

# Multiplexed Protein Quantitation in *Saccharomyces cerevisiae* Using Amine-reactive Isobaric Tagging Reagents\*<sup>§</sup>

Philip L. Ross<sup>‡</sup>, Yulin N. Huang<sup>‡</sup>, Jason N. Marchese<sup>‡</sup>, Brian Williamson<sup>‡</sup>, Kenneth Parker<sup>‡</sup>, Stephen Hattan<sup>‡</sup>, Nikita Khainovski<sup>‡</sup>, Sasi Pillai<sup>‡</sup>, Subhakar Dey<sup>‡</sup>, Scott Daniels<sup>‡</sup>, Subhasish Purkayastha<sup>‡</sup>, Peter Juhasz<sup>‡</sup>, Stephen Martin<sup>‡</sup>, Michael Bartlett-Jones<sup>§</sup>, Feng He<sup>¶</sup>, Allan Jacobson<sup>¶</sup>, and Darryl J. Pappin<sup>‡\*\*</sup>

**We describe here a multiplexed protein quantitation strategy that provides relative and absolute measurements of proteins in complex mixtures. At the core of this methodology is a multiplexed set of isobaric reagents that yield amine-derivatized peptides. The derivatized peptides are indistinguishable in MS, but exhibit intense low-mass MS/MS signature ions that support quantitation. In this study, we have examined the global protein expression of a wild-type yeast strain and the isogenic *upf1Δ* and *xrn1Δ* mutant strains that are defective in the nonsense-mediated mRNA decay and the general 5' to 3' decay pathways, respectively. We also demonstrate the use of 4-fold multiplexing to enable relative protein measurements simultaneously with determination of absolute levels of a target protein using synthetic isobaric peptide standards. We find that inactivation of Upf1p and Xrn1p causes common as well as unique effects on protein expression. *Molecular & Cellular Proteomics* 3:1154–1169, 2004.**

An initial step in the systematic investigation of cellular processes is the identification and measurement of expression levels of relevant sets of proteins. Recently, quantitative approaches utilizing MS and a host of stable isotope-labeling chemistries have emerged (reviewed in Refs. 1 and 2), offering a departure from traditional techniques employing comparative two-dimensional gel electrophoresis. The ICAT quantitative labeling strategy (3, 4) is perhaps the best-characterized method for relative protein quantitation using MS. Other elegant approaches use cell-culture enrichment with a stable isotope-labeled amino acid, including arginine (5), lysine (6), tyrosine (7), and leucine (8), for *in vivo* incorporation of a mass

difference to support relative quantitation. This circumvents potential difficulties surrounding chemical labeling downstream in a comparative experiment. All of these methods impart a mass difference as the basis for quantitation by measurement of relative peak areas of MS and/or MS/MS mass spectra. There are, however, a number of limitations imposed by mass-difference labeling. The mass-difference concept for many practical purposes is limited to a binary (2-plex) set of reagents, and this makes comparison of multiple states (e.g. several experimental controls or time-course studies) difficult to undertake. Multiple 2-plex datasets can be combined after separate analyses, but there is a high likelihood that different sets of peptides and proteins will be identified between each experiment. In addition, the use of mass-difference labels increases MS complexity, and this problem increases with numbers of a multiplexed set. Finally, the cysteine-selective affinity strategy for reduction of sample complexity (ICAT) is not amenable to identification of post-translationally modified peptides, as the majority of post-translational modification (PTM)<sup>1</sup>-containing peptides are discarded at the affinity step.

We have developed a multiplexed set of reagents for quantitative protein analysis that place isobaric mass labels at the N termini and lysine side chains of peptides in a digest mixture. The reagents are differentially isotopically labeled such that all derivatized peptides are isobaric and chromatographically indistinguishable, but yield signature or reporter ions following CID that can be used to identify and quantify individual members of the multiplex set. Absolute quantitation of targeted proteins can also be achieved using synthetic peptides tagged with one of the members of the multiplex reagent set.

In this study, we make use of a 4-fold (4-plex) multiplex strategy to simultaneously determine relative protein levels in three yeast strains and provide a demonstration of the ability to measure the absolute quantity of specific target proteins

From <sup>‡</sup>Applied Biosystems, Framingham, MA 01701; <sup>§</sup>Cancer Research UK, London WC2A 3PX, United Kingdom; <sup>¶</sup>Department of Molecular Genetics and Microbiology, University of Massachusetts, Medical School, Worcester, MA 01655; and <sup>||</sup>Section of Proteomics, Imperial College, Hammersmith Campus, London W12 0NN, United Kingdom

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<sup>1</sup> The abbreviations used are: PTM, post-translational modification; TCEP, Tris-(2-carboxyethyl)phosphine; TEAB, triethylammonium bicarbonate; NHS, *N*-hydroxy succinimide; SCX, strong cation exchange.

through the use of internal peptide standards. Of particular interest is validation of quantitation via a peptide-based workflow whereby protein extraction, digestion, and labeling are performed in parallel, prior to mixing labeled samples for chromatography and MS. A well-characterized system such as yeast provides the opportunity to validate some novel aspects of this quantitative methodology. In this study, we have examined the global protein expression of a wild-type yeast strain and the isogenic *upf1Δ* and *xrn1Δ* mutant strains that are defective in the nonsense-mediated mRNA decay and the general 5' to 3' decay pathway, respectively (9, 10). A variety of global changes are observed, including consistent up-regulation of a common set of proteins involved in amino acid biosynthetic pathways in both *upf1Δ* and *xrn1Δ* strains, and specific down-regulation of proteins of the translation apparatus in the *xrn1Δ* strain.

#### MATERIALS AND METHODS

**Protein Mixture**—A protein mix consisting of equimolar amounts of BSA,  $\alpha$ -casein, alcohol dehydrogenase, lysozyme,  $\beta$ -galactosidase, and serotransferrin (all from Sigma, Milwaukee, WI) was reduced (2 mM Tris-(2-carboxyethyl)phosphine (TCEP), 37 °C, 1 h), alkylated (5 mM iodoacetamide, 37 °C, 2 h) and digested with trypsin (1:20 w/w, 50 mM triethylammonium bicarbonate (TEAB), 37 °C, 18 h). Four equal aliquots were treated each with one of the four isotopically enriched methylpiperazine acetic acid *N*-hydroxy succinimide (NHS) ester reagents by adding 0.5 mg of reagent in ethanol to 50  $\mu$ l of peptide solution (70% v/v final ethanol) and allowed to react for 30 min at room temperature. The four reactions were combined in various proportions, evaporated to dryness, and quantities representing 500 fmol of each component analyzed by LC-MS/MS as described below.

**Yeast Protein Extraction and Labeling**—Log phase cells (75% log;  $2.1 \times 10^7$  cells/ml) were harvested, frozen, and mechanically lysed by grinding over dry ice. Crude cell lysate was prepared by suspending frozen cellular material (100 mg wet weight) in 1 ml of lysis buffer (0.1 M TEAB, 0.1% v/v Triton X-100, 6 M guanidine), vortexing (1 min), sonicating (5  $\times$  30 s), and pelleting insoluble debris by centrifugation at  $13,000 \times g$  for 5 min. Final measured protein concentrations were 3–3.4 mg/ml. Protein was reduced (2 mM TCEP, 37 °C, 1 h), alkylated (5 mM iodoacetamide, 37 °C, 2 h), and precipitated by the addition of 6 volumes of cold acetone (dry ice 20 min). Protein was collected by centrifugation ( $13,000 \times g$ , 5 min), dried in air, and frozen at  $-80$  °C. For digestion, protein was resuspended in digestion buffer (100 mM TEAB, 0.05% w/v SDS) to a final concentration of 1 mg/ml (total protein measured by bicinchoninic acid assay (Sigma, St. Louis, MO)). Equal aliquots (500  $\mu$ g) from each lysate were then digested with trypsin overnight at 37 °C (Sigma; 1:40 w/w added at 0 and 2 h) and lyophilized.

**Labeling with Multiplex Reagents**—Synthesis of the four derivatization reagents is discussed elsewhere (11). For each yeast strain, 150  $\mu$ g of total protein was resuspended in 100  $\mu$ l of labeling buffer (0.25 M TEAB, 75% ethanol), after which 1 mg of each isotopically enriched methylpiperazine acetic acid NHS ester was added (1% w/v final) and allowed to react at room temperature for 30 min. Residual reagent was quenched by adding 300  $\mu$ l of water and allowing excess reagent to completely hydrolyze over an additional 30 min, then the three labeled samples were mixed and lyophilized.

**Cation Exchange Chromatography**—The combined peptide mixture was separated by strong cation exchange (SCX) chromatography on an Agilent 1100 HPLC system using a PolySulfoethyl A column ( $4.6 \times 100$  mm, 5  $\mu$ m, 300 Å). Sample was dissolved in 4 ml of SCX

loading buffer (25% v/v ACN, 10 mM  $\text{KH}_2\text{PO}_4$ , pH 3, with phosphoric acid) and loaded and washed isocratically for 20 min at 0.5 ml/min to remove excess reagent. Peptides were eluted with a linear gradient of 0–500 mM KCl (25% v/v ACN, 10 mM  $\text{KH}_2\text{PO}_4$ , pH 3) over 15 min at a flow rate of 1 ml/min, with fractions collected at 1-min intervals.

**LC-MS Analysis**—Peptide separation was performed on an Ultimate™ chromatography system (Dionex-LC Packings, Hercules, CA) equipped with a Probot MALDI spotting device. Individual SCX fractions containing  $\sim 10$   $\mu$ g of protein material were injected and captured onto a  $0.3 \times 5$ -mm trap column (3- $\mu$ m  $\text{C}_{18}$  (Dionex-LC Packings, Hercules, CA)) and then eluted onto a  $0.1 \times 150$ -mm analytical column (3- $\mu$ m  $\text{C}_{18}$  (Dionex-LC Packings)) using an automated binary gradient (800 nl/min) from 95% buffer A (2% ACN, 0.1% TFA) to 45% buffer B (85% ACN, 5% isopropanol, 0.1% TFA) over 35 min, then 45–90% B in 5 min. For MALDI MS/MS analysis, column effluent was mixed in a 1:2 ratio with MALDI matrix (7 mg/ml- $\alpha$ -cyano-4-hydroxycinnamic acid) through a 25-nl mixing tee (Upchurch Scientific, Oak Harbor, WA) and spotted in  $16 \times 16$  spot arrays. MALDI plates were analyzed on an ABI 4700 (Applied Biosystems, Framingham, MA) proteomics analyzer. Peptide CID was performed at a collision energy of 1 kV and a collision gas pressure of  $\sim 1.5 \times 10^{-6}$  Torr. For electrospray analysis, an Ultimate LC system interfaced to a Qstar Pulsar™ (Applied Biosystems-MDS Sciex) mass spectrometer was used. The LC conditions were similar to those used for LC-MALDI, with peptides separated at a flow rate of 300 nl/min over a 75- $\mu$ m  $\times$  150-mm  $\text{C}_{18}$  column (Pepmap; Dionex) using a 2-h gradient of 5–35% B (A, 2% ACN/0.1% formic acid; B, 98% ACN/0.1% formic acid). Survey scans were acquired from  $m/z$  300–1,500 with up to three precursors selected for MS/MS from  $m/z$  90–2,000 using dynamic exclusion. A rolling collision energy was used to promote fragmentation, typical average values for doubly charged ions were 41 and 56 V for  $m/z$  600 and 900, respectively, and for triply charged ions typical average values were 29 and 43 V for  $m/z$  600 and 900, respectively. The collision energy range was  $\sim 20\%$  higher than that used for unlabeled peptides to overcome the stabilizing effect of the basic N-terminal derivative and achieve equivalent fragmentation.

**Data Analysis and Interpretation**—Peptide and protein identifications were performed using the Mascot search engine (ver. 1.9; Matrix Science, London, United Kingdom) (12). Database searching was restricted to tryptic peptides of yeast (Swiss-Prot version 42.5; 4,924 *Saccharomyces cerevisiae* sequences; 138,922 total sequences). S-acetamido, N-terminal, and lysine modifications were selected as fixed, methionine oxidation as variable, one missed cleavage allowed and precursor error tolerance at  $<50$  ppm. Full trypsin specificity (N- and C-terminal) was also applied. Signature-ion peak areas from the isobaric tags were extracted from the 4700 or QSTAR raw data and matched to identified peptides using prototype software tools. The complete list of identified peptides was then housed in an Access (Microsoft, Redmond, WA) database for grouping of results into proteins and calculation of ratios and standard deviation. Abundance ratio calculations included corrections for overlapping isotopic contributions (both natural and enriched  $^{13}\text{C}$  components).

