
YGL008C	*PMA1*	Small molecule transport	37	0.19	0.08	41	**0.57**	**0.04**	**0**
YHR190W	*ERG9*	Sterol metabolism	2	0.32	0.02	5	1.1	0.23	0.48
YDL066W	*IDP1*	TCA cycle	2	0.61	0.07	12	**0.64**	**0.05**	**0.02**
YKR039W	*GAP1*	Transport	11	N			**0.18**	**0.03**	**0**
YDR497C	*ITR1*	Transport	2	N			1.2	0.15	0.19
YDL198C	*YHM1*	Transport	3	0.5	0.08	15	0.68	0.11	0.02
YHR049W	*FSH1*	Unknown	2	N			**0.15**	**0.06**	**0**
YJL171C	YJL171C	Unknown	2	0.2	0.05	25	**0.43**	**0.12**	**0.01**
YLR364W	YLR364W	Unknown	2	0.46	0.03	6	0.81	0.15	0.18
YMR090W	YMR090W	Unknown	2	0.29	0.03	10	**0.27**	**0.1**	**0**
YOR332W	*VMA4*	Vacuolar acidification	5	0.41	0.05	13	0.71	0.27	0.23

Transcriptome data obtained from identical culture conditions (Boer *et al*, 2003) are presented with their corresponding proteins.
Matching protein and transcript changes are indicated in a gray background.
[a]Amount of peptides used for protein quantification.
[b]Average of C/N peptide ratios.
[c]Coefficient of variation.
[d]Average of ratios of carbon-limited over nitrogen-limited transcript levels (triplicate experiments).
[e]*P*-value resulting from a *t*-test between nitrogen- and carbon-limited cultures. Significantly changed transcript levels (ratio greater than 1.5 or smaller than 0.66 and *P*-value below 0.05) are indicated in bold.

Table III Functional categories over-represented among the differentially expressed proteins

MIPS functional category	Number of genes in category	*P*-value
Upregulated under glucose limitation		
Energy	18	5.1×10^{-13}
Metabolism	18	4.1×10^{-8}
C-compound and carbohydrate metabolism	16	2.2×10^{-9}
Mitochondria	12	3.2×10^{-5}
Peroxisome	9	1.6×10^{-11}
Upregulated under ammonia limitation		
Energy	10	2.3×10^{-5}
Metabolism	24	3.6×10^{-7}

Significance was estimated with FunSpec (http://funspec.med.utoronto.ca/) based on the hypergeometric distribution of the MIPS functional categories of the differentially expressed proteins compared to the yeast proteome (*P*-value smaller than 0.01, Bonferroni correction applied).

and the tricarboxylic acids cycle (17 out of 25), as well as for several amino acids metabolic pathways (glutamate and glutamine, five proteins out of six; leucine, five out of six; lysine, six out of seven; and methionine, seven out of 10).

Discussion

The adaptation of *S. cerevisiae* to different nutrient limitations, namely carbon and nitrogen, was studied in depth at the proteome level. Steady-state chemostat cultures at fixed growth rate were used to grow the cells under tightly controlled conditions. Chemostat cultivation provides an elegant tool that allows the investigation of the influence of a single nutrient limitation under steady-state conditions while all other parameters remain constant, in contrast to batch cultivation where the growth parameters change continuously. In order to relatively quantify proteins expression levels, yeast proteins were metabolically labeled in chemostat cultures

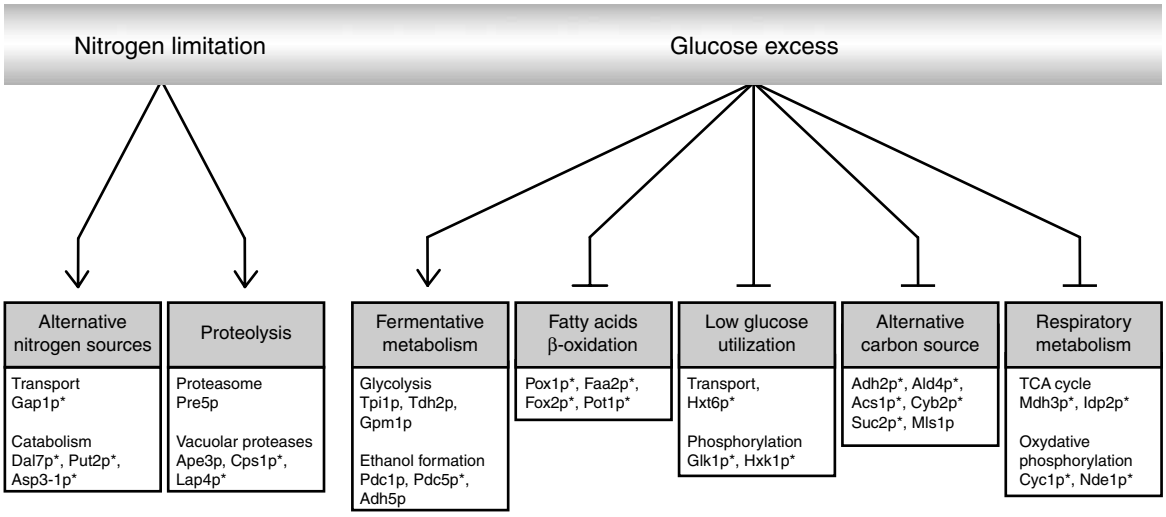

Figure 6 Sorting of the differentially expressed proteins as a function of the specific response to either glucose excess or ammonia limitation. The asterisk highlights a matching change in transcript level (C/N ratio greater than 1.5 or smaller than 0.66 and *P*-value below 0.05) (Boer *et al*, 2003). Arrows indicate a positive regulation, whereas bars indicate negative control.

using either ^{14}N or ^{15}N as a stable isotope followed by 1D gel electrophoresis and nanoflow-LC-MS/MS.

