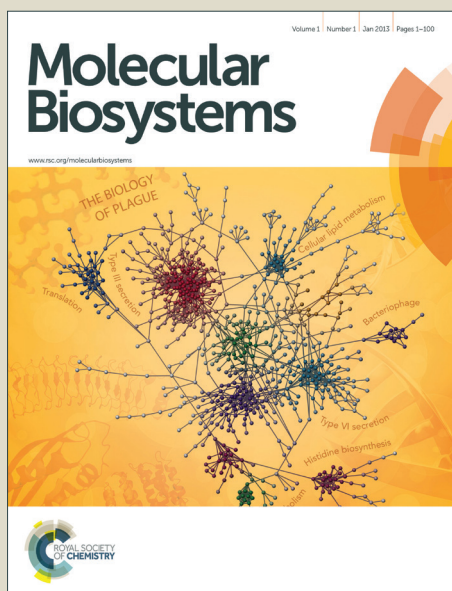


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A global investigation of gene deletion strains that affect premature stop codon bypass in yeast, *Saccharomyces cerevisiae*

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Abstract

Protein biosynthesis is an orderly process that requires a balance between rate and accuracy. To produce a functional product, the fidelity of this process has to be maintained from start to finish. In order to systematically identify genes that affect stop codon bypass, three expression plasmids pUKC817, pUKC818 and pUKC819, were integrated into the yeast non-essential loss-of-function gene array (~5000 strains). These plasmids contain three different premature stop codons (UAA, UGA and UAG, respectively) within the *LacZ* expression cassette. A fourth plasmid, pUKC815 that carries the native *LacZ* gene was used as a control. Transformed strains were subjected to large-scale β -galactosidase lift assay analysis to evaluate production of β -galactosidase for each gene deletion strain. In this way 84 potential candidate genes that affect stop codon bypass were identified. Three candidate genes, *OLA1*, *BSC2*, and *YNL040W*, were further investigated, and were found to be important for cytoplasmic protein biosynthesis.

Introduction

Protein biosynthesis has been extensively studied over the past few decades. Although much has been learned, details of the genes that regulate and influence this process require further investigation. The list of new genes that affect protein biosynthesis continues to grow.¹⁻³ Recent large-scale investigations suggest that there exist other uncharacterized factors that influence protein biosynthesis.¹⁻⁴

To produce functional proteins, the accuracy of protein biosynthesis is tightly regulated from start to finish. Errors in start codon selection during initiation, misincorporation of amino acids during elongation or inability of the ribosome to detect a stop codon during translation termination can alter the integrity of the protein biosynthesis process.

Efficient termination is an important step in ensuring accuracy of the synthesized products.⁵⁻⁶ A key determinant of the precision of translation termination is the ability of release factors to correctly recognize the stop codons. Reduced cellular levels of eukaryotic release factor 1 (eRF1) has been linked to higher stop codon bypass.⁷ Similarly premature stop codon mutants of *SUP45* codes for eRF1, are reported to exhibit increased frequency of stop codon bypass.⁸ Nonsense suppressor tRNAs can also increase the bypass of stop codons by incorporating amino acids into a growing polypeptide chain when a stop codon is reached.⁹

In the current study, we used the yeast non-essential gene knockout (also known as loss-of-function) collection to identify genes that affect stop codon bypass. To this end, 84 candidate genes were identified that once deleted, increased the synthesis of full length protein directed from an mRNA with premature stop codon. Many of these genes have unknown functions. We further studied the activity of three of these genes, *OLA1*, *BSC2* and *YNL040W*.

Materials and Methods

Strains

The Yeast deletion and overexpression set (*Saccharomyces cerevisiae*, mating type “a” (BY4741)) were used for large-scale investigation. Yeast mating type “α” (BY7092) was used for secondary gene knockout.¹⁰ DH5α strain of *Escherichia coli* was used to propagate plasmids.¹¹

Media and Antibiotics

Standard rich (YPD) and synthetic complete (SC) media were used as growth media for yeast. LB (Lysogeny broth) was used to growth *E.coli*. Antibiotics were used in the following concentrations: Paromomycin (18 mg/ml) and Cycloheximide (45 ng/ml) for drug sensitivity analysis; G418, Geneticin (200 mg/ml), ClonNat, Nourseothricin, (100mg/ml) and Ampicillin (50 mg/ml) for selective growth media.^{1, 10, 12}

Plasmids

Expression plasmids pUKC817, pUKC818 and pUKC819, contain premature stop codons UAA, UGA and UAG, respectively, within the LacZ expression cassette; pUKC815 has no premature stop codon and was used as a control.¹²⁻¹³ These plasmids were used to transform in to the yeast non- essential gene knockout collection using a modified synthetic genetic array analysis approaches^{10,12}. Translation rate was assessed using plasmids p416 (Gal-inducible LacZ expression cassette) and pAM6 (CoCl₂-inducible LacZ expression cassette) as described by.^{12,14-15} pAG25 was used as a template in PCR reactions to propagate NAT resistance gene.