#### RESULTS AND DISCUSSION

**Features of Multiplexed Tagging Chemistry**—The components of the multiplexed derivatization chemistry are introduced in Figs. 1 and 2. In preliminary studies, we used a reduced and alkylated protein digest mixture as a simple model system to validate the labeling protocol and the usage of MS/MS signature ions for quantitation. A digest mixture of six proteins was split into four identical aliquots. Each was

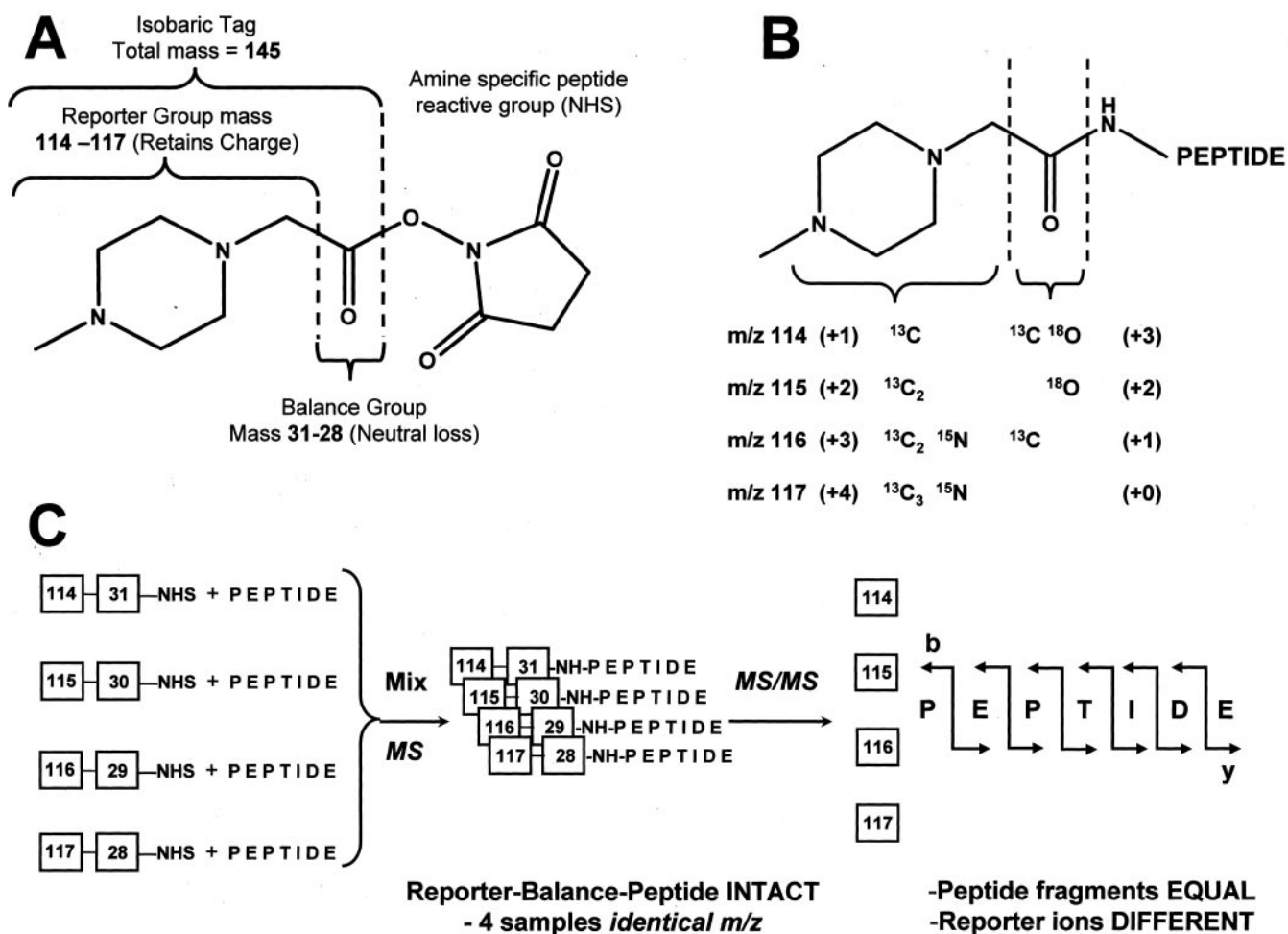


FIG. 1. A, diagram showing the components of the multiplexed isobaric tagging chemistry. The complete molecule consists of a reporter group (based on *N*-methylpiperazine), a mass balance group (carbonyl), and a peptide-reactive group (NHS ester). The overall mass of reporter and balance components of the molecule are kept constant using differential isotopic enrichment with <sup>13</sup>C, <sup>15</sup>N, and <sup>18</sup>O atoms (B), thus avoiding problems with chromatographic separation seen with enrichment involving deuterium substitution. The number and position of enriched centers in the ring has no effect on chromatographic or MS behavior. The reporter group ranges in mass from *m/z* 114.1 to 117.1, while the balance group ranges in mass from 28 to 31 Da, such that the combined mass remains constant (145.1 Da) for each of the four reagents. B, when reacted with a peptide, the tag forms an amide linkage to any peptide amine (N-terminal or  $\epsilon$  amino group of lysine). These amide linkages fragment in a similar fashion to backbone peptide bonds when subjected to CID. Following fragmentation of the tag amide bond, however, the balance (carbonyl) moiety is lost (neutral loss), while charge is retained by the reporter group fragment. The numbers in parentheses indicate the number of enriched centers in each section of the molecule. C, illustration of the isotopic tagging used to arrive at four isobaric combinations with four different reporter group masses. A mixture of four identical peptides each labeled with one member of the multiplex set appears as a single, unresolved precursor ion in MS (identical *m/z*). Following CID, the four reporter group ions appear as distinct masses (114–117 Da). All other sequence-informative fragment ions (b-, y-, etc.) remain isobaric, and their individual ion current signals (signal intensities) are additive. This remains the case even for those tryptic peptides that are labeled at both the N terminus and lysine side chains, and those peptides containing internal lysine residues due to incomplete cleavage with trypsin. The relative concentration of the peptides is thus deduced from the relative intensities of the corresponding reporter ions. In contrast to ICAT and similar mass-difference labeling strategies, quantitation is thus performed at the MS/MS stage rather than in MS.

then labeled with one of the four isotopically labeled tags, and the derivatized digests combined in mixtures of varying proportions. The multiplex isobaric tags produce abundant MS/MS signature ions at *m/z* 114.1, 115.1, 116.1, and 117.1, and the relative areas of these peaks correspond with the proportions of the labeled peptides. We have found that this mass range also has minimal contamination with background low-mass fragments produced from CID fragmentation of

peptides using either MALDI or ESI-based tandem mass spectrometers.

The mass shift imposed by isotopic enrichment of each signature ion is balanced with isotopic enrichment at the carbonyl component of the derivative, such that the total mass of each of the four tags is identical. Thus any given peptide labeled with each of the four tags has the same nominal mass, an important characteristic that provides a

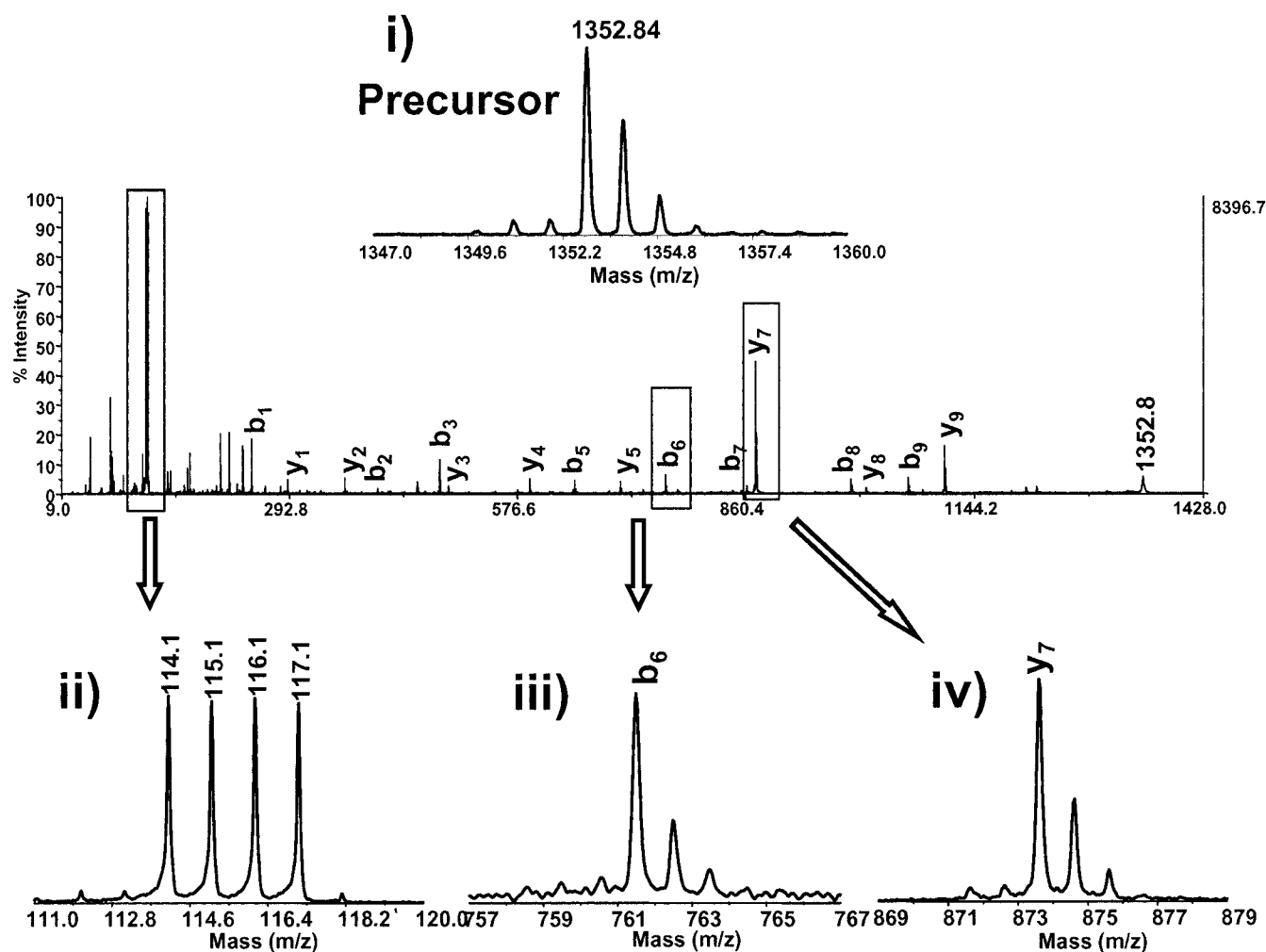


FIG. 2. Example MS/MS spectrum of peptide TPHPALTEAK from a protein digest mixture prepared by labeling four separate digests with each of the four isobaric reagents and combining the reaction mixtures in a 1:1:1:1 ratio. Components of the spectrum illustrated are (i) isotopic distribution of the precursor ( $[M+H]^+$ ,  $m/z$  1352.84), (ii) low mass region showing the signature ions used for quantitation, (iii) isotopic distribution of the  $b_6$  fragment, and (iv) isotopic distribution of the  $y_7$  fragment ion. The peptide is labeled by isobaric tags at both the N terminus and C-terminal lysine side chain. The precursor ion and all the internal fragment ions (e.g. type  $b$ - and  $y$ -) therefore contain all four members of the tag set, but remain isobaric. The example shown is the spectrum obtained from the singly charged  $[M+H]^+$  peptide using a 4700 MALDI TOF-TOF analyzer, but the same holds true for any multiply charged peptide analyzed with an ESI-source mass spectrometer.

sensitivity enhancement over mass-difference labeling. With isobaric peptides, the MS ion current at a given peptide mass is the sum of ion current from all samples in the mixture, so there is no splitting of MS precursor signal and no increase in spectral complexity by combining two or more samples (Figs. 1 and 2). The use of isobaric peptides circumvents the ambiguity encountered when trying to identify differentially labeled peptide pairs (e.g. with ICAT), a task which is further complicated by the fact that many such pairs will be separated in mass by more than one labeled residue. The sensitivity enhancement is carried over into MS/MS spectra, because all of the peptide backbone fragments ions are also isobaric (Fig. 2). One potential drawback of this approach is that MS/MS spectra must be acquired, which requires more analysis time than performing result-dependent analysis only on differentially ex-

pressed peptide pairs in MS (e.g. with ICAT). We feel, however, that the ability to identify more proteins with increased confidence and greater peptide coverage outweighs this disadvantage.