Stable isotope metabolic labeling has been applied to the yeast *S. cerevisiae* in previous proteome studies. Different stable isotopes such as ^{15}N (Oda *et al*, 1999; Washburn *et al*, 2002) and labeled amino acids (Berger *et al*, 2002; Jiang and English, 2002) have been incorporated into yeast by metabolic labeling. In most studies, batch cultivation was used to grow the yeast cells. Exceptions are the studies performed by Pratt *et al* (2002a,b), in which chemostat cultivation was used, whereby they showed that it is possible to label chemostat-cultivated yeast with decadeuterated leucine ($^2H_{10}$-Leu) as stable isotope. The *S. cerevisiae* strain used by Pratt *et al* was a leucine auxotroph. Here, we report for the complete incorporation of ^{15}N in proteins of the prototrophic *S. cerevisiae* strain CEN.PK113-7D cultivated in steady-state chemostat cultures.

Reproducibility and validation of the quantitative proteomics data

The duplicate analysis of the tryptic digests with nano-LC-MS/MS increased the number of protein identifications by approximately 25%. Liu *et al* (2004) have shown that several MudPIT analyses are necessary to achieve more than 95% confidence of analytical completeness in the proteome analysis. Therefore, subsequent replicate analysis of our samples might still increase the number of protein identifications, however at the expense of time. Still, our data set of 759 proteins provides already a clear insight into the global changes in the yeast proteome under the applied different environmental conditions. This protein set is less biased compared to 2D gel-based analyses, as we identified proteins with extreme isoelectric points (e.g. very basic protein, $pI > 9$) and extreme molecular weights (e.g. $Mw < 15$ kDa and $Mw > 150$ kDa) as well as very hydrophobic proteins, for example, integral plasma membrane proteins. As previously described, such shotgun proteomics approaches predominantly identify the most abundant proteins. Nevertheless, many proteins with low CAI (below 0.2) were identified in our data set.

By performing an inverse isotope labeling experiment, we showed that the influence of biological and analytical variation on protein expression levels is minimal under our experimental conditions (Figure 4), which we expect may be largely attributed to the fact that we use well-controlled chemostat cultures. In essence, all proteins that we could quantify by using at least three peptide pairs showed a good correlation between the ratio determined in the isotope labeling and the inverse isotope labeling experiment. We consider such inverse labeling experiment to be useful for validation of quantitative proteomics data. This set of 171 proteins provides a high-quality, validated quantitative proteome data set. The quality of this data set exceeds most current ICAT studies whereby most proteins are quantified only on one or two peptides per protein.

Yeast proteome adaptation to nutrient limitation

Owing to the broad accessibility of microarrays, most genome-wide approaches are so far based on transcriptome analysis. Glucose repression in yeast, for instance, has mainly been studied at the level of transcription with a few exceptions using 2D gel electrophoresis (Boy-Marcotte *et al*, 1998; Haurie *et al*, 2004), and so far no proteome analysis investigated response to nitrogen starvation or limitation. The development of high-throughput and reliable LC-MS/MS approaches makes proteomics an accessible tool for quantitative physiology. In the present study, we measured the relative expression levels of 759 proteins during nitrogen and carbon limitation (Supplementary Table SII). Using stringent statistical criteria, we identified a robust subset of 102 differentially expressed proteins (Tables IIA and B). In response to glucose excess, many genes known to be transcriptionally repressed displayed a corresponding decrease in protein expression. Nitrogen limitation in chemostats has so far only been described at the

transcriptome level, and other studies focus on nitrogen starvation rather than limitation. Our data set revealed that, at the protein level, *S. cerevisiae* limited for nitrogen displayed two responses previously described during nitrogen starvation, that is, the expression of proteins involved in nitrogen source scavenging and in protein turnover (Figure 6). For the sake of data reliability, we limited our analysis to proteins identified with two peptides or more. However, many proteins identified with one single peptide also showed the expected change in expression in response to carbon and nitrogen limitation, such as proteins involved in oxidative phosphorylation (Cox1p, Cox8p, Cox9p) and utilization of alternative carbon sources (Jen1p, Dld1p, Icl1p), which were all upregulated under carbon limitation.

In our data set, we also identified and relatively quantified proteins that have unknown or poorly defined biological function. Some of these proteins were found upregulated under carbon or nitrogen limitation (Tables IIA and B). These proteins might be important for survival under carbon and nitrogen limitation and further research focused on this group of proteins may provide clues about their biological function.

