Delayed Correlation of mRNA and Protein Expression in Rapamycin-treated Cells and a Role for Ggc1 in Cellular Sensitivity to Rapamycin*

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To identify new molecular targets of rapamycin, an anticancer and immunosuppressive drug, we analyzed temporal changes in yeast over 6 h in response to rapamycin at the transcriptome and proteome levels and integrated the expression patterns with functional profiling. We show that the integration of transcriptomics, proteomics, and functional data sets provides novel insights into the molecular mechanisms of rapamycin action. We first observed a temporal delay in the correlation of mRNA and protein expression where mRNA expression at 1 and 2 h correlated best with protein expression changes after 6 h of rapamycin treatment. This was especially the case for the inhibition of ribosome biogenesis and induction of heat shock and autophagy essential to promote the cellular sensitivity to rapamycin. However, increased levels of vacuolar protease could enhance resistance to rapamycin. Of the 85 proteins identified as statistically significantly changing in abundance, most of the proteins that decreased in abundance were correlated with a decrease in mRNA expression. However, of the 56 proteins increasing in abundance, 26 were not correlated with an increase in mRNA expression. These protein changes were correlated with unchanged or down-regulated mRNA expression. These proteins, involved in mitochondrial genome maintenance, endocytosis, or drug export, represent new candidates effecting rapamycin action whose expression might be post-transcriptionally or post-translationally regulated. We identified GGC1, a mitochondrial GTP/GDP carrier, as a new component of the rapamycin/target of rapamycin (TOR) signaling pathway. We determined that the protein product of GGC1 was stabilized in the presence of rapamycin, and the deletion of the GGC1 enhanced growth fitness in the presence of rapamycin. A dynamic mRNA expression analysis of Δggc1 and wild-type cells treated with rapamycin revealed a key role for Ggc1p in the regulation of ribosome biogenesis and cell cycle progression under TOR control. Molecular & Cellular Proteomics 9:271–284, 2010.

Identification of gene expression changes caused by environmental perturbations leads to an understanding of molecular mechanisms involved in cell adaptation. This is especially intriguing in the case of diseased cells and their response to drug treatment. Rapamycin, an antibiotic produced from Streptomyces hygroscopicus (1) and a potent immunosuppresser, has been recently heralded for its anticancer properties (2). Rapamycin and its derivatives (rapalogs), such as the ester of rapamycin, CCI-779 (2), are now showing significant activity against a variety of cancers (3). However, various cell lines exhibit differences in their sensitivity to rapamycin under similar growth conditions (4). The molecular mechanisms underlying this resistance, which limits the therapeutic activities of rapalogs, remain to be elucidated. Rapamycin treatment induces a cell cycle arrest from G1 to G0 phase in eukaryotic cells through inhibition of the protein kinase target of rapamycin (TOR) (5). TOR is highly conserved from yeast to human and promotes cell growth in response to nutrient availability. Therefore, TOR inhibition through rapamycin treatment mimics a nutrient starvation phenotype induced by inhibition of protein synthesis (5), acquisition of thermotolerance (6), autophagy (7), and glycogen accumulation (8). Molecular mechanisms underlying protein synthesis inhibition by rapamycin involve inhibition of translation initiation (5, 8) and ribosome biogenesis (9). The inhibition of translation and activation of autophagy upon rapamycin treatment are conserved in mammalian cells in which the ribosomal kinase S6K and initiation translation factor 4EBP play essential regulatory roles.

The abbreviations used are: TOR, target of rapamycin; NSAF, normalized spectral abundance factors; MudPIT, multidimensional protein identification technology; PLGEM, power law global error model; SAF, spectral abundance factor; AD, area times densitometry; GO, gene ontology; STN, signal-to-noise ratio; PCC, Pearson correlation coefficient; TAP, tandem affinity purification; HCA, hierarchical clustering analysis.

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roles (for a review, see Ref. 10). In yeast, however, not all the molecular components controlling inhibition of ribosome biogenesis and initiation of translation have been identified, and in both yeast and mammalian cells, gaps in the rapamycin/TOR signaling pathway remain to be filled. The use of genome-wide and proteome-wide tools has led to important insights into molecular mechanisms of rapamycin action. Hardwick et al. (11) analyzed the rapamycin-induced gene expression changes in yeast over 2 h of treatment. Bandhakavi et al. (12) analyzed the rapamycin-induced protein abundance changes in yeast after 70 min of treatment and correlated these changes to previous gene expression analyses. However, these studies have been limited in their temporal scope, and it is likely that novel molecular components in the long term response of cells to rapamycin have yet to be discovered.