Gene knockout

Gene knockout was performed via homologous recombination by using the LiAc-based transformation method described by.¹⁶ In this approach gene of interest is removed and replaced with a NAT antibiotic resistant marker. Knockout strains were confirmed using colony PCR analysis (Supplementary file 1) and were used for small-scale follow-up experiments.

Primers

All primers used in this study have been described in supplementary file 1.

qRT-PCR

High quality extracted RNA were converted to cDNA using iscript cDNA synthesis kit (Biorad) according to manufacturer's instruction. Quantitative PCR was performed using iQSybergreen master-mix kit (Biorad) according to manufacturer's instruction. qPCR amplification and detection was performed on a Rotor Gene 3000 (Corbett Research). Thermo cycler conditions were set to the following: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 30s-60°C for 30s-72°C for 30s and a final 72°C for 10 min.¹⁷⁻¹⁸ PGK-1 is used as a positive control in real time PCR experiments.¹⁹

Genetic material extractions

Total RNA was extracted using RNeasy Mini Kit (QIAGEN) according to manufacturer's instruction. Plasmids were extracted from *E.coli* using pure link quick plasmid kit (Invitrogen) and from yeast using yeast plasmid kit (Omega Bio-Tek) according to manufacturer's instruction.

β -galactosidase assay

A large-scale β -galactosidase assay using X-gal was carried out as described.²⁰⁻²² The final incubation step was continued until an average of ~ 5 colonies per plate (approximately 384 colonies) turned blue (approximately 45 minutes). Quantitative β -galactosidase assay was performed using ONPG (O-nitrophenyl- α -D-galactopyranoside) as described by.^{13,23} The rate of protein biosynthesis was measured using inducible reporter systems p416 and pAM6^{12,15}. Yeast strains transformed with expression plasmids were grown to OD₆₀₀: 0.8-1 and induced by addition of either 2% galactose (p416) or 40 μ M cobalt chloride (pAM6) for 4 hours. β -galactosidase activity was then quantified.

Spot test (drug sensitivity analysis)

Yeast cells were grown in liquid media to mid-log phase and diluted to an OD₆₀₀ of 0.01, 15 μ l of this suspension and three subsequent 10 fold dilutions were plated onto solid media containing translation inhibitory drugs (paromomycin and cycloheximide). Media with no antibiotics was used as a control. All plates were incubated at 30°C for 1-2 days in 3 replications.

Synthetic genetic array and phenotypic suppression array analyses

Synthetic genetic array (SGA) analysis was performed on the basis of yeast haploid double gene deletion mutant growth defects. Colony size is measured as the basis for a growth deficiency. The query genes OLA1, BSC2 and YNL040W were replaced with the nourseothricin-resistance (NAT) marker forming gene deletion strains in the MAT α strain, BY7092. The deletion strains were separately crossed to two target sets of deletion mutant arrays from the MAT α BY4741 library. One of the sets contained gene deletion mutants for protein biosynthesis genes (selected on the basis of GO terms) and the other contained deletion mutant for random genes, minus those in set one, and used as a control. After a few rounds of selection using G418 + NAT selective media, haploid double mutant progeny were obtained and analyzed for growth defects using colony size measurements.²⁴⁻²⁵ The experiment was repeated three times and those interactions that were found in at least two experiments were considered for confirmation using random spore analysis.¹⁰ Phenotypic suppression array (PSA) analysis was performed as described by.^{1,26}

Results and Discussion

Identification of gene deletions that affect expression of β -galactosidase gene with premature stop codons