Yeast protein complexes

As proteins often do not function on their own, but as part of larger protein complexes, we examined in our data how subunits of some well-described yeast protein complexes are regulated under nitrogen and carbon limitation. Expression levels of subunits that are part of large protein complexes were examined in more detail (Table IV). Interestingly, all seven identified subunits of the vacuolar H^+-ATPase showed increased expression under nitrogen limitation and six of the seven quantified subunits of cytochrome *c* oxidase showed an increase in expression under carbon limitation. However, most of the proteins from these complexes, with the exception of Vma4p, were excluded from our analysis owing to our strict criteria. The response to carbon or nitrogen limitation of these two complexes is however more than likely. Indeed the cytochrome *c* oxidase complex, involved in oxidative phosphorylation, is known to be repressed by glucose (Perlman and Mahler, 1974). The vacuolar ATPase ensures the acidification of these organelles, which is necessary for the export to the cytosol of the amino acids generated by the very active (and upregulated) vacuolar proteases (Sato *et al*, 1984). Similarly, most of the identified proteins of the proteasome seem to be upregulated under nitrogen limitation, whereas only Pre5p has been considered as significantly changed. These data suggest that proteins involved in complexes should not be considered individually, but rather in the context of their entire complex. However, for some complexes (e.g. the F0/F1 ATP synthase), opposite regulation of the different subunits makes the interpretation rather difficult. Different approaches can be used to further explore these interesting phenomena and get a more comprehensive view of the protein complex stoichiometry and dynamics under different nutrient-limited conditions. For example, a protein complex can be purified under different nutrient limitations with affinity-based techniques such as tandem affinity purification (Rigaut *et al*, 1999) or single-step affinity purification with the Flag epitope tag (Einhauer and Jungbauer, 2001) and the protein content and expression levels of the complex can be determined. Both methods have shown to be excellent tools for the characterization of protein complexes in large-scale protein interaction studies in yeast (Gavin *et al*, 2002).

Comparison of mRNA and protein expression levels

In order to obtain a more complete image of the cellular response of *S. cerevisiae* to carbon and nitrogen limitation, we used the transcriptome data previously described by Boer *et al* (2003), which were obtained under identical cultivation conditions and are available at http://www.nutrient-limited.bt.tudelft.nl. A scatter plot comparing the high-quality protein and transcript expression ratios for all 278 proteins identified with five peptides or more reveals a strong positive correlation compared to previous studies (Figure 7A). The positive Spearman rank correlation coefficient (Sr) of 0.55 calculated for these 278 gene products is among the highest coefficients described for yeast so far. Griffin *et al* (2002) and Washburn *et al* (2003) found weakly positive coefficients of 0.21 (n=245 loci) and 0.45 (n=678) respectively from yeast cultures grown in shake flasks. The use of chemostats in the present study could reasonably explain this rather good correlation between changes in transcript and protein levels. Indeed, the high reproducibility of steady states is likely to result in more accurate quantification, and therefore better correlation of all the measurements made in independent chemostats. The improved quality of transcriptome analysis using chemostat cultivations has been previously demonstrated (Piper *et al*, 2002). In contrast to Washburn *et al* (2003), we did not observe a specific deviation in the scattering of the ratios (Figure 7A), as they all appear to be relatively evenly spread around the perfect positive correlation line (x=y). When using all 278 proteins quantified with five peptides or more, we can therefore not conclude on the prominence of translational or transcriptional control in response to carbon and nitrogen limitation.

However, when plotting the subset of proteins differentially expressed, two very different regulation patterns emerged. The Spearman rank coefficient of the loci upregulated in carbon-limited cultures dramatically increased to 0.71. Additionally, the 'on/off' proteins only detected under carbon limitation, proteins that could not be plotted in Figure 7B, also showed a strong concomitant increase in the level of their transcript counterparts (Table IIA). This good correlation between changes in transcript and protein levels demonstrated that this upregulation in protein expression in response to glucose limitation is mainly exerted at the transcriptional level. Glucose repression is indeed known to act at the transcriptional level via several DNA-binding proteins (e.g. Mig1p, Mig2p, Adr1p) (Gancedo, 1998). In agreement with genome-wide studies both in dynamic or steady-state growth conditions (DeRisi *et al*, 1997; Boer *et al*, 2003; Daran-Lapujade *et al*, 2004), we observed that genes involved in the utilization of alternative carbon source were largely transcriptionally controlled. We further investigated four of these gene products: Faa2p, Pox1p, Fox2p and Pot1p (also known as Fox3p). These proteins catalyze five of the six reactions

Table IV Relative expression levels of proteins in some well-characterized protein complexes