Strategies have been described that employ isobaric peptide derivatives (13). To date, reaction of these reagents with complex peptide mixtures has not been shown. The use of a discreet, highly abundant, low-mass MS/MS signature ion as described in this work provides an unambiguous coding system without introducing additional sources of complexity into the mass spectrum. Finally, we use tags that generate abundant signature ions under MS/MS conditions optimal for peptide fragmentation.

The reagents described here contain *N*-methylpiperazine, a moderately strong base that conveys useful properties to the

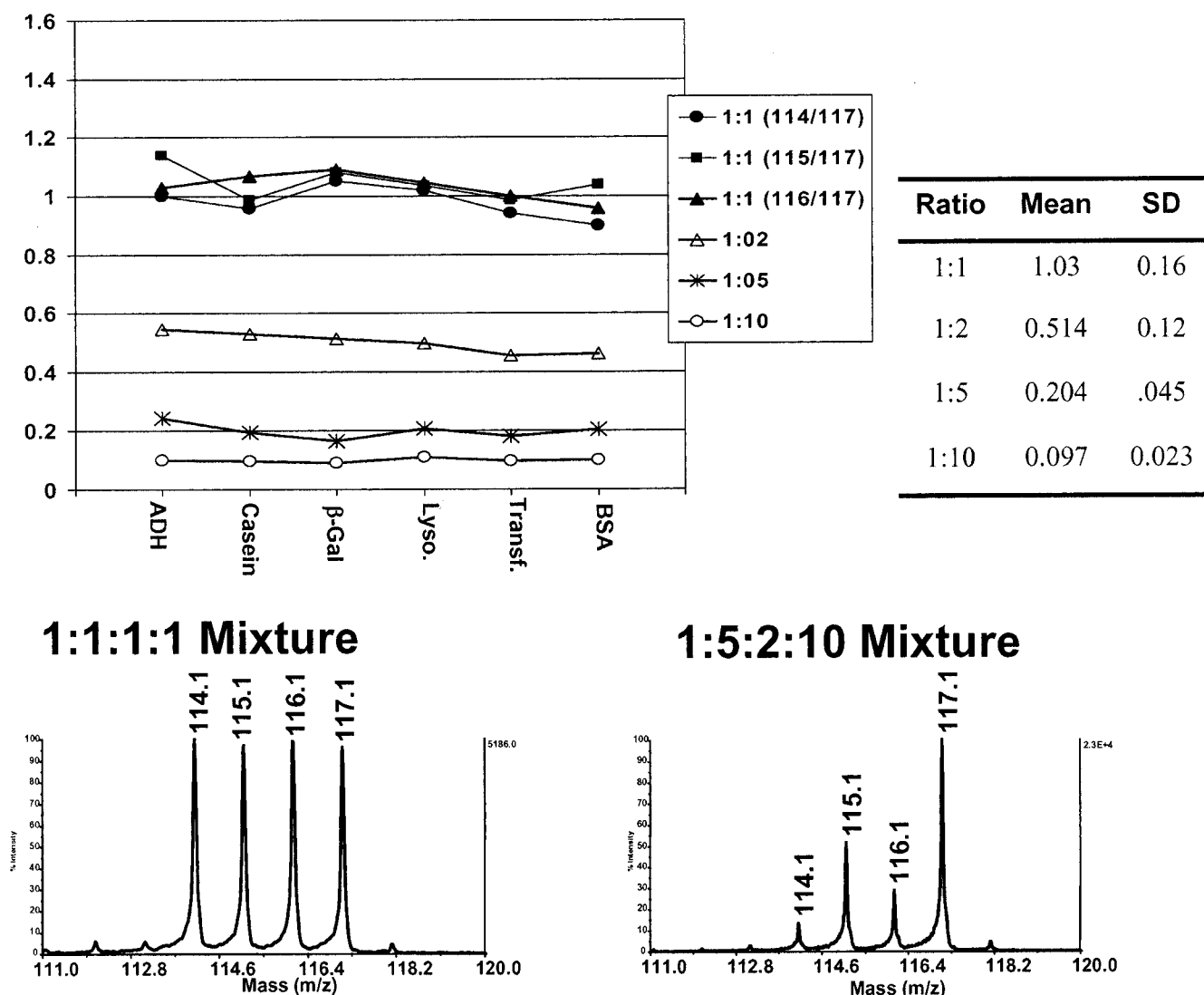


FIG. 3. Summary of relative protein measurements for (i) 1:1:1:1 and (ii) 1:5:2:10 mixtures of a six-protein digest. Ratios were expressed relative to the peak area at 117.1. Insets show examples of the signature ion regions from peptides mixed 1:1:1:1 and 1:5:2:10. Intra-protein averages are compiled from the top-ranked significant peptides labeled at N termini and lysine side chains (Mascot score > 95% confidence, rank = 1).

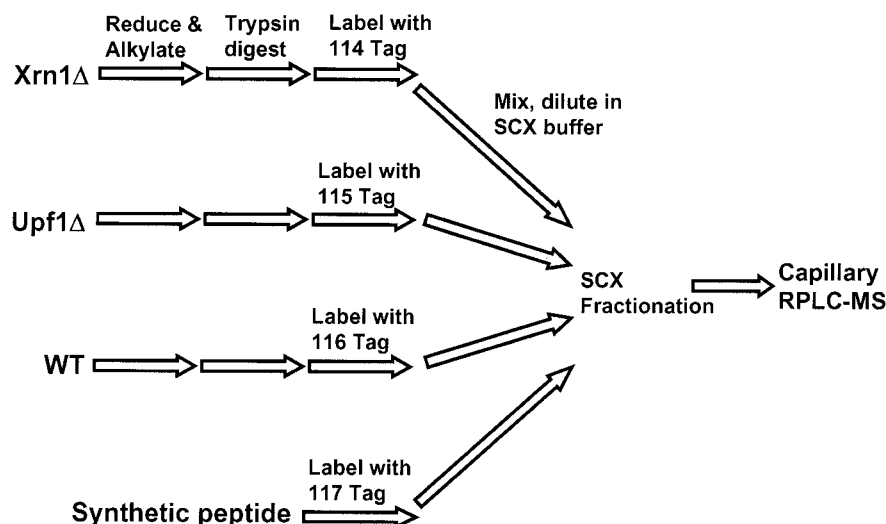
tagged peptides. The use of cyclic amines as N-terminal peptide derivatives to simplify the interpretation of MS/MS spectra has been described previously (14,15). We find that the tags behave similarly in both MALDI and ESI, with a tendency to form more abundant and complete b- and y-ion series, while also reducing the proportion of ion current going into less informative fragmentation pathways.

Our findings with the six-protein digest mixture show that the derivatization reaction itself is quite straightforward, requiring a simple room temperature reaction of ~30 min. Residual reagent is easily quenched by the addition of one or more volumes of water prior to mixing, as decreasing the organic (ethanol) concentration accelerates hydrolysis of residual reagent. Hydrolysis is essentially complete within an additional 30 min at room temperature. Comparison of protein

digest mixtures of known proportions (Fig. 3) gave accurate ratios (<6% error) and standard deviations less than 23% across two orders of magnitude. All observed peptides were confirmed as fully derivatized at N termini and lysine side chains.

**Multiplex Analysis of Whole-Yeast Lysate**—The protocol for labeling the tryptic peptides from whole-yeast lysate is outlined in Fig. 4. They consist of total protein reduction and alkylation, digestion with trypsin, and derivatization of total peptide with the isobaric reagents. These steps are performed separately (in parallel) before sample mixing and analysis by SCX chromatography and capillary reverse-phase LC-MS. Because the *N*-hydroxysuccinimide-reactive group used for derivatization reacts rapidly with primary or secondary amines (such as Tris buffer), TEAB was chosen as the reaction buffer.

FIG. 4. Depiction of overall workflow used for multiplexed comparative analysis of the wild-type, *upf1Δ*, and *xrn1Δ* strains. Equivalent amounts of whole-cell lysates were treated in parallel workflows employing standard protocols for reduction and alkylation, trypsin digestion, and derivatization (see “Materials and Methods”). A number of internal standards were derivatized with a fourth reagent, and the four samples were mixed, combined with cation exchange loading buffer and subjected to SCX chromatography followed by capillary reverse-phase HPLC coupled to MALDI-MS/MS or ESI-MS/MS.



As with any protein or peptide derivatization strategy, completeness of reaction and elimination of side reactions are a primary concern. Conditions were optimized using standard protein mixes as described above to drive the derivatization of N termini and lysine  $\epsilon$ -amines to completion. We made use of database searching (Mascot) (12) with variable modifications at N termini, lysine, and tyrosine to explore the extent of possible side reactions and establish completeness of reaction. Under optimal conditions, we observed a minimal degree (<3%) of tyrosine derivatization and a similar percentage (<3%) of unlabeled N termini or lysine  $\epsilon$ -amino groups. Reaction with serine or threonine was not observed, and any possible reaction with cysteine was blocked by prior reduction and alkylation.

For SCX chromatography, we observed a minor increase in general retention time of peptides compared with similar, unlabeled yeast digests, indicating a small increase in pI. Excess hydrolyzed reagent is not retained by the SCX column and can be efficiently removed by isocratic washing prior to eluting peptides with a salt gradient. No other complications were apparent in either SCX or reverse-phase chromatography. An important attribute is that peptides differentially labeled with each member of the multiplex set must perfectly co-elute to preserve the quantitative relationship. Using LC-MALDI, we monitored signature ion patterns across the elution profiles of a variety of peptides varying by length and extent of derivatization (*i.e.* one or more lysines). There was no observed discrepancy in any of the ratios across the elution profile in any of the peptides studied.

**Protein Identification**—Proteins were identified on the basis of having at least one peptide whose individual ion score was above the 95% confidence threshold ( $p < 0.05$ ) and also identified as the top-ranked matching sequence for that spectrum (12). We further restricted the number of identifications made by imposing spectrum and peptide-level nonredundancy such that any given peptide and any given MS/MS

spectrum was linked to only one protein. Using these criteria, 1,217 unique proteins were identified from ~4,500 peptides. More importantly, 685 of these proteins were identified by two or more of these significant peptides. Further statistical analysis for determining up- or down-regulation of protein expression levels was performed only on this latter group of 685 proteins (*i.e.* protein identifications made on single, unique peptides were discarded).

**Protein Quantitation**—The individual ratios of identified peptides from the *upf1Δ* and *xrn1Δ* strains compared with the wild-type strain were computed from signature ion peak areas using the formula:  $\text{area}(\text{mutant}) / (\text{area}(\text{mutant}) + \text{area}(\text{wild type}))$ . All protein expression values thus fall between 0 and 1, with a “no-change” 1:1 ratio = 0.5. The global all-peptide average and standard deviations were 0.503 ( $\pm 0.084$ ) and 0.485 ( $\pm 0.044$ ) for the *xrn1Δ* and *upf1Δ* strains, respectively. These values indicate a high degree of consistency between these three strains, suggesting that the parallel, peptide-based workflow did not introduce significant variability into the measurements. Peptide ratios were also grouped into proteins and averaged to arrive at protein-level ratios for those 685 proteins having two or more significant scoring peptides. The average of the protein-level standard deviations was 0.055 and 0.034 for the *xrn1Δ* and *upf1Δ* strains, respectively. In other words, there was a high degree of concordance between individual peptides contributing to the relative quantitation of any given protein.