To gain more insights into rapamycin molecular action, we performed a temporal analysis of gene and protein expression changes in *Saccharomyces cerevisiae* over 6 h of rapamycin treatment. We correlated protein abundance with mRNA expression changes and integrated gene expression patterns with functional profiling. The mRNA abundance changes were analyzed by microarray, and protein abundance changes were analyzed using a quantitative proteomics approach that combines normalized spectral abundance factors (NSAFs) with multidimensional protein identification technology (MudPIT) (13–15). Protein abundance changes were quantified using a power law global error model (PLGEM) recently shown to be an effective statistical tool to analyze NSAF-based proteomics data sets (15). Our goal was to identify additional proteins involved in the rapamycin response and to investigate how temporal changes at the protein level were reflected in changes at the transcriptional level in response to rapamycin treatment, which could give more insights into gene expression regulation during rapamycin treatment.

**EXPERIMENTAL PROCEDURES**

Rapamycin Treatment and Cell Collection—The *S. cerevisiae* strain BY4741 (MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) (16) was grown to middle log-phase (optical density at 600 nm, 0.5–1.0) in rich medium consisting of 100 ml of 10× concentrated BioExpress 1000 medium containing amino acids enriched for the isotopes 16O or 15N (Cambridge Isotope Laboratories, Andover, MA). 0.2 mg/liter uracil; 1.8 g/liter yeast nitrogen base without amino acids and ammonium sulfate, and 2% dextrose. In a fashion similar to Hardwick et al. (11), rapamycin was added to a final concentration of 100 nm, and 500–ml aliquots of cells were collected at 0, 20, 40, 60, 120, 240, and 360 min of treatment. The cells were collected by filtration using a 90-mm nitrocellulose filter of 0.5-m pore size (Whatman) and stored at −80 °C until RNA and proteins were extracted. The effect of rapamycin on cell growth was measured by optical density at 600 nm in untreated and treated cells over 6 h of rapamycin treatment and is shown in supplemental Fig. 1. Rapamycin treatment was performed in four replicated experiments on either 14N- or 15N-grown cells. mRNA and protein abundance changes were analyzed by cDNA microarrays and MudPIT as described below. For comparative gene expression analysis of wild-type and Δggc1 (his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ggc1Δ::kanMX) strains in response to rapamycin, the *S. cerevisiae* strain BY4741 and Δggc1 were grown in 14N and treated as described before. Cells were collected at 1, 2, and 4 h of rapamycin treatment by filtration using a 90-mm nitrocellulose filter of 0.5–μm pore size (Whatman) and stored at −80 °C. RNA was extracted for further cDNA microarrays analysis as described below.

**RNA Extraction, cDNA Microarray, and Affymetrix GeneChip Analysis**—For the large scale temporal analysis of mRNA expression changes during rapamycin treatment, four biological replicates for cDNA microarray analysis of *S. cerevisiae* strain BY4741 over 6 h of rapamycin treatment were generated: *S. cerevisiae* poly(A)+ RNA was extracted from 0.5–1.25 mg of total RNA using the Illustra mRNA purification kit (GE Healthcare, 77-9258-02) or the Oligotex mRNA Midi kit (Qiagen, 70042). Labeling and hybridization were carried out essentially as described previously (17). Hybridization was performed on an in-house array printed using a High Speed Linear Servo Arrayer. Two samples (treated and untreated) at five time points (0, 20, 40, 60, and 120 min) were measured in quadruplicate on two-channel spotted arrays with two normal and two dye swap. The time series was later expanded with 4- and 6-h time points measured in triplicate. Microarray images were acquired with a GenePix 4000B scanner (Axon Instruments, Foster City, CA). For image analysis, SpotReader (Niles Scientific, Portola Valley, CA) or GenePix pro 6.0 software was used (Axon Instruments). Data were processed in R using the limma package from Bioconductor (18). Measurements were transformed by within-array normalization, scaling all arrays to the 75th percentile of green channel intensity across all arrays and then normalizing between arrays using green channel quantiles. Microarray data are available at ArrayExpress (http://www.ebi.ac.uk/microarray-as/ae/) under accession number E-TABM-639.

For cDNA microarrays analysis of *S. cerevisiae* strain BY4741 and Δggc1 treated with rapamycin, yeast total RNA was prepared from 100 ml of cells. RNA was purified using hot acid phenol as described previously (19). Concentration and quality of RNA were determined by spectrophotometer and Agilent bioanalyzer analysis (Agilent Technologies, Inc., Palo Alto, CA). For array analysis, labeled mRNA targets were prepared from 150 ng of total RNA using the MessageAmp III RNA Amplification kit (Applied Biosystems/Ambion, Austin, TX) according to the manufacturer’s specifications. Array analysis was performed using Affymetrix GeneChip Yeast Genome 2.0 Arrays processed with the GeneChip Fluidics Station 450 and scanned with a GeneChip Scanner 3000 7G using standard protocols. Resulting CEL files were analyzed using RNA (20) and limma (18) in the R statistical environment. Affymetrix GeneChip data are available at ArrayExpress (http://www.ebi.ac.uk/microarray-as/ae/) under accession number E-TABM-639.