In order to identify novel genes involved in stop codon bypass (read-through) three plasmids, pUKC817, pUKC818 and pUKC819 that carry different premature stop codons, UAA, UGA and UAG, respectively within a β -galactosidase reporter gene were used to transform into the yeast non-essential gene deletion array (~ 5000 strains). The plasmid pUKC815 which carries the native β -galactosidase gene (used as a control) was also transformed for a total of approximately 20,000 strain transformations. Systematic transformation was accomplished by the modification of a large-scale mating approach that was originally designed to study high throughput genetic interactions in yeast.¹⁰ In this method, a query

yeast strain of “ α ” mating type carrying a selectable marker (in this case URA selection derived from a plasmid) is crossed with the gene deletion array strains of “ a ” mating type. After several rounds of selection, gene deletion strains of “ a ” mating type carrying the selectable marker of the query strain are selected. In this way, an array of strains each carrying a specific gene deletion together with a target plasmid is generated. The arrayed colonies are tested for their ability to produce functional β -galactosidase using a large-scale colony lift assay on the basis of X-gal hydrolysis. The premature stop codon within the β -galactosidase gene would prevent the production of full length functional β -galactosidase. Under high-fidelity, the premature stop codon is recognized, mediating the synthesis of a short non-functional polypeptide. Only when the premature stop codon is bypassed, a full length β -galactosidase protein is produced. Deletion of genes that affect premature stop codon recognition would allow the bypass of stop codons and result in increased production of full length β -galactosidase. Figure 1 illustrates a representative β -galactosidase array analysis where blue colonies are those that produced higher levels of functional β -galactosidase, recognizable by visual inspection. Each lift assay was repeated 3 times and those colonies that were detected in 2 or 3 experiments were selected. In this way 84 gene deletion strains were identified that produced blue color for one or more of the plasmids with premature stop codons.

To confirm that our large-scale screening correctly identified those colonies that produced functional β -galactosidase, we alphabetically ordered and selected the first, tenth, twentieth, thirtieth, ... deletion strains (9 in total) to follow up analysis using a small scale (low-throughput) quantitative liquid based assay on the basis of ONPG hydrolysis. We observed that 7 of 9 gene deletion strains produced levels of functional β -galactosidase, which were statistically higher than that produced by a control strain. This suggests an overall sensitivity of approximately 78% for our screen. The list of the genes that were identified in our screen is found in supplementary file 2. Among these we find a number of expected genes such as *RPL31A* that codes for large ribosomal subunit protein L31A, *NAM7* that codes for Upf1 protein required for efficient termination at nonsense codons, *NOP58* that codes for Nop58 protein involved in pre-rRNA processing, etc.

We subjected the gene deletion strains identified via our large-scale screening to sensitivity analysis against two antibiotics, cycloheximide and paromomycin. Cycloheximide interferes with the translocation step of protein biosynthesis^{1,27} and paromomycin has been linked to codon misreading.²⁸ Using serial dilution spot test analysis, decreasing cell concentration of the 84 target strains were subjected to sub-inhibitory concentrations of drugs; 45 ng/ml of cycloheximide and 18 mg/ml of paromomycin. Approximately 69% of the identified gene deletion strains showed sensitivity to one or both drugs (supplementary file 2). This compares to approximately ~26% of the entire set of yeast non-essential gene deletion strains that show sensitivity to at least one of these drugs.^{1,12}

To improve understanding of the activity of the identified genes, we performed Gene Ontology (GO) enrichment analysis to cluster these genes on the basis of the function or the cellular process in which they participate. GO analysis indicates that, 19% of our selected genes are involved in protein biosynthesis pathways (P-value: 2.40E-8), 12% are involved in regulation of nucleic acids synthesis/stability (P-value: 3.51E-02), 7% are involved in cell wall/membrane synthesis (P-value: 3.33E-02), 6% are involved in sulfate synthesis (P-value: 5.72E-06) and 5% are involved in signal transduction (P-value: 1.07E-02). In addition 31% of the genes were found to have either unknown function and/or unknown cellular process in which they participate. Some of the clustering observed here might be expected. It is not surprising to detect protein biosynthesis related genes. Mutations in these genes can influence the overall process of translation, and hence the ability of the cell to correctly recognize different codons. Similarly, deficiency in regulation of nucleotide stability through nonsense-mediated mRNA decay could explain an increase in protein expression from an mRNA template with premature

stop codon, by preventing the mRNA from degradation. The less expected categories are cell wall/membrane synthesis, sulfate synthesis and signal transduction. A cross-communication between protein biosynthesis and cell wall/membrane might be explained by a proposed regulation of translation initiation fidelity which is mediated by stress.²⁹ Similarly, very recently cell wall integrity has been connected to protein biosynthesis through the activity of eukaryotic translation initiation factor 5A (*eIF5A*).² Sulfur modification of translation machinery has been linked to fidelity of translation³ and hence can support an enrichment of sulfate pathway genes among those that affect stop codon bypass. Lastly, enrichment for signal transduction genes may be explained by the activity of signal transduction pathway in the control of eukaryotic protein biosynthesis.³⁰