From the global mean and standard deviation for each strain we identified proteins whose average expression ratios fell outside of  $\pm 1$  standard deviation from the global mean. We further excluded proteins whose individual standard deviation (*i.e.* between peptides) was greater than 0.1. From this, a list of up- and down-regulated proteins was generated for the *xrn1Δ* and *upf1Δ* strains (Table I). Using these criteria, 62 and 48 proteins were considered up-regulated and 23 and 39 proteins down-regulated in *upf1Δ* and *xrn1Δ*, respectively

TABLE I  
Summary of significant protein expression changes in multiplex *Xrn1Δ-Upf1Δ-WT* comparative study

Protein name	Count <sup>a</sup>	Xrn1Δ ratio <sup>b</sup>	Xrn1Δ S.D.	Upf1Δ ratio <sup>b</sup>	Upf1Δ S.D.	Xrn1Δ fold change <sup>c</sup>	Rep <sup>d,e</sup>
<b>Xrn1Δ-up</b>							
(P22943) 12-kDa heat shock protein (glucose and lipid-regulated protein)	5	0.792	0.069	0.453	0.084	3.806	Yes
(P23776) Glucan 1,3-β-glucosidase I/II precursor (EC 3.2.1.58)	4	0.732	0.021	0.536	0.066	2.738	Yes
(P03965) Carbamoyl-phosphate synthase, arginine-specific, large chain (EC 6.3.5.5)	11	0.682	0.041	0.546	0.049	2.148	Yes
(P56628) 60S ribosomal protein L22-B	2	0.681	0.078	0.526	0.044	2.138	Yes
(P06208) 2-isopropylmalate synthase (EC 2.3.3.13) (α-isopropylmalate synthase)	15	0.68	0.047	0.546	0.038	2.125	Yes
(P49334) Mitochondrial import receptor subunit TOM22	2	0.679	0.037	0.584	0.11	2.118	Yes
(P04806) Hexokinase A (EC 2.7.1.1) (Hexokinase PI)	3	0.67	0.075	0.466	0.053	2.031	Yes
(P00890) Citrate synthase, mitochondrial precursor (EC 2.3.3.1)	2	0.66	0.048	0.528	0.036	1.969	Yes
(P17709) Glucokinase (EC 2.7.1.2) (glucose kinase) (GLK)	2	0.656	0.082	0.494	0.067	1.906	Yes
(P39726) Glycine cleavage system H protein, mitochondrial precursor	2	0.654	0.099	0.576	0.12	1.892	Yes
(Q00055) Glycerol-3-phosphate dehydrogenase [NAD+] 1 (EC 1.1.1.8)	2	0.651	0.018	0.459	0.053	1.868	Yes
(P46992) Hypothetical 43.0-kDa protein in CPS1-FPP1 intergenic region	2	0.649	0.011	0.537	0.0074	1.85	
(Q12019) Midasin (MIDAS-containing protein)	2	0.646	0.044	0.512	0.052	1.826	
(P04076) Argininosuccinate lyase (EC 4.3.2.1) (arginosuccinase) (ASAL)	4	0.642	0.054	0.569	0.061	1.797	Yes
(P00498) ATP phosphoribosyltransferase (EC 2.4.2.17)	5	0.628	0.054	0.537	0.037	1.689	Yes
(Q08965) Ribosome biogenesis protein BMS1	2	0.625	0.016	0.521	0.014	1.665	Yes
(P40482) Protein transport protein Sec24 (abnormal nuclear morphology 1)	2	0.615	0.075	0.525	0.05	1.595	Yes
(P00812) Arginase (EC 3.5.3.1)	3	0.614	0.019	0.567	0.032	1.59	Yes
(P06168) Ketol-acid reductoisomerase, mitochondrial precursor (EC 1.1.1.86)	18	0.613	0.099	0.525	0.057	1.587	Yes
(Q04182) ATP-dependent permease PDR15	2	0.563	0.045	0.535	0.016	1.293	Yes
(P33416) Heat shock protein 78, mitochondrial precursor	2	0.614	0.015	0.515	0.044	1.581	
(P31539) Heat shock protein 104	10	0.612	0.041	0.534	0.03	1.579	
(P37291) Serine hydroxymethyltransferase, cytosolic (EC 2.1.2.1) (serine methylase)	14	0.612	0.063	0.515	0.04	1.578	Yes
(P22768) Argininosuccinate synthase (EC 6.3.4.5) (citrulline-aspartate ligase)	3	0.611	0.0053	0.572	0.015	1.574	Yes
(P07273) Transcription elongation factor S-II (DNA strand transfer protein α)	3	0.61	0.094	0.531	0.011	1.563	Yes
(P38777) Hypothetical 27.3-kDa protein in AAP1-SMF2 intergenic region	4	0.609	0.069	0.534	0.027	1.561	
(P39522) Dihydroxy-acid dehydratase, mitochondrial precursor (EC 4.2.1.9) (DAD)	10	0.606	0.061	0.505	0.037	1.54	Yes
(Q04869) Hypothetical 38.2-kDa protein in PRE5-FET4 intergenic region	3	0.606	0.041	0.493	0.028	1.536	Yes
(P40215) Hypothetical 62.8-kDa protein in RPS16A-TIF34 intergenic region	3	0.605	0.078	0.457	0.057	1.534	Yes
(P47119) HAM1 protein	3	0.603	0.050	0.488	0.011	1.516	Yes
(P00937) Anthranilate synthase component II (EC 4.1.3.27)	2	0.602	0.008	0.49	0.008	1.515	Yes
(P53332) Hypothetical 34.3-kDa protein in TAF145-YOR1 intergenic region	2	0.602	0.0047	0.475	0.007	1.512	
(P42940) Probable electron transfer flavoprotein β-subunit (β-ETF)	2	0.601	0.043	0.553	0.087	1.509	Yes
(P17505) Malate dehydrogenase, mitochondrial precursor (EC 1.1.1.37)	5	0.600	0.03	0.491	0.035	1.504	Yes
(P00128) Ubiquinol-cytochrome C reductase complex 14-kDa protein (EC 1.10.2.2)	4	0.60	0.034	0.476	0.055	1.5	Yes
(P18239) ADP,ATP carrier protein 2 (ADP/ATP translocase 2)	3	0.6	0.049	0.479	0.054	1.5	Yes
(P06634) Probable ATP-dependent RNA helicase DED1	25	0.598	0.052	0.491	0.037	1.49	Yes
(P32454) Aminopeptidase II (EC 3.4.11.-) (YscII)	2	0.598	0.061	0.532	0.027	1.487	Yes
(P00427) Cytochrome c oxidase polypeptide VI, mitochondrial precursor (EC 1.9.3.1)	2	0.593	0.021	0.478	0.02	1.457	Yes
(Q04636) Hypothetical 63.0-kDa protein in DAK1-ORC1 intergenic region	2	0.591	0.029	0.563	0.048	1.445	Yes
(P38715) Probable oxidoreductase GRE3 (EC 1.-.-.-)	2	0.591	0.008	0.475	0.011	1.443	Yes
(P27616) Phosphoribosylamidoimidazole-succinocarboxamide synthase (EC 6.3.2.6)	3	0.587	0.079	0.517	0.047	1.421	
(P04046) Amidophosphoribosyltransferase (EC 2.4.2.14)	2	0.586	0.023	0.54	0.0097	1.418	Yes
(P23542) Aspartate aminotransferase, cytoplasmic (EC 2.6.1.1) (transaminase A)	5	0.585	0.044	0.523	0.021	1.407	Yes
(P33734) Imidazole glycerol phosphate synthase hisHF (IGP synthase)	3	0.584	0.046	0.533	0.019	1.403	Yes
(P07256) Ubiquinol-cytochrome c reductase complex core protein I, mitochondrial precursor	2	0.582	0.027	0.46	0.036	1.393	Yes
(P10591) Heat shock protein SSA1 (heat shock protein YG100)	6	0.581	0.075	0.54	0.038	1.384	Yes
(P34227) Hypothetical 29.5-kDa protein in SEF1-KIP1 intergenic region	2	0.58	0.038	0.524	0.033	1.383	Yes

TABLE I—continued

Protein name	Count <sup>a</sup>	Xrn1Δ ratio <sup>b</sup>	Xrn1Δ S.D.	Upf1Δ ratio <sup>b</sup>	Upf1Δ S.D.	Xrn1Δ fold change <sup>c</sup>	Rep <sup>d,e</sup>
<b>Xrn1Δ-down</b>							
(P40029) Peptide methionine sulfoxide reductase (EC 1.8.4.6)	2	0.299	0.0244	0.454	0.0089	0.446	Yes
(P05747) 60S ribosomal protein L29 (YL43)	11	0.342	0.036	0.471	0.02	0.546	Yes
(P07215) Metallothionein precursor (Cu-MT) (copper chelatin)	3	0.35	0.056	0.562	0.024	0.565	Yes
(P14796) 60S ribosomal protein L40 (CEP52)	4	0.354	0.046	0.441	0.022	0.576	Yes
(P05748) 60S ribosomal protein L15-A (YL10) (L13) (RP15R) (YP18)	12	0.37	0.056	0.47	0.028	0.618	Yes
(P05755) 40S ribosomal protein S9-B (S13) (YS11) (RP21) (YP28)	2	0.373	0.015	0.463	0.0056	0.625	
(P02293) Histone H2B.1	2	0.378	0.029	0.431	0.044	0.64	Yes
(Q12213) 60S ribosomal protein L7-B (L6B) (YL8B)	8	0.382	0.094	0.457	0.016	0.696	Yes
(P53297) PAB1-binding protein 1	6	0.383	0.092	0.457	0.025	0.653	Yes
(P87262) 60S ribosomal protein L34-A	2	0.385	0.076	0.489	0.003	0.706	Yes
(P41057) 40S ribosomal protein S29-A (S36) (YS29)	6	0.388	0.07	0.47	0.013	0.668	Yes
(P04650) 60S ribosomal protein L39 (L46) (YL40)	2	0.389	0.019	0.445	0.0017	0.67	Yes
(P14126) 60S ribosomal protein L3 (YL1) (RP1) (trichodermin resistance protein)	25	0.389	0.069	0.472	0.031	0.671	Yes
(P10614) Cytochrome P450 51 (EC 1.14.13.70) (CYPLI) (P450-LIA1)	6	0.39	0.066	0.497	0.03	0.671	Yes
(P47100) Transposon Ty1 protein B	16	0.399	0.077	0.514	0.023	0.672	Yes
(P26786) 40S ribosomal protein S7-A (RP30)	2	0.399	0.013	0.474	0.009	0.672	Yes
(P05743) 60S ribosomal protein L26-A (YL33)	5	0.399	0.060	0.44	0.038	0.722	Yes
(P46784) 40S ribosomal protein S10-B	6	0.393	0.081	0.461	0.025	0.731	Yes
(Q03834) MUTS protein homolog 6	2	0.3937	0.075	0.513	0.12	0.734	Yes
(P41805) 60S ribosomal protein L10 (L9)	23	0.394	0.061	0.463	0.017	0.684	Yes
(P40150) Heat shock protein SSB2	2	0.397	0.015	0.442	0.011	0.693	Yes
(P54780) 60S ribosomal protein L15-B (YL10) (L13) (RP15R) (YP18)	3	0.403	0.034	0.51	0.023	0.764	Yes
(P47098) Transposon Ty1 protein B	4	0.403	0.055	0.527	0.057	0.765	Yes
(Q01855) 40S ribosomal protein S15 (S21) (YS21) (RP52) (RIG protein)	5	0.404	0.045	0.474	0.041	0.714	Yes
(Q02753) 60S ribosomal protein L21-A	3	0.405	0.056	0.471	0.012	0.716	Yes
(P23301) Eukaryotic translation initiation factor 5A-2 (eIF-5A 2) (eIF-4D)	5	0.405	0.078	0.468	0.024	0.718	Yes
(P29547) Elongation factor 1-γ 1 (EF-1-γ 1)	5	0.41	0.064	0.445	0.032	0.733	Yes
(P07282) 40S ribosomal protein S25 precursor (S31) (YS23) (RP45)	5	0.412	0.066	0.452	0.021	0.739	Yes
(P38693) Acid phosphatase PHO12 precursor (EC 3.1.3.2)	4	0.414	0.098	0.416	0.05	0.744	
(P39939) 40S ribosomal protein S26-B	4	0.415	0.049	0.477	0.0055	0.749	Yes
(P06106) MET17 protein [includes O-acetylhomoserine sulfhydrylase (EC 2.5.1.49)]	8	0.416	0.085	0.474	0.024	0.75	Yes
(P14832) Peptidyl-prolyl <i>cis-trans</i> isomerase (EC 5.2.1.8) (PPlase) (rotamase)	9	0.416	0.055	0.524	0.05	0.751	Yes
(P23254) Transketolase 1 (EC 2.2.1.1) (TK 1)	11	0.417	0.055	0.478	0.04	0.753	Yes
(P46990) 60S ribosomal protein L17-B (YL17-B)	3	0.418	0.043	0.487	0.027	0.757	Yes
(P05740) 60S ribosomal protein L17-A (YL17-A)	9	0.419	0.037	0.458	0.035	0.761	Yes
(P07281) 40S ribosomal protein S19-B (S16B) (YS16) (RP55)	2	0.419	0.0082	0.455	0.012	0.762	Yes
(P04912) Histone H2A.2	2	0.42	0.005	0.379	0.051	0.763	Yes
(P25631) Hypothetical 24.7-kDa protein in ARE1-THR4 intergenic region	3	0.42	0.06	0.509	0.014	0.763	Yes
<b>Upf1Δ-up</b>							
(P22768) Argininosuccinate synthase (EC 6.3.4.5) (citrulline-aspartate ligase)	3	0.611	0.005	0.572	0.016	1.435	Yes
(P04076) Argininosuccinate lyase (EC 4.3.2.1) (arginosuccinase) (ASAL)	4	0.642	0.054	0.569	0.061	1.417	Yes
(P00812) Arginase (EC 3.5.3.1)	3	0.614	0.019	0.567	0.032	1.404	Yes
(P30822) Exportin 1 (chromosome region maintenance protein 1)	2	0.57	0.074	0.565	0.068	1.397	Yes
(Q04636) Hypothetical 63.0-kDa protein in DAK1-ORC1 intergenic region	2	0.591	0.029	0.563	0.048	1.383	
(P00815) Histidine biosynthesis trifunctional protein	4	0.672	0.121	0.562	0.044	1.377	Yes
(P07215) Metallothionein precursor (Cu-MT) (copper chelatin)	3	0.35	0.056	0.562	0.024	1.376	Yes
(P53337) Hypothetical 35.0-kDa protein in BGL2-ZUO1 intergenic region	2	0.563	0.056	0.557	0.034	1.348	
(P38886) 26S proteasome regulatory subunit RPN10	4	0.533	0.084	0.555	0.055	1.34	Yes
(P53111) Hypothetical 38.1-kDa protein in RCK1-AMS1 intergenic region	2	0.582	0.155	0.554	0.043	1.33	Yes
(P42940) Probable electron transfer flavoprotein β-subunit (β-ETF)	2	0.601	0.043	0.553	0.087	1.326	
(P13587) Sodium transport ATPase 1 (EC 3.6.3.7)	2	0.549	0.017	0.548	0.022	1.301	
(P03965) Carbamoyl-phosphate synthase, arginine-specific, large chain (EC 6.3.5.5)	11	0.682	0.041	0.546	0.05	1.29	Yes
(P06208) 2-isopropylmalate synthase (EC 2.3.3.13) (α-isopropylmalate synthase)	15	0.68	0.047	0.546	0.038	1.287	Yes
(P14906) Translocation protein (NPL1 protein)	2	0.535	0.039	0.546	0.046	1.285	Yes
(P49089) Asparagine synthetase [glutamine-hydrolyzing] 1 (EC 6.3.5.4)	11	0.561	0.054	0.542	0.025	1.268	Yes