**Protein Extraction**—Untreated and treated cells from four biological replicates were resuspended in lysis buffer (310 mM sodium fluoride, 3.45 mM sodium orthovanadate, 12 mM EDTA, 250 mM sodium chloride, and 100 mM sodium carbonate) and broken using silica glass beads by 10 cycles consisting of 1 min of vortexing at 2,500 rpm followed by a 30-s incubation at 4 °C. Unbroken cells were removed by centrifugation for 20 min at 4,000 × g at 4 °C. The supernatant was transferred to a 50-ml centrifuge tube, and soluble proteins were separated from the crude membrane fraction by centrifugation for 1 h at 22,000 × g at 4 °C. The supernatant containing the soluble protein extract was collected and stored at −80 °C. Membrane proteins were further extracted as described previously (14). Protein concentration for soluble and membrane protein extracts was determined by bicinchoninic acid (BCA) assay (Pierce). MudPIT analyses were performed on 500 and 70–500 μg, respectively, of total 14N- and 15N-labeled soluble and membrane proteins each mixed at a 1:1 ratio before TCA precipitation.

**MudPIT Analysis**—Protein mixtures were TCA-precipitated, urea-denatured, reduced, alkylated, and digested with endoprotease...
Lys-C followed by modified trypsin digestion (both from Roche Applied Science) as described previously [21]. Peptide mixtures were loaded onto a split phase column connecting a 250-μm-diameter fused silica microcapillary column packed with 5-μm C_{18} reverse phase (Aqua, Phenomenex) and strong cation exchange particles (Partisphere SCX, Whatman) to an 8-cm 100-μm-diameter reverse phase tip column (22). Loaded microcapillary columns were placed in line with a Quaternary Agilent 1100 series HPLC pump and an LTQ linear ion trap mass spectrometer equipped with a nano-LEC electro-spray ionization source (ThermoFinnigan). A fully automated 12-step MudPIT run was performed as described previously (14). Each full MS scan (from 400 to 1600 m/z range) was followed by five MS/MS events using data-dependent acquisition where the five most intense ions from a given MS scan were subjected to CID.

**MS/MS Data Processing and Data Filtering—**RAW files were extracted into m2 file format (23) using RAW_Xtract (24). Proteins were identified by database searching using SEQUEST software (25). Briefly, no enzyme specificity was imposed during searches, setting a mass tolerance of 3 amu for precursor ions and of 0 amu for fragment ions. In all searches, cysteine residues were considered to be fully carboxamidomethylated (+57 Da statically added). No variable modifications were searched. Tandem mass spectra were searched against a database containing 14,176 protein sequences combining 6,911 S. cerevisiae proteins (from the National Center of Biotechnology Information March 3, 2006 release), 177 common contaminants such as keratin and immunoglobulins, and their corresponding 7,088 decoy sequences (National Center for Biotechnology Information March 3, 2006 release). The lists of detected peptides and proteins were sorted and selected using DTASelect (26) with the following criteria set: spectra/peptide matches were only retained if they had a DeltaCn of at least 0.1; minimum XCorr of 1.5 for singly, 2.5 for doubly, and 3.0 for triply charged spectra; and maximum Sp rank of 10. In addition, peptides had to be fully tryptic and at least 7 amino acids long. Peptide hits from multiple runs were compared using CONTRAST (28) and contrast-report (27). Proteins that were subsets of others were removed using the parsimony option in DTASelect (26). Proteins that were identified by the same set of peptides (including at least one peptide unique to such protein group to distinguish between isoforms) were grouped together, and then one accession number was considered as representative of each protein group such that only non-redundant proteins are reported. The false discovery rate was calculated as the number of spectra matching randomized peptides multiplied by 2 and divided by the total number of spectra as described before (28, 29); it ranged between 0.16 and 0.32 for all MudPIT runs.

Protein abundances were estimated using NSAF values calculated from the spectral counts of each identified protein (14). To account for the fact that larger proteins tend to contribute more peptide/spectra, spectral counts were divided by protein length to provide a spectral abundance factor (SAF). SAF values were then normalized against the sum of all SAF values in the corresponding run, allowing the comparison of protein levels across different runs. No particular thresholds or outlier removal steps were applied prior to SAF calculation. SAFs were calculated separately for the 14N and 15N spectral count values extracted from the merged data set using an in-house program, NSAF_NRatios.exe (Tim Wen). The peptide and protein lists and their NSAF values are provided in supplemental Table 1, A and B. For subsequent statistical analysis, all data sets were further processed to retain proteins that were identified in at least three replicate experiments.