Protein complex	Protein	Systematic name	Protein				Transcript	
			C/N[a]	s.d.	CV[b] (%)	No. of peptides[c]	C/N[d]	*P*-value
Arp2/3	Arp3p	YJR065C	0.87			1	1.02	0.89
(4/6)	Arc15p	YIL062C	0.75			1	1.04	0.75
	Arc18p	YLR370C	0.40	0.4	105	2	1.26	0.17
	Arc19p	YKL013C	0.38			1	1.08	0.52
Cytochrome *bc*1	Cor1p	YBL045C	1.37	0.5	34	10	2.16	0.13
(7/10)	Cyt1p	YOR065W	1.07	0.2	21	5	1.15	0.52
	Qcr10p	YHR001W	1.76	1.5	88	2	1.2	0.06
	Qcr2p	YPR191W	1.43	0.4	29	24	1.41	0.04
	Qcr6p	YFR033C	1.50	0.1	8	2	1.25	0.07
	Qcr7p	YDR529C	1.22	0.2	17	11	1.34	0.08
	Rip1p	YEL024W	1.00	0.4	37	3	1.42	0.04
Cytochrome *c* oxidase	Cox1p	Q0045	1.75			1	*	
(7/11)	Cox2p	Q0250	1.71	0.8	44	3	*	
	Cox4p	YGL187C	1.59	0.7	45	6	1.38	0.02
	Cox5ap	YNL052W	1.60	0.5	31	6	1.46	0.13
	Cox6p	YHR051W	1.32	0.4	30	8	1.45	0.07
	Cox8p	YLR395C	2.12			1	1.30	0.03
	Cox9p	YDL067C	1.53			1	1.05	0.47
F0/F1 ATP synthase	Atp1p	YBL099W	1.10	0.4	37	33	1.15	0.12
(10/18)	Atp15p	YPL271W	1.17	0.5	41	3	1.50	0.01
	Atp17p	YDR377W	0.43			1	1.09	0.45
	Atp2p	YJR121W	1.07	0.5	43	49	1.21	0.28
	Atp20p	YPR020W	1.78	1.2	66	4	1.59	0.12
	Atp3p	YBR039W	1.28	0.4	33	7	1.40	0.08
	Atp4p	YPL078C	0.98	0.04	4	6	1.40	0.10
	Atp5p	YDR298C	1.17	0.2	20	12	1.11	0.23
	Atp7p	YKL016C	1.35	0.3	21	14	1.49	0.07
	Tim11p	YDR322C	1.48	0.4	27	6	1.51	0.01
Proteasome	Pre1p	YER012W	0.96	0.4	37	4	0.85	0.06
(22/36)	Pre10p	YOR362C	0.32			1	0.82	0.28
	Pre2p	YPR103W	1.02	0.2	20	2	0.85	0.02
	Pre3p	YJL001W	0.82	0.4	47	7	1.09	0.53
	Pre4p	YFR050C	0.56			1	1.07	0.35
	Pre5p	YMR314W	0.64	0.001	0	2	0.97	0.80
	Pre6p	YOL038W	0.82	0.2	20	4	1.16	0.22
	Pre9p	YGR135W	0.52	0.1	28	2	0.74	0.02
	Pup1p	YOR157C	0.76			1	0.69	0.06
	Pup2p	YGR253C	0.69	0.1	10	3	0.87	0.21
	Pup3p	YER094C	0.61	0.2	30	4	0.79	0.07
	Rpn1p	YHR027C	0.94	0.3	30	2	0.85	0.03
	Rpn10p	YHR200W	0.67			1	1.03	0.90
	Rpn12p	YFR052W	0.78	0.1	13	3	0.85	0.21
	Rpn2p	YIL075C	0.75	0.4	60	3	0.70	0.06
	Rpn5p	YDL147W	0.72			1	0.83	0.23
	Rpn8p	YOR261C	0.83			1	0.88	0.13
	Rpt1p	YKL145W	0.37			1	0.96	0.58
	Rpt2p	YDL007W	0.77			1	0.69	0.01
	Rpt4p	YOR259C	0.88	0.2	24	3	0.78	0.04
	Rpt5p	YOR117W	1.42			1	1.00	0.96
	Rpt6p	YGL048C	1.05			1	1.04	0.63
Vacuolar H+ ATPase	Tfp1p	YDL185W	0.35	0.1	41	20	0.44	0.01
(7/14)	Vma13p	YPR036W	0.20			1	0.86	0.18
	Vma2p	YBR127C	0.44	0.2	40	19	0.73	0.03
	Vma4p	YOR332W	0.41	0.1	13	5	0.71	0.23
	Vma5p	YKL080W	0.62	0.3	41	3	0.60	0.02
	Vma6p	YLR447C	0.37	0.2	59	4	0.58	0.01
	Vma8p	YEL051W	0.30			1	0.56	0.00
Pyruvate dehydrogenase	Lat1p	YNL071W	1.10	0.04	4	3	1.58	0.01
(5/5)	Lpd1p	YFL018C	1.34	0.2	14	5	1.40	0.02
	Pda1p	YER178W	1.15			1	1.03	0.85
	Pdb1p	YBR221C	0.83	0.1	13	3	0.93	0.50
	Pdx1p	YGR193C	1.25	0.01	1	2	0.88	0.37

In the left column, the name of the protein complex is indicated with, in parentheses, the amount of identified proteins compared to the total amount of proteins in the complex. The quantified subunits of the complex found in the proteome analysis are indicated on the right of the protein complex name together with their systematic name. The relative protein expression ratio carbon versus nitrogen limitation (ratio C/N) is indicated as well as the protein quantification class as described in Materials and methods.
Transcriptome data are obtained from identical culture conditions (Boer *et al*, 2003).
*Mitochondrial genes, so not present on microarray.
[a]Average of C/N peptide ratios.
[b]Coefficient of variation.
[c]Number of peptides used for protein quantification.
[d]Average of mRNA ratios of carbon-limited over nitrogen-limited transcript levels (triplicate experiments).