**Multiplexed Quantitation Using Isobaric Reagents**

TABLE I—continued

Protein name	Count <sup>a</sup>	Xrn1Δ ratio <sup>b</sup>	Xrn1Δ S.D.	Upf1Δ ratio <sup>b</sup>	Upf1Δ S.D.	Xrn1Δ fold change <sup>c</sup>	Rep <sup>d,e</sup>
(P32179) 3'(2'),5'-bisphosphate nucleotidase	2	0.564	0.071	0.541	0.049	1.264	Yes
(P10591) Heat shock protein SSA1 (heat shock protein YG100)	6	0.581	0.075	0.54	0.038	1.257	
(P04046) Amidophosphoribosyltransferase (EC 2.4.2.14)	2	0.586	0.023	0.54	0.01	1.257	Yes
(P38066) GTP cyclohydrolase II (EC 3.5.4.25)	3	0.515	0.085	0.539	0.067	1.249	
(P38891) Branched-chain amino acid aminotransferase, mitochondrial precursor	11	0.527	0.057	0.538	0.031	1.246	Yes
(P07274) Profilin	2	0.575	0.019	0.538	0.039	1.244	Yes
(P00498) ATP phosphoribosyltransferase (EC 2.4.2.17)	5	0.628	0.054	0.537	0.037	1.243	Yes
(P46992) Hypothetical 43.0-kDa protein in CPS1-FPP1 intergenic region	2	0.649	0.011	0.537	0.007	1.242	
(P38009) Bifunctional purine biosynthesis protein ADE17	2	0.701	0.127	0.537	0.048	1.241	
(P23776) Glucan 1,3-β-glucosidase I/II precursor (EC 3.2.1.58)	4	0.732	0.021	0.536	0.066	1.235	Yes
(P38080) Probable serine/threonine-protein kinase YBR059C (EC 2.7.1.-)	3	0.546	0.019	0.535	0.056	1.232	
(Q04182) ATP-dependent permease PDR15	2	0.564	0.045	0.535	0.016	1.23	
(P25294) SIS1 protein	2	0.442	0.019	0.535	0.046	1.229	
(P38765) Hypothetical 32.6-kDa protein in DAP2-SLT2 intergenic region	2	0.523	0.005	0.534	0.058	1.227	Yes
(P48353) HLJ1 protein	3	0.544	0.059	0.534	0.02	1.226	
(P38777) Hypothetical 27.3-kDa protein in AAP1-SMF2 intergenic region	4	0.609	0.069	0.534	0.027	1.225	
(P39676) Flavohemoprotein (hemoglobin-like protein) (flavohemoglobin)	8	0.513	0.083	0.534	0.013	1.223	Yes
(P31539) Heat shock protein 104	10	0.612	0.041	0.534	0.03	1.223	Yes
(P33734) Imidazole glycerol phosphate synthase hisHF (IGP synthase)	3	0.584	0.046	0.533	0.019	1.219	
(P41895) Transcription initiation factor IIF, α subunit (TFIIF-α)	2	0.512	0.039	0.533	0.064	1.219	
(P32454) Aminopeptidase II (EC 3.4.11.-) (YscII)	2	0.598	0.061	0.532	0.027	1.215	Yes
(P36013) Probable NAD-dependent malic enzyme (EC 1.1.1.38) (NAD-ME)	7	0.56	0.042	0.531	0.047	1.211	Yes
(P07273) Transcription elongation factor S-II (DNA strand transfer protein α)	3	0.61	0.094	0.531	0.011	1.208	Yes
(P38998) Saccharopine dehydrogenase [NAD <sup>+</sup> , L-lysine forming] (EC 1.5.1.7)	2	0.55	0.018	0.53	0.007	1.205	Yes
(P13663) Aspartate-semialdehyde dehydrogenase (EC 1.2.1.11)	12	0.533	0.046	0.53	0.025	1.204	Yes
(P10962) MAK16 protein	2	0.533	0.016	0.529	0.084	1.2	Yes
(P07264) 3-isopropylmalate dehydratase (EC 4.2.1.33) (isopropylmalate isomerase)	26	0.486	0.061	0.529	0.027	1.199	Yes
(Q06142) Importin β-1 subunit (karyopherin β-1 subunit) (importin 95)	4	0.548	0.045	0.529	0.026	1.198	
(P36112) Hypothetical 61.1-kDa protein in YPT52-DBP7 intergenic region	2	0.524	0.021	0.528	0.053	1.195	Yes
(P10659) S-adenosylmethionine synthetase 1 (EC 2.5.1.6)	13	0.502	0.053	0.528	0.023	1.193	Yes
(P32381) Actin-like protein ARP2	2	0.523	0.014	0.528	0.05	1.193	Yes
(P15891) Actin binding protein	7	0.563	0.061	0.528	0.067	1.192	Yes
(P51401) 60S ribosomal protein L9-B (L8) (YL11) (RP25)	2	0.518	0.009	0.527	0.008	1.191	Yes
(P47079) T-complex protein 1, θ subunit (TCP-1-θ) (CCT-θ)	5	0.548	0.063	0.527	0.048	1.189	Yes
(P40482) Protein transport protein Sec24 (abnormal nuclear morphology 1)	2	0.615	0.075	0.525	0.05	1.179	Yes
(P06168) Ketol-acid reductoisomerase, mitochondrial precursor (EC 1.1.1.86)	18	0.613	0.099	0.525	0.057	1.177	Yes
(P34227) Hypothetical 29.5-kDa protein in SEF1-KIP1 intergenic region	2	0.58	0.038	0.524	0.033	1.176	
(P14832) Peptidyl-prolyl <i>cis-trans</i> isomerase (EC 5.2.1.8) (PPIase) (rotamase)	9	0.416	0.055	0.524	0.051	1.173	
(P07342) Acetolactate synthase, mitochondrial precursor (EC 2.2.1.6)	10	0.556	0.045	0.523	0.025	1.172	
(Q06252) Hypothetical 22.2-kDa protein in TFS1-SAM1 intergenic region	2	0.459	0.087	0.523	0.027	1.17	Yes
(P32563) Vacuolar ATP synthase 95-kDa subunit (vacuolar ATPase 95-kDa subunit)	6	0.55	0.056	0.523	0.05	1.169	
(P23542) Aspartate aminotransferase, cytoplasmic (EC 2.6.1.1) (transaminase A)	5	0.585	0.044	0.523	0.021	1.169	Yes
(P43555) 47-kDa endomembrane protein precursor (endosomal P44 protein)	2	0.534	0.043	0.523	0.038	1.168	
(P47176) Branched-chain amino acid aminotransferase, cytosolic (EC 2.6.1.42)	6	0.521	0.073	0.522	0.027	1.165	Yes
(Q08965) Ribosome biogenesis protein BMS1	2	0.625	0.016	0.521	0.014	1.159	
<b>Upf1Δ-down</b>							
(Q03667) Hypothetical 16.7-kDa protein in CDC5-MVP1 intergenic region	2	0.469	0.18	0.344	0.084	0.549	Yes
(P43579) Hypothetical 78.8-kDa protein in HSP12-HXT10 intergenic region	2	0.577	0.027	0.376	0.032	0.634	
(P04912) Histone H2A.2	2	0.42	0.005	0.379	0.051	0.642	
(P87108) Mitochondrial import inner membrane translocase subunit TIM10	2	0.502	0.044	0.41	0.012	0.732	Yes
(P38693) Acid phosphatase PHO12 precursor (EC 3.1.3.2)	4	0.414	0.098	0.416	0.05	0.751	Yes
(P15565) N(2),N(2)-dimethylguanosine tRNA methyltransferase, mitochondrial prec.	2	0.516	0.125	0.417	0.005	0.753	Yes

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TABLE I—continued

Protein name	Count <sup>a</sup>	Xrn1Δ ratio <sup>b</sup>	Xrn1Δ S.D.	Upf1Δ ratio <sup>b</sup>	Upf1Δ S.D.	Xrn1Δ fold change <sup>c</sup>	Rep <sup>d,e</sup>
(P32591) Transcription regulatory protein SWI3 (SWI/SNF complex component SWI3)	2	0.452	0.118	0.417	0.087	0.755	
(P02309) Histone H4	4	0.474	0.06	0.42	0.038	0.762	
(P21538) DNA-binding protein REB1 (QBP)	2	0.499	0.018	0.42	0.023	0.762	
(P30624) Long-chain-fatty-acid-CoA ligase 1 (EC 6.2.1.3)	2	0.527	0.001	0.421	0.051	0.768	
(P15019) Transaldolase (EC 2.2.1.2)	3	0.487	0.057	0.424	0.035	0.775	
(P32386) ATP-dependent bile acid permease	2	0.513	0.006	0.425	0.027	0.78	
(P25644) Topoisomerase II-associated protein PAT1	3	0.503	0.067	0.425	0.012	0.78	
(P07263) Histidyl-tRNA synthetase, mitochondrial precursor (EC 6.1.1.21)	4	0.485	0.064	0.426	0.065	0.783	Yes
(P53720) SMM1 protein	2	0.578	0.089	0.427	0.049	0.785	
(P19358) S-adenosylmethionine synthetase 2 (EC 2.5.1.6)	3	0.463	0.051	0.428	0.092	0.79	Yes
(P02293) Histone H2B.1	2	0.378	0.029	0.431	0.044	0.801	
(Q04439) Myosin-5 isoform	2	0.493	0.018	0.432	0.049	0.804	Yes
(P32610) Vacuolar ATP synthase subunit D (EC 3.6.3.14) (V-ATPase D subunit)	3	0.531	0.014	0.433	0.066	0.807	
(Q04493) Prefoldin subunit 5	2	0.489	0.011	0.434	0.027	0.81	Yes
(P00942) Triosephosphate isomerase (EC 5.3.1.1) (TIM)	14	0.486	0.145	0.436	0.039	0.817	Yes
(P00330) Alcohol dehydrogenase I (EC 1.1.1.1) (YADH-1)	21	0.489	0.161	0.438	0.097	0.822	Yes
(P04911) Histone H2A.1	3	0.427	0.03	0.411	0.032	0.735	

<sup>a</sup> Number of top-ranked, significant peptides, including duplicate sequences from separate MS/MS spectra.