**Chemical Genomic Profiling—**The deletion library of 62 MATα haploid deletion strains were grown at 30 °C to middle log phase (optical density at 600 nm, 1.0) in 200 μl of YPD + G418 (200 mg/liter). The plates were then transferred to new 96-well microtiter plates, and six 10-fold serial dilutions were performed using the Span-8 pod of the Biomek FX with YPD as the diluent. Each serial dilution plate was immediately pinned in quadruplicate onto one YPD – rapamycin Omnitray and one YPD + rapamycin (100 nm final concentration) using Singer RoTor HDA (Singer instruments). Plates were incubated for 4 days at 30 °C and scanned using a UMAX power lock 1120 scanner (Amersham Biosciences). Area and densitometry (AD) values were generated by processing growth plate images with the digital image processing software Axiovision (Carl Zeiss). Two replicate experiments were performed. One AD value was attributed to each dilution (1–6), and the sum of the six AD product was used to compare growth fitness between each deletion strain and the wild-type strain (BY4741) with and without rapamycin. The growth fitness difference was measured by -fold change ratio as follows:

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\text{ADsum(mutant)/ADsum(BY4741)}_{rapa} = \frac{\text{ADsum(mutant)}}{\text{ADsum(BY4741)}}_{rapa}
\]

To determine a significant growth defect in response to rapamycin treatment, statistical analysis by two-way analysis of variance was applied on the ADsum ratios of deletion strains and wild-type strain BY4741 with and without rapamycin from two replicated experiments. Strains were then considered significantly sensitive or resistant if affected by drug treatment at a maximum p value threshold of 0.1.

**Statistical Analyses—**For the PLGEM analysis, NSAF data sets were imported into the R environment for statistical computing, and the Bioconductor package plgem (15, 30) was used to fit a PLGEM to the individual data sets, evaluate the goodness of fit of the model to the data, and detect differentially abundant proteins. The PLGEM fit to each data set is provided in supplemental Fig. 2, and PLGEM analysis of the quantitative proteomics data set is provided in supplemental Table 2. To quantify yeast growth fitness, we defined “AD ratio” as the ratio of the AD values for each ORF to the AD value for wild type on the same plate. AD ratios eliminate plate to plate variation and were used as response variables in the analysis. We performed two-way analysis of variance to test whether there was significant interaction between the ORF and drug as follows: y_{ijk} = μ + ORF_i + drug_j + ORF_i × drug_j + e_{ijk} where y_{ijk} is the AD ratio for the ith ORF, jth drug treatment, and the kth experiment for i = 1, 2, ..., 200, j = 1, 2 indicating with/without drug treatment, and k = 1, 2; μ is the overall mean; and ORF, drug, and ORF × drug, respectively, represent ORF, drug, and their interaction effect. Significant ORF by drug interaction effect (p value <0.1) indicated that ORFs have a different growth response to drug treatment. We further tested growth difference between wild-type and deletion strains with and without drug treatment for each ORF. The Benjamini-Yekutiel multitest correction method (31) was applied to control the false discovery rate. Clustering analyses were performed in MeV 4.0 (32), and gene ontology (GO) enrichment term analyses were performed using the FATIGO+ function in babelomics (33).

**Construction of Tandem Affinity Purification (TAP)-tagged Ggc1 in Yeast—**The protein Ggc1 was TAP-tagged following the procedure of Puig et al. (34). The TAP tag containing two IgG binding domains of Staphylococcus aureus Protein A, a tobacco etch virus protease cleavage site, and calmodulin binding peptide (558 bp) and a selection marker URA3 (804 bp) from Kluyveromyces lactis were fused to the GGC1 ORF (903 bp) at the C-terminal end to maintain expression.
of Ggc1p at its native level. The native GGC1 gene was replaced by the TAP-tagged GGC1 by homologous recombination into the genome of S. cerevisiae. The DNA fragment used for this recombination, containing the TAP tag, the C-terminal part of Ggc1 ORF, and a region of similarity of the yeast genome, was generated by PCR using the plasmid pBS1539 as a template (34) and the following primers (Integrated DNA Technologies): forward, 5′-AACGTGTCTTCTCATTTTGCGT'TAGCTCAATTTGATACCTGATAACCTGTCGTCGAAATCCTAGGAAAAGAACTG-3′; and reverse (complement), 5′-TATTGTTATACATTTATATTCTACAAGCTGAATGCCAAGGAATAGAACTAGTAACTTAGG-3′. The forward primer was specific to the C-terminal section of Ggc1 (without stop codon), whereas the reverse primer was specific to the yeast genome (region after the stop codon of Ggc1 ORF) and the TAP tag marker cassette. The resulting PCR fragment of 2.2 kb was purified and transformed into S. cerevisiae strain BY4741 competent cells. Recombinant strains containing the TAP-tagged GGC1 were selected on synthetic defined medium depleted for uracil (SD – Ura).