Little information is known about the remaining 31% of the genes. The list of these includes *OLA1*, a conserved gene whose product is homologous to a sub-family of A(G)TPases linked to translation in human cells,³¹ *BSC2*, an uncharacterized gene that was previously found to contain a region (± 50 nucleotides surrounding the stop codon) that promotes stop codon bypass³² and *YNL040W*, an uncharacterized open reading frame. Knockout mutant strains for *OLA1*, *BSC2* and *YNL040W* genes showed sensitivity to both cycloheximide and paromomycin (Figure 2). Re-introduction of these genes into their corresponding gene knockout strains reversed their sensitive phenotypes (Figure 2). We further investigated the activity of these genes.

***OLA1*, *BSC2* and *YNL040W* affect protein biosynthesis**

Observed influence of gene deletions for *OLA1*, *BSC2* and *YNL040W* on premature stop codon bypass was confirmed using low-throughput β -galactosidase assay on the basis of ONPG hydrolysis. As indicated in Figure 3A, deletion of each of the target genes increased the level of β -galactosidase expression derived from all three expression cassettes carrying different premature stop codons. The observed differences were comparable to those for *rp131a Δ* strain used as a positive control. Alteration in mRNA content can also explain differences in gene expression. To examine this possibility, the content of β -galactosidase mRNAs for the gene deletion strains was investigated using qRT-PCR analysis. No statistically significant difference between the content of mRNAs for the target and control strains were observed (Figure 3B). This indicates that the observed increase in expression of β -galactosidase mRNAs with premature stop codons appears to be at the protein biosynthesis level and not at the mRNA content level.

Integrity of protein biosynthesis is maintained through a balance between the accuracy and the rate of translation. Next we asked whether deletions of *OLA*, *BSC2* and *YNL040W* had any effect on the rate of translation. This was evaluated using two inducible β -galactosidase expression systems (p416 and pAM6). It was observed that deletion of *YNL040W* significantly reduced the rate of protein biosynthesis measured by β -galactosidase expression (Figure 4A). Interestingly, however, deletion of *OLA1* and *BSC2* increased the level of protein biosynthesis. *rp131a Δ* was used as a positive control. As a possible source of alteration of gene expression, the content of β -galactosidase mRNA was investigated as above. The content of β -galactosidase mRNA was found to be constant in all gene deletion strains suggesting that the observed differences appear to be independent of mRNA content (Figure 4B). Considering that deletions of *OLA1*, *BSC2* and *YNL040W* affected the stop codon bypass (above), one way to explain these results is that *OLA1* and *BSC2* may participate in maintaining a balance between the quantity and quality of protein biosynthesis. Deletions of *OLA1* and *BSC2* increased the rate of protein biosynthesis, but decreased the ability of the cell to recognize stop codons. The effect of *YNL040W* deletion seems to be more general, reducing both the rate of translation and recognition of premature stop codons.

To better investigate the involvement of *OLA1*, *BSC2* and *YNL040W* in protein biosynthesis we, studied the genetic interactions they made with other genes that influence translation. It is possible that