<sup>b</sup> Calculated as area(mutant)/(area(mutant) + area(wild type)), with expression change ranging from 0 to 1, where no change = 0.5.

<sup>c</sup> Fold changes were calculated with respect to the global peptide average, 0.503 for Xrn1Δ and 0.485 for Upf1Δ.

<sup>d</sup> Yes indicates that protein was identified in preliminary 2-plex experiments, as in Footnote 2.

<sup>e</sup> Yes indicates that protein was identified in ICAT experiments (16).

(Table I). We compared the current set of differentially expressed proteins with relative expression data from a 2-plex pilot scale study<sup>2</sup> of a separate batch of protein lysates from the wild-type and *xrn1Δ* strains. This comparison revealed that 86% of up-regulated and 79% of down-regulated proteins (Table I) were identified in the preliminary experiment as up- or down-regulated, respectively. We also compared the current set of differentially expressed proteins to that identified using ICAT for relative protein quantitation in the *upf1Δ* strain (16). Because of the substantial difference between workflows, there were proteins unique to each experiment; for example many proteins having two or fewer cysteines (including all major histones) were not observed in the ICAT dataset. However, comparison of the relative expression of proteins common to both experiments proved to be a useful validation of our approach. Of the 85 differentially expressed proteins we observed in the *upf1Δ* strain, 49 were also observed by ICAT, and 42 of these were concordant with respect to up- or down-regulation. The average number of peptides identified per protein also increased from ~2 peptides/protein in the ICAT study to 4.5 peptides/protein in the current work.

An additional feature of these comparative experiments was an observed increase in the number of lysine-containing tryptic peptides. The ratio of lysine to arginine-terminated tryptic peptides identified increased from 0.79 in comparable

whole-yeast experiments using underivatized (native) peptides to a value of 0.98 in this current study. This indicates that the lysine-derivatized peptides are identified more frequently, possibly due to higher ionization efficiency when labeled with the basic piperazine tags. For the entire set of ~4,500 high-significance peptides, more than 97% were fully modified at the N terminus or lysine side chains. Incomplete and tyrosine side chain reactions thus comprised <3% of identified peptides.

The experiment also demonstrated absolute quantitation of selected proteins (Fig. 5A). In this example, 1 pmol of a synthetic tryptic peptide (ILESHDIVPPEVR from carbamoyl phosphate synthetase) was labeled with the 117-reporter isobaric reagent and combined with the yeast strains prior to cation exchange chromatography. The peptide was identified automatically during the course of the experiment, and the intensity of the synthetic peptide-derived signature ion at *m/z* 117.1 was used to calculate an absolute value. We can estimate using the molecular weight (135,417) of carbamoyl phosphate synthetase that there were ~26 ng of this protein in the original 150 μg of wild-type yeast lysate. This calculates to ~45,000 copies per cell in the wild-type strain, rising to ~98,000 copies/cell in *xrn1Δ*. This approach, where added internal peptide standards remain isobaric, is significantly different from the absolute quantitation (AQUA) approach (17), where a mass-difference approach is employed through isotopic enrichment of the synthetic peptides. Finally, we were also able to perform a more specific analysis to confirm the deletion of the Xrn1 protein in the *xrn1Δ* yeast strain (Fig. 5B),

<sup>2</sup> Pilot studies were conducted as a 2-plex experiment using 100 μg of total protein with similar conditions for protein extraction, digestion, labeling, chromatography, and MS.

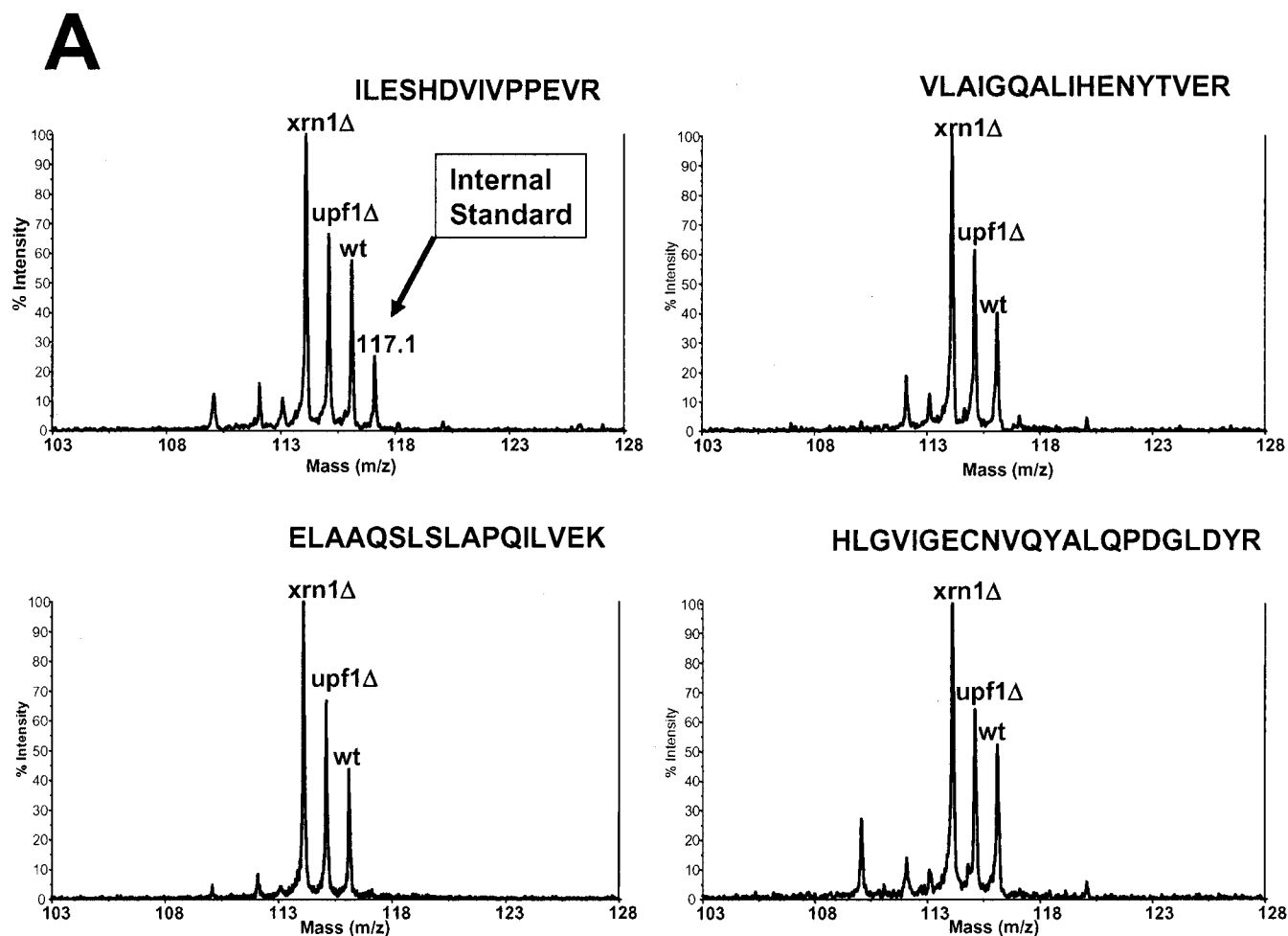


FIG. 5. Example signature ion regions from four MS/MS spectra illustrating the degree of consistency in relative quantitative measurement of (A) an up-regulated protein (carbamoyl phosphate synthetase). Illustrated are the signature ion regions of four identified tryptic peptides (from a total of 11) that show consistency of measurement between the three strains. For all 11 peptides identified, the fold increase of this protein in the *xrn1Δ* strain was 2.15 relative to wild type, with an S.D. of <12%. This protein was also used to demonstrate an absolute quantitative measurement using an internal spiked synthetic tryptic peptide (ILESHDVIVPPEVR) labeled with the 117-reporter isobaric reagent. *B*, highlighted signature ion region showing confirmation of deletion of the Xrn1p protein in the *xrn1Δ* yeast strain by quantitative measurement of the identified tryptic peptide IGPMEAIATVFPVTGLVR (derivatized *m/z* 2015.1) that corresponds to residues 861–878 of the native Xrn1 protein. This peptide (and hence protein) is clearly absent only in the *xrn1Δ* strain.

where the absence of a signature ion at *m/z* 114.1 established the loss of this protein.

**Proteins Up-regulated in the *upf1Δ* and *xrn1Δ* Strains**—The data clearly indicate that deletion of the Xrn1 and Upf1 proteins produces similar changes to the protein phenotype (Fig. 6). Of the 48 proteins considered to be up-regulated in the *xrn1Δ* strain, 23 are seen to be also up-regulated in the *upf1Δ* strain. The magnitude of increases is, however, two to three times greater in the *xrn1Δ* strain. Similarity in protein up-regulation also extends to ontological comparison of these two mutant strains (Fig. 6). Up-regulated proteins were grouped according to biological function as described (18). Significant groups were chosen by comparison with those identified proteins that were not significantly up- or down-regulated ( $p < 0.05$ ). Closer inspection reveals that many of

the proteins are involved in amino acid biosynthesis (including many aspects of amine and nitrogen metabolism). Enzymes involved in the biosynthesis of each of the 20 common amino acids as well as enzymes involved in general nitrogen and amine metabolism (e.g. urea cycle and general metabolism of amine groups) are up-regulated in both *xrn1Δ* and *upf1Δ* strains. This confirms the findings of the earlier ICAT study comparing the wild-type and *upf1Δ* strains, where up-regulation of proteins involved in the urea cycle and amino acid metabolism (particularly arginine) was noted (16). Other metabolic changes seen in both *upf1Δ* and *xrn1Δ* strains include up-regulation of enzymes involved in pantothenate and CoA biosynthesis, starch and sucrose metabolism, and purine and pyrimidine metabolism.