The mRNA abundance changes of ~6,000 ORFs were measured over 6 h of rapamycin treatment and resulted in the repression of 366 genes and induction of 291 genes at a minimum threshold of 2-fold change. Genes changing in abundance were clustered based on -fold change and sorted by cellular function (supplemental Fig. 3). During 2 h of treatment, rapamycin repressed genes encoding ribosomal proteins and nucleotide biosynthesis enzymes (supplemental Table 3 and Fig. 3, group 1) and induced expression of genes involved in detoxification, energy regeneration, and autophagy (supplemental Table 3 and Fig. 3, group 7). These changes at early time of rapamycin treatment, especially the inhibition of cytosolic ribosomes gene expression, are in good concordance with earlier reports (11, 37). Moreover, gene expression analysis at 4 and 6 h of rapamycin treatment specifically showed a decrease in expression of genes involved in lipid metabolism (supplemental Table 3 and Fig. 3, group 9) and an increase in expression of genes involved in amino acid and vitamin biosynthesis and components of the respiratory chain complex IV (supplemental Table 3 and Fig. 3, groups 3 and 8).

Protein abundance changes in response to rapamycin were analyzed in data sets containing a total of 773, 733, 706, 737, 692, 701, and 670 proteins identified respectively at 0-, 20-, 40-, 60-, 120-, 240-, and 360-min time points in treated and untreated conditions. NSAF values were calculated from spectral counts generated from MudPIT runs and used as a measure of relative protein abundance within protein mixtures (14) (supplemental Table 1A). To identify protein abundance changes, we used the statistical method PLGEM previously shown to significantly improve the analysis of NSAF data sets and allow for automated data analyses (15). PLGEM fit equally well on all data sets with correlation coefficient values ranging from 0.85 and 0.99 and adjusted R² values >0.98 (supplemental Fig. 2). The signal-to-noise ratio (STN), incorporating the standard deviation calculated from PLGEM, was used to compare protein abundance changes between treated and untreated samples at any given time point and over the entire time course of the experiments (supplemental Table 2). The number of proteins changing in abundance at each time point was calculated at a maximum p value threshold of 0.01. Most of the significant changes were observed at 4 and 6 h of rapamycin treatment, indicating that rapamycin-induced protein abundance changes do not occur immediately after rapamycin is added (Fig. 1). Among the 85 proteins identified as significantly changing in response to rapamycin, 29 and 56 were identified as decreasing and increasing in abundance, respectively (Fig. 1).

To identify cellular effects induced by rapamycin treatment, proteins with changes in abundance in response to rapamycin were sorted by cellular function. Proteins whose abundance decreased were mostly involved in translation, including 84% of cytosolic ribosomal proteins (Table I and Fig. 2). This supports the fact that inhibition of protein synthesis through translation is one of the main inhibitory effects induced by rapamycin. Nucleotide and lipid metabolism might also be affected as suggested by the decrease in abundance of nucleotide biosynthesis (Ade17 and Ura1) and inositol metabolism enzymes (Ino1) (Table I). Protein synthesis was clearly inhibited; therefore, it was surprising to observe that among the total number of proteins differentially expressed 66% or 56 proteins were increasing in abundance. Proteins whose level increased belonged to diverse cellular processes (Table I and Fig. 2A). Therefore, the observed changes in abundance, either decreasing or increasing, were specific for a certain type of cellular function (Fig. 2A). This specificity was also observed for cellular localization where mitochondrial proteins, vacuolar proteins, and punctate composite proteins were enriched in the list of proteins whose abundance in-
creases, whereas proteins whose abundance decreases were mostly cytoplasmic (Fig. 2B).

As observed by microarray analysis (Ref. 11 and supplemental Table 3), we confirmed at the protein level the inhibition of ribosomal protein synthesis and the activation of the tricarboxylic acid cycle, autophagy, heat shock, and retrograde responses (Fig. 1 and Table I). These conclusions support the reliability of our method to perform large scale quantitative proteomics analysis. Moreover, we specifically observed at the protein level changes of proteins...
involved in vesicle-mediated transport or endocytosis, drug export, regulation of cellular osmolarity, and mitochondrial genome maintenance (Fig. 1 and Table I). It was very striking to observe that most of the changes at the protein level occurred at late time of rapamycin (4 and 6 h), whereas mRNA expression of most of the genes changed rapidly.
after rapamycin was added (20 min) (Fig. 3A). To gain more insights into gene expression regulation during rapamycin treatment, we investigated how protein abundance changes correlated with mRNA expression changes over the time course of rapamycin treatment.

Temporal Comparison of mRNA and Protein Abundance Changes in Response to Rapamycin Treatment—The mRNA and protein abundance changes were compared over 6 h of rapamycin treatment and at each time point. For an accurate temporal comparison, genes were retained only if identified at each individual time point and in at least three replicated experiments. A total of 328 mRNA-protein pairs were then analyzed whose expression ratios (supplemental Table 4A) are illustrated in Fig. 3A. The Pearson correlation coefficient (PCC) was used to measure the correlation between mRNA and protein expression changes. Initially, the correlation became higher as a function of time where the PCC increased from 0.03 at the zero time point to 0.58 at 4 h of rapamycin treatment (Fig. 3B). However, there was a temporal delay in the highest correlation of mRNA and protein expression changes. Protein abundance at 6 h of treatment was best correlated with mRNA changes at 1 and 2 h of rapamycin treatment with a PCC of 0.60 (Fig. 3B). We conclude that in the context of rapamycin-treated cells protein abundance changes correlated the highest with mRNA changing at early time points of treatment with a delay of 3–5 h.