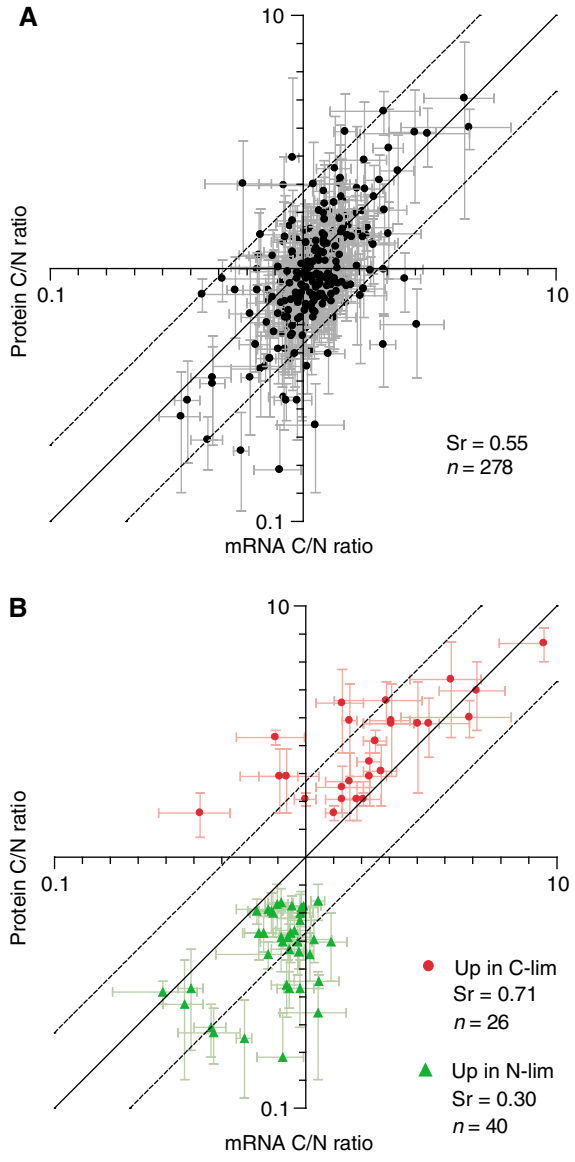

Figure 7 Scatter plot of protein ratios versus mRNA expression ratios between carbon- and nitrogen-limited chemostats. The mRNA expression ratios were measured using oligonucleotide microarrays from rigorously identical culture conditions and were previously published by Boer *et al* (2003). The plotted $x=y$ line indicates data points showing perfect correlation between mRNA and proteins abundances. Dashed lines indicate a two-fold deviation between mRNA and protein expression ratios. (**A**) All proteins quantified on the basis of five peptide pairs or more (n=278) were plotted. (**B**) Proteins that are differentially expressed are plotted (see Tables IIA and B). As 'on/off' proteins were excluded from statistical analysis, only the remaining 26 proteins upregulated under glucose limitation and 40 proteins upregulated under ammonia limitation were plotted.

involved in fatty acids β-oxidation (van Roermund *et al*, 2003) (Figure 8A). The expression of this peroxisomal pathway, which enables *S. cerevisiae* to use fatty acids as carbon and energy sources, has been intensively studied. Repression by glucose of these loci has been mainly investigated at the level of transcription and shows that, in the presence of glucose, *FAA2*, *POX1*, *FOX2* and *POT1* transcript levels are very low or undetectable, whereas they are high with non-repressive carbon sources (Karpichev and Small, 1998). The quantitative PCR analysis performed with these four transcripts further confirmed this response to carbon limitation or excess in chemostat cultures (Supplementary Table SIII). Although reports about protein expression are scarce, they nevertheless indicate good correlations between transcripts and protein levels for this class of proteins. For instance in the presence of excess glucose, *FOX2* and *POT1* transcripts are expressed at very low levels and none of the corresponding proteins are expressed (Hutchins *et al*, 1999; Ohlmeier *et al*, 2004). When grown with non-repressive carbon source (glycerol), yeast cells display a concomitant and strong increase in both mRNA and protein levels (Ohlmeier *et al*, 2004). The current data, including our work, do not indicate any potential post-transcriptional regulation of the expression of genes involved in β-oxidation when cells are grown in steady state. Conversely, active peroxisomal protein degradation has been observed upon addition of glucose to non-repressed yeast cells (Hutchins *et al*, 1999). However, this dynamic phenomenon, known as pexophagy, enables the rapid degradation of undesirable proteins and is not likely to occur in steady-state growing cells.

Conversely, the upregulation of proteins under nitrogen limitation poorly correlated with their corresponding transcripts, and the Spearman rank correlation coefficient was significantly lower (Sr=0.30; Figure 7B). Indeed, whereas protein levels were increased 1.5- to three-fold, most of the transcript expression levels remained unchanged, which indicates strong post-transcriptional control in response to nitrogen limitation. For instance, despite major changes in protein level, the glycolytic transcripts were unchanged, which corroborates previous chemostat studies that showed that major changes in glycolytic fluxes and glycolytic proteins in response to various carbon sources were not controlled at the transcriptional level (Daran-Lapujade *et al*, 2004; Kolkman *et al*, 2005). We further investigated two proteins, *GDH1* encoding the major isoenzymes of NADP-linked glutamate dehydrogenase and *GLN1* encoding glutamine synthetase. These two reactions constitute the major pathway for the assimilation of ammonia in *S. cerevisiae* (Figure 8B). *GDH1* is a critical gene at the interface between carbon and nitrogen metabolism and its expression is therefore strongly regulated by transcription factors involved in carbon (Hap complex) (Dang *et al*, 1996) and nitrogen metabolism (nitrogen catabolic response (NCR)), Gcn4p, Leu3p (Hu *et al*, 1995; Riego *et al*, 2002). *GDH1* transcription is repressed by glucose, but induced by nitrogen limitation via the NCR. This antagonistic and complex regulation reasonably explains why both microarray and qPCR analysis showed a constant mRNA level independent of the glucose and ammonia concentrations in the chemostat culture (Supplementary Table SIII). However, in agreement with our findings, several studies specifically dedicated to *GDH1* (Dang *et al*, 1996; DeLuna *et al*, 2001; Riego *et al*, 2002) as well as large-scale studies (Griffin *et al*, 2002) observed discrepancies between changes in transcript and protein levels and/or enzyme activities for *GDH1*, suggesting the post-transcriptional regulation of its expression. Similarly, changes in *GLN1* transcript and protein levels showed poor correlations in large-scale studies (Griffin *et al*, 2002; Washburn *et al*, 2003). Our data do not reveal whether these higher protein levels resulted from a faster protein