Our data indicated that deletion of the Xrn1 and Upf1 pro-

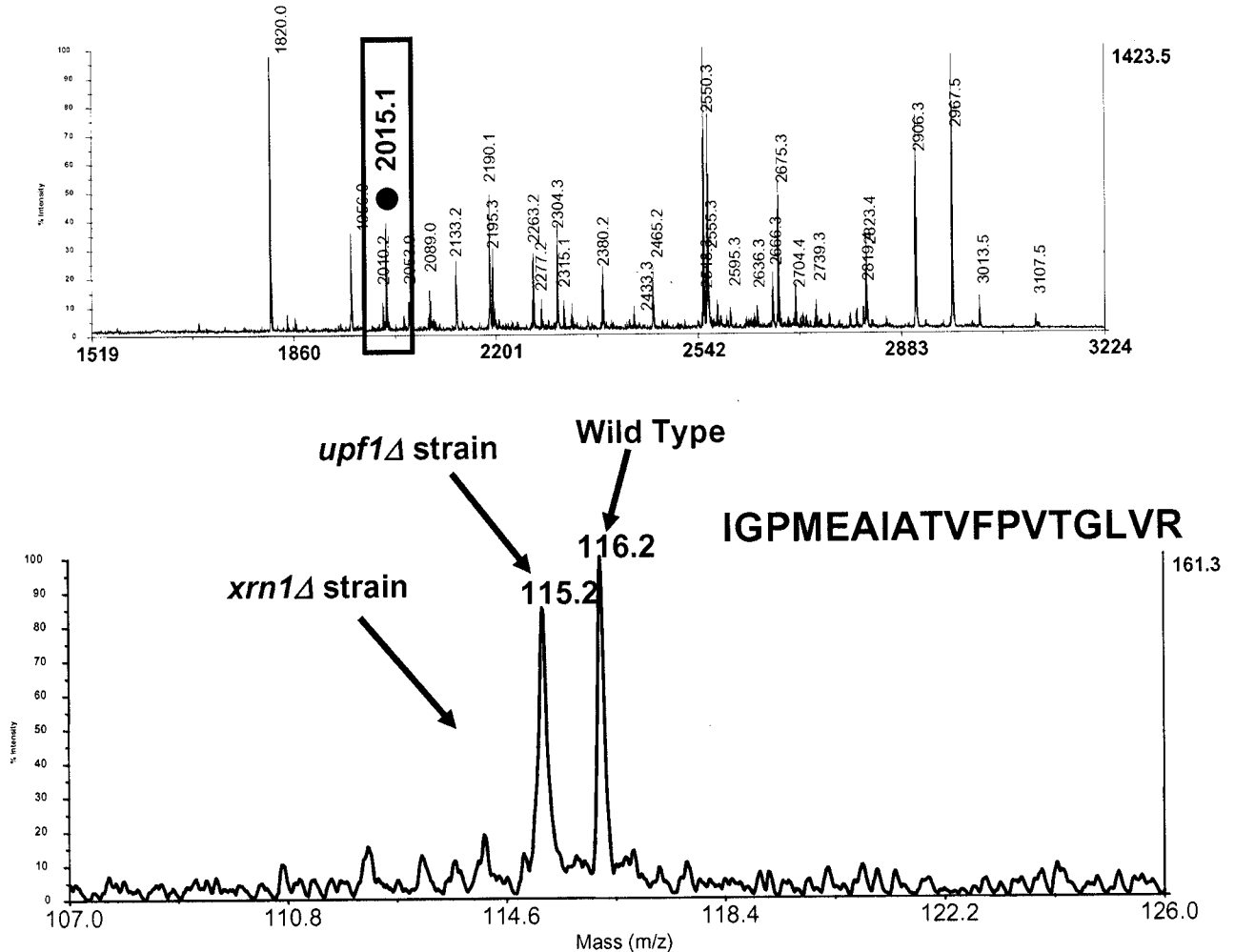
**B**


FIG. 5—continued

teins also yields distinct protein phenotypes. Previous *xrn1Δ* and *upf1Δ* deletion or mutation studies have shown that removal or inhibition of the activities of these two proteins resulted in significantly increased levels of cellular mRNA (19). One pathway of mRNA decay in yeast involves poly(A) shortening followed by decapping and then 5' to 3' decay. Cells devoid of Xrn1p therefore accumulate mRNAs that have shortened or no poly(A) tails and which may lack the 5' cap structure (20). We find that the *xrn1Δ* strain showed specific up-regulation of the ribosome biogenesis protein BMS1 required for the maturation of the 40S ribosomal subunit (21), the SC24 protein required to promote the transport of secretory, membrane, and vacuolar proteins from the endoplasmic reticulum to the Golgi complex (22), and the RNA polymerase II transcription elongation factor TFS2 required for efficient transcription elongation past template-encoded arresting sites. The *upf1Δ* strain showed up-regulation of proteins such

as the Sec63 protein important for protein assembly in the nucleus and endoplasmic reticulum (23), the Sis1 protein required for normal initiation of translation (24), the polymerase II transcription factor TFIIIF that promotes transcriptional elongation (25), and the t-complex protein 1 that acts as a molecular chaperone for protein folding.

**Proteins Down-regulated in the *xrn1Δ* and *upf1Δ* Strains—**In contrast to the general similarity of proteins up-regulated in the *xrn1Δ* and *upf1Δ* strains, those proteins that are significantly down-regulated appear to be quite different. Of the 39 proteins down-regulated in the *xrn1Δ* strain, only three are common to the *upf1Δ* strain. Ontological classification (18) also indicates that the effects of these two mutant strains are substantially different (Table I, Fig. 6). More than half of the proteins down-regulated in *xrn1Δ* cells are structural components of the ribosome or are factors involved in protein synthesis. These include the poly(A)-binding protein Pab1p that

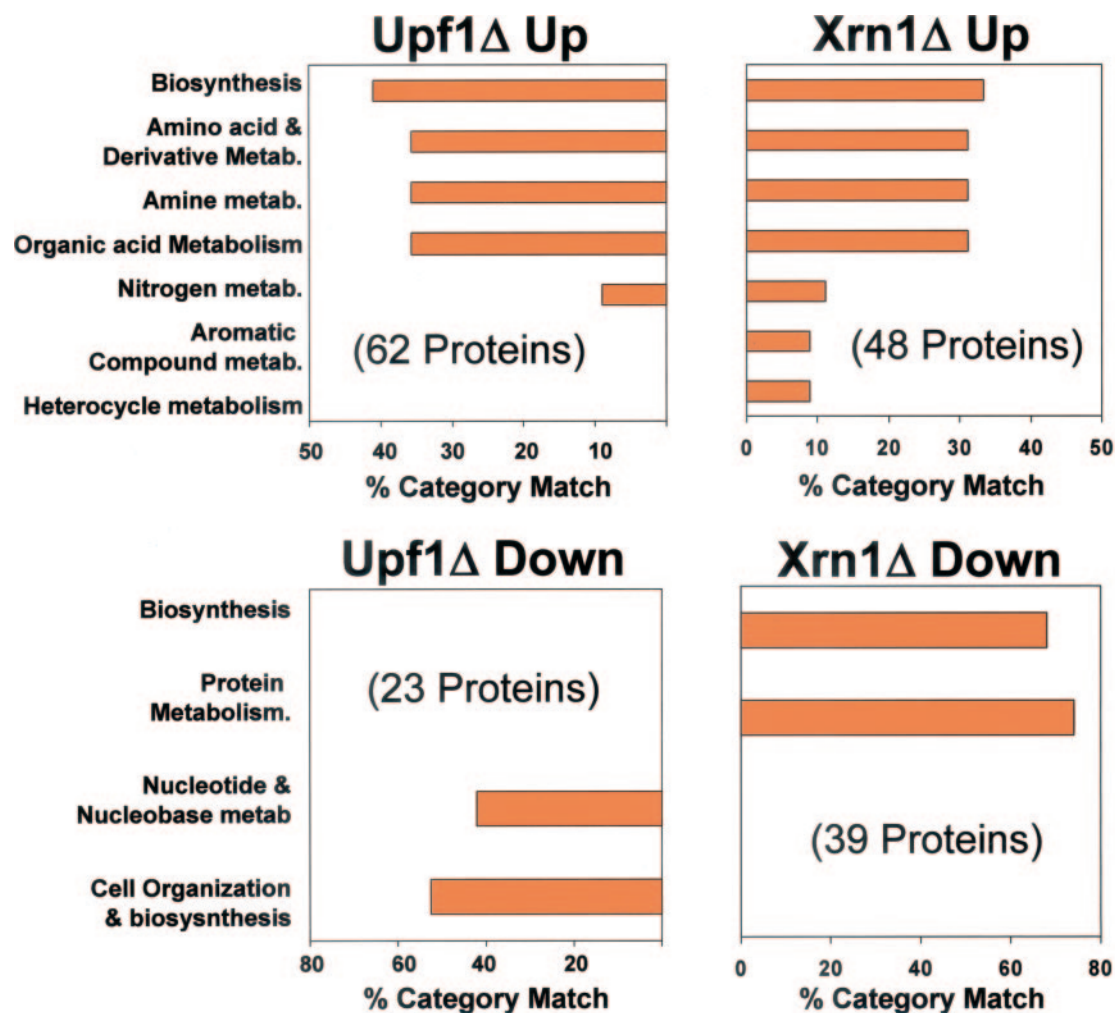


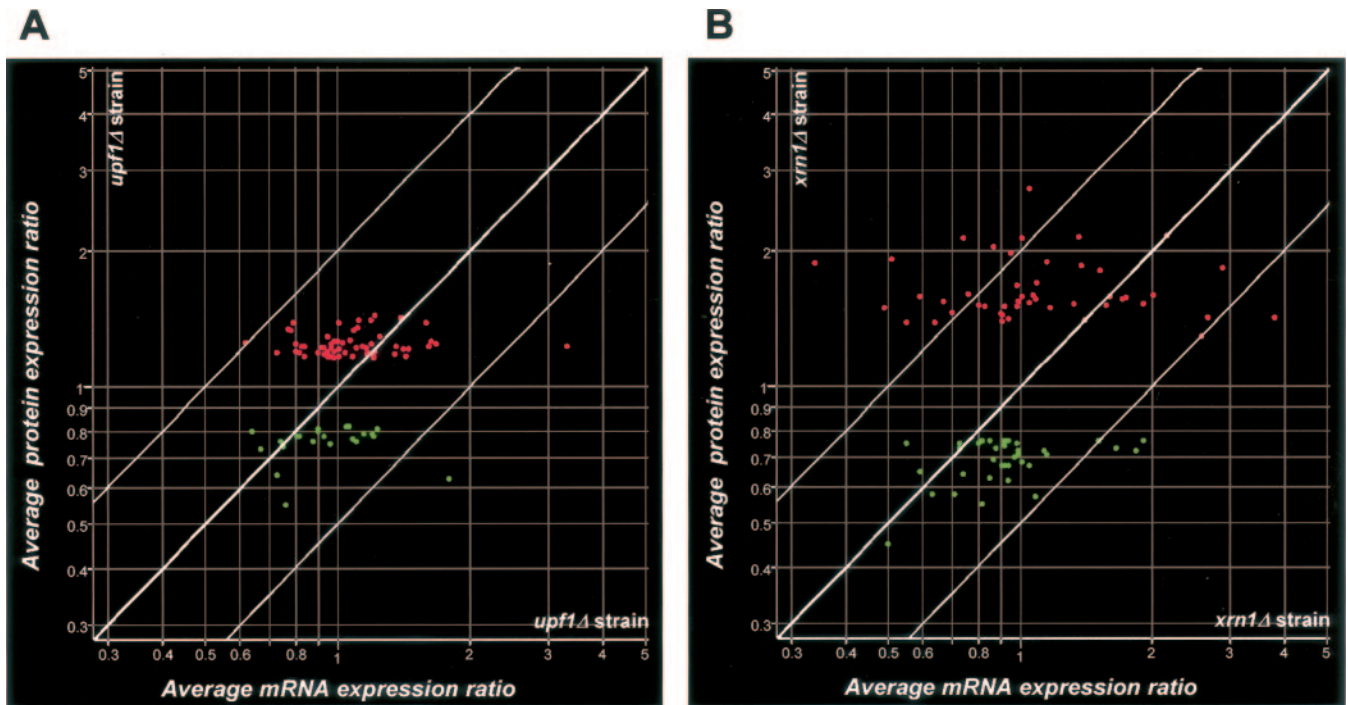
FIG. 6. Display of ontology profile from an ontology search (18) of significant up- and down-regulated proteins observed in the multiplex yeast experiment. Protein classification groups were identified as significant by comparison with the remaining identified proteins that were not significantly up- or down-regulated ( $p < 0.05$ ). This approach determines whether the chosen up- or down-regulated proteins are distributed randomly with respect to the classification of the remaining (~600) identified proteins whose expression levels do not change.

also serves as a scaffold for a series of post-transcriptional regulatory factors involved in mRNA 3' processing, export, translation, and turnover (26), the initiating factor eIF-5A (which may have a role in regulating exonucleolytic decay (27)), and the elongation factor-1 $\gamma$  subunit.

In contrast, many of the down-regulated proteins in the *upf1* $\Delta$  strain are involved in DNA replication or RNA transcription. Approximately one-third of the proteins down-regulated in *upf1* $\Delta$  cells are involved in chromosome and chromatin structure (histones) or transcriptional regulation. These include the Swi3 protein, which is a global activator of transcription required for the induced expression of a large number of genes (28), and Reb1 (29), a sequence-specific DNA-binding protein that recognizes sites within both the enhancer and the promoter regions of the rRNA genes as well as sites upstream of many genes transcribed by RNA polymerase II. Also down-regulated are the Trm1 and METL (S-adenosyl methionine synthetase) proteins, which are required for methylation of

cellular tRNAs (30). While the basis for these observations is unclear, it is possible that the down-regulation of proteins involved in protein translation in *xrn1* $\Delta$  cells reflects a regulatory circuit responding to the loss of Xrn1p's role in pre-rRNA processing (31, 32) and that the up-regulation of proteins involved in amino acid biosynthesis in both mutant strains may occur in these strains as a consequence of restoring functional translation of several endogenous nonsense-containing mRNAs encoding enzymes in histidine, arginine, and leucine biosynthesis (33).