To identify in which cellular functions this delayed correlation would apply, gene expression ratios were compared with protein expression ratios at the single gene level. After clustering analysis, 12 patterns of correlation between mRNA and protein expression changes were defined (supplemental Fig. 4 and Table 4B). This supports the fact that the relation between mRNA and protein expression changes is not linear, including patterns of gene and protein expression changing either in the same direction or in opposite directions. For ribosomal proteins and genes encoding nucleotide biosynthesis enzymes, mRNA and protein abundance changes were both found to decrease in abundance and were highly correlated in a delayed fashion (Fig. 3C, group 4, and supplemental Table 4B, group 4). The same delayed abundance correlation was observed for proteins involved in retrograde response (Dld3p), heat shock response (Hsp104p), or proteolysis process (Pep4p) whose abundance increased (Fig. 3C, group 1, and supplemental Table 4B, group 1). However, for some proteins, the protein abundance had a higher increase than mRNA changes (supplemental Fig. 4, group 3) or was not correlated with an increase of mRNA expression (Fig. 3C and supplemental Fig. 4, groups 6, 10, and 12). This was the case for mitochondrial proteins involved in retrograde response (Idh1p, Idh2p, and Aco1p), vesicle-mediated transport (Gdi1p), and drug export (Pdr5p) (supplemental Table 4B). Among this category of proteins, we also observed enrichment for mitochondrial, punctate composite, or cell periphery proteins (Fig. 4B).

After rapamycin was added (20 min) (Fig. 3A). To gain more insights into gene expression regulation during rapamycin treatment, we investigated how protein abundance changes correlated with mRNA expression changes over the time course of rapamycin treatment.

**Fig. 2.** Cellular functions of proteins involved in rapamycin response. Proteins that were significantly changing in abundance at a p value threshold of 0.01 are sorted by biological process (GO terms)(A) and cellular localization (B) (Yeast GFP (green fluorescent protein) Fusion Localization Database). Proteins decreasing and increasing in abundance are colored in blue and yellow, respectively. In both panels, the number of proteins is shown on the y axis, and either biological process or cellular localization is shown on the x axis.

Some of these proteins and their associated cellular functions
are described for the first time as being involved in the rapamycin response. They could represent new molecular components promoting or resisting rapamycin action and illustrate the significance of performing temporal proteomics analyses in tandem with mRNA expression analyses.

Chemical Genomic Profiling of Deletion Strains for Proteins Involved in Rapamycin Response—To determine whether these proteins might be functionally relevant for rapamycin-mediated effects and whether abundance changes confer rapamycin resistance or sensitivity, the growth fitness of strains deleted for the 85 genes cited above was tested in the absence or presence of rapamycin. By correlating protein abundance changes with rapamycin sensitivity of the deletion strains, we could determine whether proteins would promote or act against the drug effect. Because 23 of these gene deletions were lethal, we compared the growth of a total of 62 viable deletion strains with the wild-type strain BY4741 (Fig. 5 and supplemental Fig. 5 and Table 5). Strains were considered resistant or sensitive if they grew more or less, respectively, than the wild-type strain in the presence of rapamycin. Of the four strains that lacked genes encoding ribosomal proteins, cells were either more resistant (Rps1b and Rpp2a) or sensitive (Rpl12b and Rps8a) to rapamycin than the wild-type strain (Fig. 5 and supplemental Table 5B). This confirmed
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![Graph A](image1.png)

**Fig. 4.** Number of proteins and their localization within different patterns of correlation between mRNA and protein abundance changes. **A,** the number of proteins identified with the 85 proteins identified from the PLGEM analysis (y axis) were sorted by pattern of mRNA and protein abundance changes (x axis) in which mRNA is down-protein is down, mRNA is up-protein is up, mRNA is up-protein is not changing, mRNA is not changing, mRNA is down-protein is not changing. **B,** localization of proteins belonging to patterns of correlation between mRNA and protein abundance changes in which mRNA is down-protein is down (blue), mRNA is up-protein is down (green), mRNA is up-protein is up (orange), mRNA is not changed-protein is up (red), and mRNA is up-protein is up (dark red). Prot, protein; down, decreasing in expression or abundance; up, increasing in expression or abundance; no change, no change in expression or abundance.

The main role of ribosomes and protein translation in the rapamycin response but also opened the question of different roles for ribosome constituents in response to rapamycin treatment. Although most of the ribosomal subunits decreased in abundance, Rpl40a was shown to be more abundant in treated conditions (Fig. 1).