alterations in the expression of two genes result in a phenotype that is not easily explained in light of the phenotypes caused by alteration of individual gene expressions. In this case, the two genes are said to form a genetic interaction.^{10,33} Genetic interactions can be used to investigate functional association between two genes. The most commonly studied form of genetic association is explained by negative genetic interactions.^{10,12,25} In a given pathway “A”, deletion of a target gene “A1” may have a negligible phenotypic consequence due to the presence of a parallel pathway “B” that compensates for the absence of “A1” gene product. Similarly, deletion of a gene in pathway “B” can be compensated by pathway “A”. However, deletion of two genes, one in pathway A (for example, gene “A1”) and the other in pathway B (for example, gene “B2”), can leave both pathways inactive and hence cause a phenotypic consequence (synthetic sick phenotype) that cannot be explained by the phenotypes for individual gene deletions for genes “A1” and “B2” alone. In this way, genes “A1” and “B2” are described to form a negative genetic interaction. These interactions can provide insight into a higher level association between gene functions. In this manner, function(s) of uncharacterized genes may be studied by the genetic interactions that they make with other genes with known functions.^{12,25,34} To this end, we investigated the synthetic sick interaction that *OLA1*, *BSC2* and *YNL040W* formed with two sets of 384 gene deletion strains. Set one contained an array of deletion mutant strains for genes associated with the process of protein biosynthesis and set two contained strains with deletion mutations for random genes (excluding those involved in protein biosynthesis), used as a control. To study negative genetic interactions, gene deletion strains for target genes were generated in an “ α ” mating type strain. Then, the query strains were crossed with the array of gene deletion strains of “ a ” mating type. After a few rounds of selection, double gene deletion mutants in “ a ” mating type were selected. In this way 768 double gene deletion mutants were generated for each target gene. The fitness of double gene deletion strains were quantified and analyzed by colony size measurements.²⁴⁻²⁵ To improve coverage, we combined our interaction data with those previously reported (<http://drygin.cbr.utoronto.ca>). A complete list of interactions is presented in supplementary file 3. Illustrated in Figure 5A, *OLA1* formed negative interactions with a number of interesting genes including ribosomal protein encoding genes (for example, *RPL12A* and *RPL15B*), *GCN1* that codes for a positive regulator of translation initiation factor eIF2 by Gcn2, and *EFT1* that codes for elongation factor 2 involved in ribosomal translocation. Further clustering of the interacting genes into different functional categories on the basis of GO terms showed enrichment for 4 clusters. The most significant enrichment belonged to genes involved in regulation of translation (P-value: 2.27E-07). This was followed by ribosome biogenesis (P-value: 4.37E-05), amino acid metabolism (P-value: 1.26E-03) and RNA binding (P-value: 5.11E-03). Similarly, *BSC2* interacted with a number of protein biosynthesis related genes; clustering of the interacting partners showed enrichment for genes involved in regulation of translation (P-value: 8.94E-04) (Figure 5B). *YNL040W*s interactors were enriched for ribosome biogenesis gene (P-value: 1.21E-02) (Figure 5C). Re-introduction of *OLA1*, *BSC2* and *YNL040W* into a representative set of corresponding double mutant strains, reversed the sick phenotype observed for the double mutant strains (Supplementary file 4).

Next we examined the ability of the overexpression of *OLA1*, *BSC2* and *YNL040W* genes to compensate the phenotypes of different gene deletion mutants in response to cycloheximide treatment. For this, phenotypic suppression analysis (PSA) was used. This is a similar approach to the genetic interaction analysis method above with the exception that a compensatory effect of the overexpression of a target gene is sought. If the overexpression of a target gene compensates a phenotype caused by the absence of another gene, a functional link between the two genes is assumed.^{1,12,26} To this end, the strains in the two gene deletion arrays described above (one for protein biosynthesis related genes and the other random) were separately transformed with overexpression plasmids for *OLA1*, *BSC2*, and *YNL040W*, in

addition to an empty plasmid. Gene deletion strains along with those transformed with overexpression plasmids were grown in the presence of a sub-inhibitory concentration of cycloheximide (45 ng/ml). Those gene deletion strains that showed sensitivity to the drug treatment, but whose sensitivity was compensated by one of the target overexpressed genes, were identified on the basis of colony size measurement. Positive hits were confirmed using spot test analysis (Figure 6). It was observed that overexpression of *OLA1* gene compensated for sensitivity to cycloheximide caused by deletions of *RPS11B*, *RPS8A* and *CPA1* genes. *RPS11B* and *RPS8A* code for small ribosomal subunit proteins S11 and S8, respectively. S11 forms part of the mRNA exit tunnel and S8 is part of a bridge between 40S and 60S subunits.³⁵ *CPA1* codes for carbamoyl phosphate synthetase involved in arginine biosynthesis. *BSC2* overexpression compensated for gene deletion for *RPS24B* which codes for small ribosomal subunit S24B. Overexpression of *YNL040W* compensated for the deletions of *DEP1* and *SIN3*, both of which code for components of Rpd3L histone deacetylase complex shown to affect the expression of rDNA genes.³⁶ The phenotypic compensations observed here further connect the activity of the target genes to protein biosynthesis.