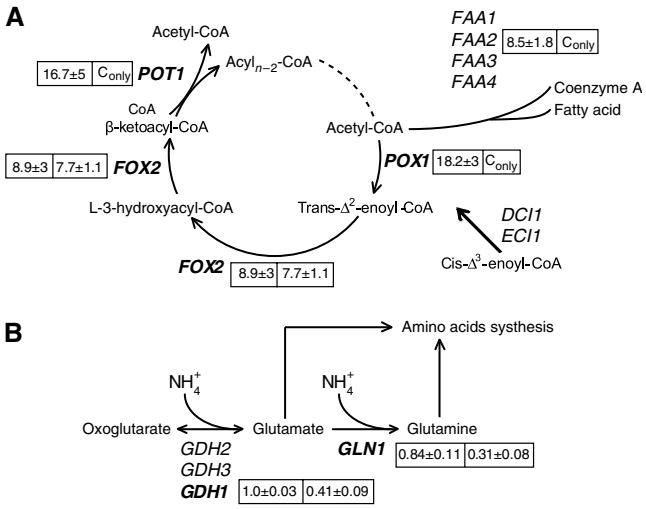

Figure 8 Changes in expression of transcripts and proteins (carbon versus nitrogen limitation) involved in fatty acids β-Oxidation (**A**) and ammonia incorporation into organic nitrogen (**B**). Proteins quantified in this study with their corresponding microarray mRNA level (left box) and protein level (right box) are indicated in bold.

synthesis, a slower degradation or both. It is however reasonable to assume that, under conditions where nitrogen is scarce, and probably intracellular amino acids as well, the cells tightly control translation and degradation in order to minimize spoilage. For instance, it has been well described that Gcn4p, DNA-binding protein involved in the activation of amino acids biosynthetic pathways, is controlled in response to amino-acid starvation both by increased translational efficiency and by stabilization of the protein (Hinnebusch, 2005). Gcn4p response is not elicited in mineral media with ammonia as the sole nitrogen source; however, it is reasonable to assume that ammonia availability triggers a similar mechanism for the regulation of the abundance of Gdh1p and Gln1p, two essential proteins for ammonia assimilation. The regulation of Gcn4p translational efficiency is a very complex mechanism, for which amino-acid starvation is mainly sensed via the loading of tRNA. In chemostat, ammonia limitation via lowered intracellular amino acids concentrations could in a similar way enhance Gdh1 and Gln1 translational efficiency.

We cannot analyze all proteins independently; however, some other loci displaying a complex regulation are worth mentioning. For instance, *PDC1* (pyruvate decarboxylase) and *APE3* (vacuolar protease) expression appeared to be strongly controlled at the post-transcriptional level (*PDC1*: $C/N_{mRNA}=0.81\pm0.2$, $C/N_{protein}=0.16\pm0.08$, *APE3*: $C/N_{mRNA}=1.12\pm0.3$, $C/N_{protein}=0.24\pm0.11$). More peculiarly, some loci were regulated in opposite direction at the mRNA and protein levels, such as *LYS20* (lysine metabolism; $C/N_{mRNA}=2.08\pm0.2$, $C/N_{protein}=0.5\pm0.15$). These observations underline the need for multilevel analysis in yeast systems biology and for further research into the post-transcriptional mechanisms underlying the observed differences.

Materials and methods

Strain and culture conditions

Wild-type *S. cerevisiae* strain CEN.PK113-7D (*MAT***a**) (van Dijken *et al*, 2000) was grown at 30°C in 2-l chemostats (Applikon), with a working volume of 1.0 l as described by van den Berg *et al* (1996). Cultures were fed with a defined mineral medium that limited growth by either carbon or nitrogen with all other growth requirements in excess and at a constant residual concentration. The defined mineral medium composition was based on that described by Verduyn *et al* (1992). The medium contained the following components: carbon-limited, $(NH_4)_2SO_4$ 19 mM and glucose 42 mM; nitrogen-limited, $(NH_4)_2SO_4$ 7.5 mM and glucose 330 mM. The dilution rate was set at 0.10 h^{-1}. The pH was measured online and kept constant at 5.0 by the automatic addition of 2 M KOH with the use of an Applikon ADI 1030 biocontroller. Stirrer speed was 800 r.p.m. and the airflow was 0.5 l/min. Dissolved oxygen tension was measured online with an Ingold model 34 100 3002 probe, and was above 50% of air saturation. The off-gas was cooled by a condenser connected to a cryostat set at 2°C, and oxygen and carbon dioxide were measured off-line with an ADC 7000 gas analyzer.