Among the 56 proteins whose abundance increased in response to rapamycin, 49 viable strains were tested. Among the 17 strains resulting in a significant phenotype, one strain deleted for a gene encoding the vacuolar protease Pep4, involved in autophagy, conferred rapamycin sensitivity. Although the abundance of Pep4 increased in response to rapamycin treatment (Fig. 5), we suggest that autophagy might be involved in rapamycin resistance where Pep4 might play an important role. However, 95.5% of these strains conferred resistance to rapamycin, including genes encoding mitochondrial proteins (Idh1, Idh2, Cit1, Mdh1, Por1, Atp5, and Ggc1), drug export (Pdr5), or proteolysis (Cpr1) (Fig. 5 and supplemental Table 5B). Therefore, cellular processes such as retrograde and heat shock responses, proteolysis, and mitochondrial genome maintenance are essential to promote rapamycin action or, in other terms, confer rapamycin sensitivity.

The two gene deletions that resulted in the greatest increase in resistance to rapamycin growth inhibition were *atp5*, a component of the mitochondrial F1F0 ATP synthase (38), and *GGC1*, a mitochondrial GTP/GDP carrier (39). The deletion of *atp5* and *ggc1*, respectively, resulted in 3.5- and 2.8-fold better growth fitness than wild type in the presence of rapamycin (*Atp5*: p value = 4.14 × 10^{-31}; *Ggc1*: p value = 3.53 × 10^{-31}; Fig. 5 and supplemental Table 5B). *GGC1* proved to be particularly interesting, first because of the increased growth fitness of the *ggc1* mutant but also for the anticorrelation of mRNA and protein expression changes. At the mRNA level, its expression remained unchanged until 2 h of rapamycin treatment where it began to decrease from a ratio of treated/untreated of 0.51 at 2 h to a ratio of 0.22 at 6 h (supplemental Table 3). Quantitative PCR demonstrated that the ratio of *GGC1* mRNA in treated/untreated cells was 0.32 ± 0.16 (data not shown), validating the microarray analysis. In contrast, the protein Ggc1p was barely detected with few spectral counts until 4 and 6 h of rapamycin treatment where it increased in abundance, Rpl40a was shown to be more abundant in treated conditions (Fig. 1).
Fig. 5. Integration of expression changes patterns with functional genomics analysis and Ggc1 protein stability analysis. A, growth of deletion strains significantly affected in their growth in response to rapamycin at a p value threshold of 0.1. Growth fitness was compared at six different dilutions A–F where A was undiluted and B–F ranged from $10^{-2}$ to $10^{-6}$ dilution factors starting with an optical density at 600 nm between 0.8 and 1.2 (10-fold dilution series from A–F). Two technical replicates of each dilution are shown in each panel A–F. B, correlation of mRNA and protein abundance changes and deletion strain phenotype of the 85 proteins significantly changing in abundance by PLGEM at a p value threshold of 0.01. The mRNA and protein expression ratios were compared with the -fold change difference in growth between deletion strain and wild-type strain (between rapamycin-treated and untreated conditions). Values were clustered using the HCA function in MeV 4.0 by Pearson correlation. mRNA and protein decreasing, increasing, or not changing in abundance are illustrated in blue, yellow, and black, respectively. Genes highlighted in red were statistically significant in difference of growth fitness compared with the wild type in the presence of rapamycin. Note that the expression changes are measured by a ratio. Therefore, a decrease in expression is represented by a ratio below 1. C, Ggc1-TAP was grown to an A$_{600}$ of 0.6, and cells were treated for the indicated time in the presence of 35 µg/ml cycloheximide ± 100 nM rapamycin. Cell extracts from each time point were analyzed by Western blot analysis for Ggc1-TAP and Pgi (indicated with arrows).
Rapamycin and Temporal mRNA and Protein Expression Changes

![Gene expression change analysis of wild-type and Δggc1 cells in response to rapamycin treatment.](image)

Fig. 6. Gene expression change analysis of wild-type and Δggc1 cells in response to rapamycin treatment. Gene expression changes were analyzed between wild-type and Δggc1 cells treated with or without rapamycin (100 nM) at 0, 1, 2, and 4 h. The figure shows a colored map of log2 expression ratios from genes significantly changing in ggc1 mutant compared with wild type in the presence of rapamycin. Values ranging from −3 to +3 are gradually colored from blue to black to yellow as indicated by the scale and clustered using Euclidian distance using the HCA function in MeV. Genes that were up-regulated were ribosomal genes or genes involved in ribosome biogenesis, whereas down-regulated genes were mostly involved in cell cycle progression, mitosis, and meiosis. ECCB, early cell cycle box.