*Relative Changes in mRNA and Protein Levels*—He *et al.* (19) have previously used high-density oligonucleotide microarray analysis to assess global changes in mRNA abundance in the *upf1* $\Delta$  and *xrn1* $\Delta$  strains. We have utilized this data to determine whether there is a correlation between the relative changes in protein and mRNA levels in the *upf1* $\Delta$  and *xrn1* $\Delta$  strains. Fig. 7 compares average fold changes in RNA abundance (from four independent experiments) to the rela-



**FIG. 7. Changes in mRNA levels do not correlate with changes in protein levels in *upf1Δ* and *xrn1Δ* cells.** Scatter plots were used to compare the average mRNA and protein expression ratios in *upf1Δ* (A) and *xrn1Δ* (B) cells. Expression ratios are defined as the fold-change in mutant versus wild-type cells. Average mRNA expression ratios are from He *et al.* (19). Protein expression ratios are derived from the *upf1Δ* data (85 proteins) and *xrn1Δ* data (87 proteins) listed in Table I. Pair-wise comparisons are plotted on a logarithmic scale, with the middle line indicating the line of equivalence and the outer two lines indicating 2-fold differences in expression.

tive changes in protein levels determined in our analyses of the wild-type, *upf1Δ*, and *xrn1Δ* extracts. Fig. 7A shows that, for those proteins showing significant changes in levels in *upf1Δ* cells, there is no significant correlation between mRNA and protein levels ( $r = 0.19$ ). Likewise, in *xrn1Δ* cells, proteins showing significant increases or decreases in levels also show no significant correlation with the levels of their respective mRNAs (Fig. 7B;  $r = 0.20$ ). These comparisons indicate that the levels of a small number of proteins in the *upf1Δ* and *xrn1Δ* strains are regulated post-transcriptionally, *i.e.* for some proteins there are substantive differences between the respective changes in mRNA and protein levels. In agreement with previous studies (34, 35), these measurements of protein and mRNA levels are revealing quite different and nonoverlapping aspects of the overall phenotypic changes induced by the deletion of these factors.

#### CONCLUSIONS

We have used a multiplexed peptide quantitation methodology to identify global protein expression trends in a set of isogenic yeast strains. This approach made use of a set of four isobaric peptide derivatization reagents that yield informative MS/MS spectra for peptide identification and quantitation. The derivatized peptides are indistinguishable by their MS spectra or MS/MS ion series, but exhibit intense, low-mass MS/MS signature ions that permit quantitation of mem-

bers of the multiplex set. The reagents have been incorporated into a simple workflow consisting of protein extraction, tryptic digestion, and peptide labeling followed by cation exchange fractionation. Samples are then analyzed by conventional capillary reversed-phase LC-MS/MS using MALDI or electrospray-based MS. We also demonstrated the use of 4-fold multiplexing to enable relative protein measurements in several samples simultaneously with determination of absolute levels of a target protein using synthetic isobaric peptide standards.

In this study, we used the reagents to compare global protein expression in wild-type yeast and the isogenic *upf1Δ* and *xrn1Δ* mutant strains that are defective in the nonsense-mediated mRNA decay and the 5' to 3' decay pathway. We find that inactivation of Upf1p and Xrn1p cause both common as well as distinct effects on protein expression. Both mutant strains show increased expression of a common set of proteins involved in amino acid biosynthesis and general nitrogen metabolism. The *upf1Δ* strain showed specific down-regulation of proteins involved in DNA replication and RNA transcription, whereas the *xrn1Δ* strain exhibited specific down-regulation of components of the translation apparatus, including ribosomal proteins and translation factors. Comparison between mRNA changes and protein changes of these yeast strains showed no significant correlation.

The isobaric nature of the tags permitted the simultaneous

comparison of multiple yeast strains and added synthetic peptide internal standards in a single two-dimensional-LC-MS experiment, with no increase in chromatographic or MS complexity. Importantly, ratio measurements for all the identified peptides was 100% for all strains. Measured expression ratios demonstrated high consistency, and intra-protein peptide mean and standard deviations were highly reproducible (15–17%). The mixed, multiplex nature of the experiment removes any quantitative variability from chromatography that may be seen in sequential two-dimensional LC-MS analyses of individual peptide mixtures (36), and peptide coverage is significantly increased relative to ICAT. The tagging chemistry is global in that any peptide with a free amine can be labeled and measured. This should enable strategies that seek to isolate and quantify specific classes of peptides (e.g. phosphopeptides) that were essentially impossible using the ICAT cysteine-selective chemistry.

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\*\* To whom correspondence should be addressed: Applied Biosystems, 500 Old Connecticut Path, Framingham, MA 01701. Tel.: 508-383-7484; Fax: 508-383-7813; E-mail: [pappindj@appliedbiosystems.com](mailto:pappindj@appliedbiosystems.com).

REFERENCES

1. Goshe, M. B., and Smith, R. D. (2003) Stable isotope-coded proteomic mass spectrometry. *Curr. Opin. Biotechnol.* **14**, 101–109
2. Tao, W. A., and Aebersold, R. (2003) Advances in quantitative proteomics via stable isotope tagging and mass spectrometry. *Curr. Opin. Biotechnol.* **14**, 110–118
3. Gygi, S. P., Rist, B., Gerber, S. A., Turecek, F., Gelb, M. H., and Aebersold, R. (1999) Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat. Biotechnol.* **17**, 994–999
4. Han D. K., Eng J., Zhou H., and Aebersold R. (2001) Quantitative profiling of differentiation-induced microsomal proteins using isotope-coded affinity tags and mass spectrometry. *Nat. Biotechnol.* **19**, 946–951
5. Ong, S. E., Kratchmarova, I., and Mann, M. (2003) Properties of <sup>13</sup>C-substituted arginine in stable isotope labeling by amino acids in cell culture (SILAC). *J. Proteome Res.* **2**, 173–181
6. Martinovic, S., Veenstra, T. D., Anderson, G. A., Pasa-Tolic, L., and Smith, R. D. (2002) Selective incorporation of isotopically labeled amino acids for identification of intact proteins on a proteome-wide level. *J. Mass Spectrom.* **37**, 99–107
7. Ibarrola, N., Molina, H., Iwahori, A., and Pandey, A. (2004) A novel proteomic approach for specific identification of tyrosine kinase substrates using <sup>13</sup>C-labeled tyrosine. *J. Biol. Chem.* **279**, 15805–15813
8. Ong, S. E., Blagoev, B., Kratchmarova, I., Kristensen, D. B., Steen, H., Pandey, A., and Mann, M. (2002) Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Mol. Cell. Proteomics* **1**, 376–386
9. Gonzalez, C. I., Battacharya, A., Wang W., and Peltz, S. W. (2001) Nonsense-mediated mRNA decay in *Saccharomyces cerevisiae*. *Gene* **274**, 15–25

10. Hentze, M. W., and Kulozik A. E. (1999) A perfect message: RNA surveillance and nonsense-mediated decay. *Cell* **96**, 307–310
11. Pappin, D. J. C., and Bartlett-Jones, M. (2003) Methods, mixtures, kits and compositions pertaining to analyte determination. *PCT Intl. Appl. Application No. WO 2004-US2077*
12. Perkins, D. N., Pappin, D. J., Creasy, D. M., and Cottrell, J. S. P. (1999) Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* **20**, 3551–3567
13. Thompson, A., Schafer J., Kuhn, K., Kienle, S., Schwarz, J., Schmidt G., Johnstone, R., Neumann, T., Hamon, C. (2003) Tandem mass tags: A novel quantification strategy for comparative analysis of complex protein mixtures by MS/MS. *Anal. Chem.* **75**, 1895–1904
14. Karimi-Busheri, F. Daly, G., Robins, P., Canas, B., Pappin, D. J., Sgouros, J., Miller, G. G., Fakhrai, H., Davis, E. M., Le Beau, M. M., and Weinfeld, M. (1999) Molecular characterization of a human DNA kinase. *J. Biol. Chem.* **274**, 24187–24194
15. Höss, M., Robins, P., Naven, T. J. P., Pappin, D. J. C., Sgouros, J., and Lindahl, T. A. (1999) human DNA editing enzyme homologous to the *Escherichia coli* DnaQ/MutD protein. *EMBO J.* **18**, 3868–3875
16. Parker, K.C., Patterson D., Williamson, B. Marchese, J., Graber, A., He, F., Jacobson, A., Juhasz, P., and Martin S. (2004) Depth of proteome issues: A yeast ICAT reagent study. *Mol. Cell. Proteomics* **3**, 625–659
17. Gerber, S. A., Rush, J., Stemman, O., Kirschner, M. W., and Gygi, S. P. (2003) Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 6940–6945
18. Al-Shahrour, F., Diaz-Uriarte, R., and Dopazo, J. (2004) Fatigo: A web tool for finding significant associations of Gene Ontology terms with groups of genes ([fatigo.bioinfo.cnio.es](http://fatigo.bioinfo.cnio.es)). *Bioinformatics* **20**, 578–580
19. He, F., Li, X., Spatrick, P., Casillo, R., Dong, S., and Jacobson, A. (2003) Genome-wide analysis of mRNAs regulated by the nonsense-mediated and 5' to 3' mRNA decay pathways in yeast. *Mol. Cell.* **12**, 1439–1452
20. Hsu, C. L., and Stevens, A. (1993) Yeast cells lacking 5'-3' exoribonuclease 1 contain mRNA species that are poly(A) deficient and partially lack the 5' cap structure. *Mol. Cell. Biol.* **13**, 4826–4835
21. Wegierski, T., Billy, E., Nasr, F., and Filipowicz, W. (2001) Bms1p, a G-domain-containing protein, associates with Rcl1p and is required for 18S rRNA biogenesis in yeast. *RNA* **7**, 1254–1267
22. Kurihara, T., Hamamoto, S., Gimeno, R. E., Kaiser, C. A., Schekman, R., and Yoshihisa, T. (2000) Sec24p and Isp1p function interchangeably in transport vesicle formation from the endoplasmic reticulum in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **11**, 983–998
23. Sadler, I., Chiang, A., Kurihara, T., Rothblatt, J. A., Way, J., and Silver, P. A. (1989) A yeast gene important for protein assembly into the endoplasmic reticulum and the nucleus has homology to DnaJ, an *Escherichia coli* heat shock protein. *J. Cell Biol.* **109**, 2665–2675
24. Zhong, T., and Arndt, K. T. (1993) The yeast SIS1 protein, a DnaJ homolog, is required for the initiation of translation. *Cell* **73**, 1175–1186
25. Henry, N. L., Sayre, M. H., and Kornberg, R. D. (1992) Purification and characterization of yeast RNA polymerase II general initiation factor  $\gamma$ . *J. Biol. Chem.* **267**, 23388–23392
26. Mangus, D. A., Amrani, N., and Jacobson, A. (1998) Pbp1, a factor interacting with *Saccharomyces cerevisiae* poly(A)-binding protein, regulates polyadenylation. *Mol. Cell. Biol.* **18**, 7383–7396
27. Zuk, D., and Jacobson, A. (1998) A single amino acid substitution in yeast eIF-5A results in mRNA stabilization. *EMBO J.* **17**, 2914–2925
28. Peterson, C. L., and Herskowitz, I. (1992) Characterization of the yeast SWI1, SWI2, and SWI3 genes, which encode a global activator of transcription. *Cell* **68**, 573–583
29. Ju, Q., Morrow, B. E., and Warner, J. R. (1990) REB1, a yeast DNA-binding protein with many targets, is essential for growth and bears some resemblance to the oncogene myb. *Mol. Cell. Biol.* **10**, 5226–5234
30. Thomas, D., Rothstein, R., Rosenberg, N., and Surdin-Kerjan, Y. (1988) SAM2 encodes the second methionine S-adenosyl transferase in *Saccharomyces cerevisiae*: Physiology and regulation of both enzymes. *Mol. Cell. Biol.* **8**, 5132–5139
31. Fatica, A., and Tollervey, D. (2002) Making ribosomes. *Curr. Opin. Cell Biol.* **14**, 313–318
32. Stevens, A. Hsu, C. L., Isham, K. R., and Larimer, F. W. (1991) Fragments of the internal transcribed spacer 1 or pre-rRNA accumulate in *Saccharomyces cerevisiae* lacking 5'-3' exoribonuclease 1. *J. Bacteriol.* **173**, 7024–7028

33. Maderazo, A. B., He, F., Mangus, D. A., and Jacobson, A. (2000) Upf1p control of nonsense mRNA translation is regulated by Nmd2p and Upf3p. *Mol. Cell. Biol.* **20**, 4591–4603
34. Griffin, T. J., Gygi, S. P., Ideker, T., Rist, B., Eng, J., Hood, L., and Aebersold R. (2002) Complementary profiling of gene expression at the transcriptome and proteome levels in *Saccharomyces cerevisiae*. *Mol. Cell. Proteomics* **1**, 323–333
35. Gygi, S. P., Rochon, Y., Franza, B. R., and Aebersold, R. (1999) Correlation between protein and mRNA abundance in yeast. *Mol. Cell. Biol.* **19**, 1720–1730
36. Washburn, M. P., Ulaszek, R. R., and Yates, J. R. III (2003) Reproducibility of quantitative proteomic analyses of complex biological mixtures by multidimensional protein identification technology. *Anal. Chem.* **75**, 5054–5061