The nitrogen- and carbon-limited chemostat cultures were grown twice. First time, the culture was grown with normal ammonium sulfate as the sole nitrogen source and second time with ^{15}N-enriched ammonium sulfate. All cultures were started with $(^{14}NH_4)_2SO_4$ as the sole nitrogen source (Merck, Darmstadt, Germany). In the case where ^{15}N isotope was used, the medium vessel was replaced by a new vessel containing >99.5% $(^{15}NH_4)_2SO_4$ (Isotec Inc., Miamisburg, USA) after five volume changes. After five additional volume changes, a new steady state was reached and samples for proteome analysis were taken. Steady-state samples were taken from both ^{14}N- and ^{15}N-fed cultures before 15 volume changes to avoid strain adaptation owing to long-term cultivation (Ferea *et al*, 1999). Dry weight, metabolite, dissolved oxygen and gas profiles had to be constant over at least three volume changes before sampling. Samples dedicated to proteome analysis were sampled on ice and immediately centrifuged (5 min at 0°C), washed twice with ice-cold sterile water and stored five times concentrated in water at −80°C.

Protein preparation

Yeast cells were lyophilized before protein extraction. As the starting material for protein extraction, 50 mg dry weight of cells was used. Glass beads (Sigma, acid washed, 425–600 μm) were added and the cells were disrupted by vortexing for 10 min at 4°C. After cell lysis, the yeast cells were resuspended in 500 μl of hot (95°C) SDS buffer 4 × (0.5 M Tris–HCl, pH 6.8, 10% (w/v) SDS, 100 mM dithiothreitol, supplemented with protease inhibitors (Complete, Protease Inhibitor Cocktail Tablets (Roche Diagnostics)). The sample was boiled for 10 min. Lysates were cleared by centrifugation at 3000 *g*. Protein concentrations were determined using the 2D Quant Kit (Amersham Biosciences). The protein extracts were stored in aliquots at −80°C.

Protein separation and in-gel tryptic digestion

Equal amounts of unlabeled and labeled protein extracts were mixed and 200 μg of the protein mixture was separated on a homemade 1.5-mm-thick 12% SDS–PAGE gel (Hoeffer SE600 system, Amersham Biosciences). Proteins were visualized using Coomassie blue staining. The gel lane was cut into 40 slices. Each slice was cut into small (approximately 1 mm^3) pieces. The gel pieces were washed, in-gel reduced with dithiothreitol, alkylated with iodoacetamide and digested by adding trypsin at a concentration of 10 ng/μl (overnight at 37°C) as described by Wilm *et al* (1996).

Nanoflow-LC-MS/MS

Nanoflow-LC-MS/MS analysis was performed by coupling an Agilent 1100 Series LC system (vacuum degasser, autosampler and one high-pressure-mixing binary pump without static mixer) to an LCQ^{tm} Classic Quadrupole Ion Trap mass spectrometer (Finnigan, San Jose, CA, USA) as described by Meiring *et al* (2002). Briefly, peptides were delivered to a trap column (Aqua™ C18RP (Phenomenex); 15 mm × 100 μm ID, packed in-house) at 5 μl/min 100% buffer A (A=0.1 M acetic acid). After reducing the flow to approximately 150 nl/min by a splitter, the peptides were transferred to the analytical column (Aqua™ C18RP (Phenomenex); 15 cm × 75 μm ID, packed in-house) with a linear gradient from 0 to 50% buffer B (B=0.1 M acetic acid in 80% acetonitrile) in 90 min. The column eluent was sprayed directly into the ESI source of the mass spectrometer via a butt-connected nano-ESI emitter (New Objectives). The LCQ operated in a positive ion mode, and peptides were fragmented in a data-dependent mode. One MS survey scan was followed by one data-dependent MS/MS scan. Dynamic exclusion was used for LC-MS/MS analysis. The following settings were used: repeat count was set at 2, repeat duration was set at 0.50, exclusion list size was set at 25 and the exclusion duration was set at 3.00 min.

Protein identification

The SEQUEST algorithm (Eng *et al*, 1994) was used to interpret the obtained MS/MS spectra against the complete non-redundant proteome database in FASTA format of *S. cerevisiae* from the European Bioinformatics Institute (EBI) containing 6195 entries as described by Washburn *et al* (2002). The algorithm was run twice on each data set to identify proteins first from unlabeled cultures, then from ^{15}N-labeled cultures. Therefore, two separate SEQUEST parameters files containing the masses of each amino acid were prepared, one with the masses of each amino acid set to reflect the amino acid containing all ^{14}N and the other parameters file set to reflect the masses of each amino acid containing all ^{15}N. The parameters chosen for the interpretation of MS/MS spectra were as follows: use of trypsin for proteolysis, maximum of two missed cleavages and carbamidomethyl and oxidized methionine set as fixed and variable modifications, respectively. The precursor-ion mass tolerance and the fragment-ion tolerance were both set at 1.2 Da. The program DTASelect (Tabb *et al*, 2002) was used to select the peptide identifications and to assemble the peptides into proteins. The following selection criteria were used to filter the peptide identifications to both the ^{14}N and ^{15}N result files: the minimum Xcorr was set at 1.9, 2.2 and 3.75 for 1+, 2+ and 3+ peptides, respectively, and the ΔCns was set at a minimum of 0.1 for each peptide. By searching in a reversed database, we obtained a false positive rate on peptide level of 10, 1 and 0.2% for proteins identified with one, two and three peptides, respectively. In Supplementary Table SI, detailed information about the protein identification can be found, including for each peptide match the sequence, precursor mass, charge, deltaCNs and Xcorrs.