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\( p = 5.67 \times 10^{-6} \) at 4 h of rapamycin treatment (Fig. 6). These results demonstrate that deletion of GG\( C1 \) first allows cells to derepress ribosomal genes despite the presence of rapamycin, thus potentially allowing for continued ribosomal activity and subsequently causing changes in the expression of genes involved in cell cycle progression. These results suggest a novel role for Ggc1 in the long term response of cells to rapamycin.

**DISCUSSION**

To gain more insights into rapamycin molecular action, previous studies had performed rapamycin-induced gene expression changes in yeast over 2 h of treatment (11) and rapamycin-induced protein expression changes in yeast after 70 min of treatment and correlated these protein expression changes to previous gene expression analyses (12). In this study, we carried out a temporal analysis of mRNA and protein expression over 6 h of rapamycin treatment and integrated gene expression changes with functional profiling. We have shown that the best correlation of mRNA and protein expression occurred in a temporally delayed fashion where the highest correlations were measured between 1- and 2-h mRNA expression changes and the 4- and 6-h protein expression changes. There are relatively few studies attempting to analyze large scale mRNA and protein expression, and these studies have found similar partial positive correlations of mRNA and protein expression (41–44). However, in contrast to our study, prior studies have not addressed mRNA and protein expression in a temporal fashion where multiple time points were analyzed for both mRNA and protein expression. We have especially demonstrated that incorporating the time factor when correlating mRNA and protein abundance changes can have a major impact on the correlation factor. In our study, a delay of 3–5 h was observed between correlated expression of mRNA and protein changes. This delay might be needed for proteins to be degraded or to increase in abundance, suggesting also that rapamycin effects mediated through protein activity might not be immediate after rapamycin addition. This was especially the case for ribosome biogenesis, heat shock, retrograde response, and autophagy. Those gene expression changes are essential to promote cell cycle arrest and a nutrient starvation phenotype causing rapamycin cellular sensitivity (Ref. 5 and our study). Their late protein abundance changes could directly be linked to the time needed for rapamycin to exert its effects at the cellular level. For example, cell growth inhibition induced by rapamycin treatment was first observed after 4 h of rapamycin treatment (supplemental Fig. 1). Barbet et al. (5) have also shown that the arrest of the cell cycle from G\( S \) to G\( 0 \) phase (1n DNA content) induced by rapamycin treatment was visible after 4 h of rapamycin treatment.

We also found instances where mRNA changes and protein changes were not correlated, e.g. where protein expression did change but mRNA did not change. Among the total number of proteins changing in abundance during rapamycin treatment, 66% increased in abundance, and half of these were not correlated with increased mRNA expression. Among these proteins, 50% were localized to mitochondria, cell periphery, or punctate composite. Specific examples included mitochondrial proteins involved in genome maintenance (Ggc1), osmotic stability (Por1), and drug export (Pdr5). The question arises on how protein abundance increases whereas protein synthesis is inhibited. There are several possible explanations, which include the differential regulation of mRNA expression, stability, and degradation as compared with the protein expression, stability, and degradation. In the case of Ggc1, this protein was more stable in the presence of rapamycin than in its absence. This difference in degradation rates could explain the detected increase in Ggc1 protein levels at 4 and 6 h in rapamycin-treated cells. The Ggc1 protein abundance is not necessarily increasing in rapamycin-treated cells, but it is decreasing in untreated cells. This then results in a ratio that demonstrates higher levels of Ggc1 protein in rapamycin-treated cells when compared with untreated cells.

Toward gaining additional insight into the molecular action of rapamycin, we demonstrated the importance of Ggc1 in regulating ribosomal and cell cycle gene expression in rapamycin-treated cells. Deletion of GG\( C1 \) increased by 3-fold the growth fitness in the presence of rapamycin. The deletion of GG\( C1 \) in the presence of rapamycin derepressed some ribosomal as well as ribosome biogenesis genes followed by changes in expression of cell cycle genes. This let us suggest that Ggc1 is a key player in the regulation of ribosome biogenesis and cell cycle progression in rapamycin-treated cells. Because Ggc1 is a GTP/GDP carrier essential for mitochondrial genome maintenance (39), it may be required for mitochondrial protein synthesis and DNA metabolism. Because deletion of GG\( C1 \) resulted in ribosomal gene expression derepression and changes in expression of cell cycle genes, Ggc1p could play a role in coordinating protein synthesis and cell cycle progression or be involved in what has been recently called the metabolic checkpoint during cell cycle progression (45). Schieke et al. (46) have described a similar phenomenon where mitochondrial bioenergetics was regulating G\( S \) phase cell cycle progression under TOR control in mammalian cells. Owusu-Ansah et al. (47) also described a role for mitochondrial dysfunction in the G\( S \)/S cell cycle transition in Drosophila melanogaster. Future studies investigating how Ggc1 activity and mitochondrial genome maintenance could be related to ribosome biogenesis and cell cycle progression may provide important additional insights into the mechanism of action of rapamycin and on the coordination of cell metabolism and cell cycle progression under TOR control.

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