Little information is available about the activity of yeast Ola1 protein. Here, we show that deletion of *OLA1* can increase premature stop codon bypass. It also increased the rate of protein biosynthesis measured by an inducible expression system. *OLA1* formed negative genetic interactions with a number of protein biosynthesis related genes most notably those involved in translation regulation. Its overexpression also rescued phenotypes for *RPS11B*, *RPS8A* and *CPA1*, further connecting Ola1 activity to protein biosynthesis. An involvement for Ola1 in protein biosynthesis is supported by its expression data from microarray analysis.³⁷⁻³⁸ The group of proteins with the most similar profile of expression to Ola1 protein are highly enriched for cytoplasmic translation genes (P-value = 1.29E-8). These observations are in agreement with what is known about human Ola1 (hOla1) protein. It is characterized as a member of obg family compromising a group of ancient A(G)TPases that belong to translation factor (TRAFAC) proteins.³¹

Bsc2 is another protein of unknown function. Its genomic region is reported to contain a section with a premature stop codon compatible with the Bypass of Stop Codon (BSC) mode of expression.³² Here, we report that its deletion increased the rate of premature stop codon bypass. Consequently, it appears that the Bsc2p may function in negative regulation of premature stop codon bypass and hence regulate its own expression. To our knowledge this is a unique mode of regulation of eukaryotic gene expression and demands further investigation. Interestingly, deletion of Bsc2 also increased the rate of protein synthesis further connecting the activity of Bsc2 to regulation of translation. This is further supported by negative genetic interaction data where *BSC2* interacted with a number of genes involved in regulation of translation.

YNL040W codes for a putative protein of unknown function with similarity to bacterial alanyl-tRNA synthetase. Here, we show that deletion of *YNL040W* increased the rate of premature stop codon bypass and reduced the rate of protein synthesis connecting its activity to the process of protein biosynthesis. The genetic interaction data further links *YNL040W* to ribosome biogenesis.

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Figure 1: A representative yeast gene deletion colony array that was subjected to β -galactosidase lift assay. Blue colonies indicate the presence of higher levels of β -galactosidase produced from an mRNA that carries a premature stop codon.

Figure 2: Drug sensitivity analysis. Gene knockout mutant strains for *OLA1*, *BSC2* and *YNL040W* showed sensitivity to both cycloheximide and paromomycin. Re-introductions of the deleted genes into the corresponding gene knockout mutants reversed the observed sensitivity. Yeast cells were grown to mid-log phase and diluted to an OD_{600} of 0.01, 15 μ l of this suspension and three subsequent 10 fold dilutions were plated.

Figure 3: A) β -galactosidase expression from templates that contain premature stop codons. Constructs pUKC817, pUKC818 and pUKC819 contain premature stop codon UAA, UGA and UAG, respectively, within *LacZ* expression cassette. pUKC815 contains native *LacZ* gene. β -galactosidase expression derived from constructs with premature stop codons (pUKC817, pUKC818 and pUKC819) are normalized to β -galactosidase expression from a native *LacZ* gene (pUKC815) and related to that of a control strain. B) β -galactosidase mRNA content analysis. β -galactosidase mRNA contents are related to those for the control strain. *PGK1* mRNA content was used for normalization*(P-value \leq 0.05).

Figure 4: A) Translation rate measurement. Translation rate was measured using two β -galactosidase reporter expression cassettes, p416 and pAM6, under the transcriptional control of inducible Gal1 and LORE promoters, respectively. The values are normalized to that of a control strain. B) β -galactosidase mRNA content analysis. β -galactosidase mRNA content was related to that of *PGK1* for the above inducible β -galactosidase constructs. *(P-value \leq 0.05).

Figure 5: The union (from this study and literature) of synthetic sick interactions for *OIA1* (A), *BSC2* (B) and *YNL040W* (C). *OLA1* interacted with genes that contain the following GO terms: regulation of translation (P-value: 2.27E-07), ribosomal biogenesis (P-value: 4.37E-05), amino acid metabolism (P-value: 1.26E-03) and RNA binding (P-value: 5.11E-03). B) *BSC2* interacted with gene involved in regulation of translation (P-value: 8.94E-04). C) *YNL040W* interacted with ribosomal biogenesis genes (P-value: 1.21E-02). Degree of sickness is color coded. * Represents interactions that were included from literature.

Figure 6: Representative spot test confirmation for phenotypic suppression analysis of *OLA1*. Gene deletion mutants for *RPS11B*, *RPS8A* and *CPA1A* show sensitivity to 45 ng/ml of cycloheximide. Overexpression of *OLA1* compensates the observed sensitivity. Yeast cells were grown to mid-log phase and diluted to an OD_{600} of 0.01, 15 μ l of this suspension and three subsequent 10 fold dilutions were plated.