Relative quantification

Ion chromatograms from each peptide exceeding the DTASelect criteria were extracted from the Xcalibur raw data file by a modified version of EXTRACT-CHRO. The relative protein expression ratios were calculated from peptide ion current ratios using the RelEx software as described by MacCoss *et al* (2003). The relative protein expression levels obtained by using the RelEx software were all inspected manually. If all peptides from one protein were detected in the extracted ion chromatogram of one condition but not detected in the ion chromatogram of the other condition, this protein was considered to be exclusively present in one limitation. After removal of these so-called 'on/off' proteins, the data were filtered by using the signal to noise filter (minimum S/N cutoff was set at 5) and the regression filter (regression cutoff at 1 and at 10 was set at 0.5) within the RelEx software, peptide ratio outliers were removed and the relative protein expression ratios (C/N: carbon limited/nitrogen limited) were calculated by using the RelEx software.

Data analysis

Proteins were placed into functional categories as defined by the Comprehensive Yeast Genome Database (CYGD) from the Munich Information Center for Protein Sequences database (MIPS) (Mewes *et al*, 2004) and the *Saccharomyces* Genome Database (SGD). Significance of the over-representation of functional categories among the differentially expressed proteins was estimated with FunSpec (http://funspec.med.utoronto.ca/, *P*-value smaller than 0.01, Bonferroni correction). The subcellular localization of proteins was determined using the *Saccharomyces* Genome Database (SGD; http://www.yeastgenome.org). The isoelectric points and the molecular weights of proteins were calculated using the 'Compute p*I*/Mw tool' at the ExPASy Proteomics Server (http://www.expasy.org/).

In our analysis, we only considered proteins quantified with two or more peptide pairs. The 'on/off' proteins quantified with two or more peptide pairs were automatically considered as significantly changed. The other ratios were considered as significantly changed when satisfying one of the following three categories: (i) proteins with a C/N ratio between 1.5 and 2 or between 0.5 and 0.66 had to have a CV (coefficient of variation, average ratio divided by standard deviation) smaller than 20%, (ii) proteins with a C/N ratio between 2 and 3 or between 0.33 and 0.5 had to have a CV below 30%, and finally, (iii) proteins with C/N ratio higher than 3 or lower than 0.33 had to have a CV below 50%.

Western blotting

αTBP antibodies were kindly donated by Dr Anthony Weil, α-GAP1 antiserum was kindly donated by Dr J Holthuis, α-EGD2 antibodies were kindly donated by Dr Martine Collart and α-H3 antibodies were directed against the C-terminus of histone H3 and were obtained from Abcam (Ab1791). All primary antibodies were used at 1:2000 dilutions. Total protein samples were run on 10% SDS–PAGE gels, blotted onto PVDF membranes and blocked for 1–24 h in 5% milk powder in TBST (10 mM Tris–HCl pH 8, 150 mM NaCl, 0.05% Tween 20). After incubation with the primary antibody in TBST for 1 h, blots were washed in TBST and incubated for 1 h with secondary antibody conjugated to HRP. Blots were developed using Western lightning chemiluminescence reagent (Perkin-Elmer Life Sciences Inc.)

RT–PCR

Cells were sampled and total RNA extracted as described by Piper *et al* (2002). Residual DNA was removed by DNAse I treatment (RNeasy, Qiagen) and total RNA purity and integrity was assessed on an RNA Nano Labchip with an Agilent 2100 Bioanalyzer. First strand cDNA synthesis was carried out (2 μg total RNA, 200 U M-MLV RT (Invitrogen) and 100 ng random primers (Invitrogen) according to the supplier's instructions). Quantitative real-time PCR assays were performed with the SYBR Green Jumpstart Taq ReadyMix (Sigma-Aldrich) in the DNA Engine Opticon I system (MJ Research). The complete protocol and sequence of primers used for quantitative PCR are described in Supplementary Tables SIII and SIV. Data were analyzed using the Opticon Monitor™ software package version 1.04, calculating C_t values. Subsequently, the relative expression ratios for each gene were determined according to the $2^{\Delta\Delta C_t}$ method with E (Efficiency) correction (Pfaffl (REF p2) between C- and N-limited cultures.

Supplementary information

Supplementary information is available at *Molecular Systems Biology* website (www.nature.com/msb).

Acknowledgements

We kindly acknowledge Pim Pijnappel and Marc Timmers (Utrecht UMC) for help with Western blotting analysis and Marinka Almering (TU Delft) for assistance in the q-PCR experiments. We kindly thank Anthony Weil and Martine Collart for providing αTBP antibodies and α-EGD2 antibodies, respectively. We kindly thank John Yates and Michael MacCoss for their help and useful instructions in setting up the RelEx software in our laboratory. AK acknowledges financial support by DSM. The Netherlands Proteomics Centre (http://www.netherlandsproteomicscentre.nl/) supports the work of the group of AJR Heck. Additionally, the research groups of JT Pronk and AJR Heck are part of the Kluyver Centre for Genomics of Industrial Fermentation, which is supported by the Netherlands Genomics Initiative.

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