HIGH-RESOLUTION PHENOTYPIC PROFILING OF A EUKARYOTIC RIBOSOME

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High-resolution phenotypic profiling of a eukaryotic ribosome

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To Åsa and Elias, who matter most.

ABSTRACT

All living cells contain ribosomes, complex macromolecular assemblies of ribonucleic acid (RNA) and proteins, responsible for synthesizing polypeptides in a process called translation. High-resolution three-dimensional ribosomal structures coupled with biochemical studies have paved the way in understanding the various mechanistic events during protein synthesis. However, a systematic study dealing with the individual components and features of the ribosome is lacking. Specifically, the functional connection of each ribosomal protein and individual ribosomal RNA (rRNA) modification to cellular processes outside the domains of translation has not been fully explored.

Using the yeast *Saccharomyces cerevisiae* as a model eukaryotic organism, I investigated the contribution of individual ribosomal proteins and rRNA modifications to cellular fitness during growth in optimal and stress environments. I performed high-resolution phenotypic profiling on isogenic yeast strains with individual deletions in 110 cytoplasmic ribosomal protein (cRP) genes, 67 mitochondrial ribosomal protein (mRP) genes and 65 small nucleolar RNA genes (snoRNA). SnoRNAs facilitate site-specific rRNA modifications hence, their removal results to the absence of modification on the corresponding target site on the rRNA. I utilized a high-throughput phenotyping approach wherein the growth behaviors of individual deletion mutants were monitored in liquid micro-culture environmental arrays. Three physiologically-relevant growth variables, growth lag, growth rate and growth efficiency, extracted from high-resolution growth curves allowed for the precise quantification of marginal phenotypic consequences of deletions not detectable by standard, more qualitative approaches.

Detailed analysis of growth dynamics in a plethora of perturbed environments revealed a surprising range of hitherto unknown phenotypic diversity in the ribosome ranging from extreme sensitivity to extreme resistance in essentially every tested environment. Identification of dominant trends in the chemogenetic landscape allowed for the separation of the deletion mutants into distinct stress sub clusters, resolving the phenotypes into effects on different aspects of cellular physiology. Notable was the identification of phenotypes in all the sets of deletion mutants (cRP, mRP and snoRNA knockouts) exhibiting varying degrees of sensitivity to alternative carbon sources, strongly linking many of them to respiratory functions. The mix of cRP, mRP and snoRNA deletion mutants in some stress-specific sub cluster, such as the oxidative stress sub cluster, also indicated greater than expected functional overlap. The functional specialization within the yeast ribosome revealed a new level of translational complexity suggesting the existence of compositionally distinct ribosomes which are customized according to environmental cues. The direct participation of some ribosomal protein components and snoRNAs in cellular stress response was also envisioned. The implication of these hypotheses is significant not only in the basic understanding of the inner workings of the ribosome but also in providing new avenues in explaining the underlying mechanisms in the growing number of human diseases linked to defective components of the translational machinery.

PAPERS

This dissertation is based on the following papers, referred to by Roman numerals in the text:

I. **Esguerra J**, Warringer J, Blomberg A. (2008) Functional importance of individual rRNA *2'-O*-ribose methylations revealed by high-resolution phenotyping. *RNA*. 14:649-656. Epub 2008 Feb 6.

II. **Esguerra J**, Mak R, Warringer J, Boone C, Nislow C, Blomberg A. (2008) High-resolution phenotypic profiling of non-essential box C/D and box H/ACA snoRNA gene deletion mutants. Manuscript.

III. **Esguerra J**, Warringer J, Blomberg A. (2008) Functional specialization in the eukaryotic ribosome. Submitted.

IV. **Esguerra J**, Warringer J, Sunnerhagen P, Blomberg A. (2008) General translational features of mutants lacking stress-specific classes of cytoplasmic ribosomal proteins. Manuscript.

Other papers not included but referred to in this thesis:

V. Piccinelli P, **Esguerra J**, Blomberg A, Samuelsson T. (2006) *Hunting for non-coding RNA genes in yeast.* In Computational identification of non-coding RNAs (PhD Thesis). Paul Piccinelli. Institute of Biomedicine at Sahlgrenska Academy, Göteborg University.

CONTENTS

ABBREVIATIONS

cRP	cytoplasmic ribosomal protein
mRP	mitochondrial ribosomal protein
RNA	ribonucleic acid
mRNA	messenger RNA
rRNA	ribosomal RNA
snoRNA	small nucleolar RNA
SSU	small subunit
LSU	large subunit
RPS	ribosomal protein small subunit
RPL	ribosomal protein large subunit
r-proteins	ribosomal proteins
PTC	peptidyl transferase center

PREFACE

Almost a decade and a half ago, a classic parable about a retired biochemist and a retired geneticist relates the story of how the latter would tie the hands of workers in a car factory to understand the inner workings of cars. [1] In the morning, the geneticist would randomly select a worker and tie up his hands. Then later on the day, he would go up a hill and observe the cars going out of the assembly line while he sips his afternoon beer. This way not only he could figure out the function, for example of the steering wheel (when one day he saw the cars piling up on the lawn), but could also deduce which worker was being responsible for mounting a specific part of the car. The story of course was biased against the biochemist, whose methods in learning about the functioning of cars, was described by the geneticist to consist of grounding up hundreds of cars into pieces, identifying and determining the proportion of the basic components and then remix the fractions in trying to reproduce some aspects of the car's functioning.

A year later after the publication of the said essay, a rebuttal obviously penned by a pissed biochemist appeared in the same newsletter, re-telling the parable of the two retirees, but this time from the retired biochemist's point of view. In the new parable, the geneticist almost died of car accident because he thought that one of the workers whose hands he tied up was indispensible in the smooth running of the car. Of course, it turned out later that the said worker was responsible for installing the car's seat belt.

These parables told by people from their own camps echo the seemingly disparate approaches and merits of genetics and biochemistry in the old days. Although the first story started off as a lighthearted way of a genetics professor to convince his undergraduate students about the superiority of genetic approaches over those of biochemistry, it nevertheless highlights the misgivings in failing to recognize the importance of integrating together knowledge from various fields to come up with a holistic understanding of life itself.

Today, we are witnessing an era of schizophrenic endeavors in the biological sciences, where researchers in the field find it harder and harder how to categorize themselves. A trained molecular biologist like me, performing chemo-genetic experiments and who calls the bioinformatics room his second home (the lab bench being the first) where data analyses are performed, is typical of the present day lab workhorses *a.k.a.* PhD students and post-docs.

This thesis is about hundreds of "cars", each one lacking a unique part in one of the most central components of the engine. The said parts ensure the car to run optimally, whether it is on the *autobahn* or whether it is traversing the roughest roads. What we found, among

[1] Genetics Society of America Newsletter, April 30 1993. GENErations Vol. 1, No. 3. Editor's Note: William Sullivan described the Triumph of Genetics over Biochemistry through a parable entitled "The Salvation of Doug."

others, is the strengthening of previous observations suggesting that these parts are in fact components of the turbo compartment, which is engaged most especially while road conditions are favorable and are disengaged when conditions get tough. “Test-driving” our cars in a plethora of road conditions, we surprisingly found patterns in their behavior indicating that those parts may be involved in some way or another in circuitries outside the engine ensuring the optimal running of the car in different road conditions.

Now, for fear of analogy breakdown as inevitably happens in such kind of ponderings, allow me to shift my gear into the jargons of the field.

AIMS AND FINDINGS IN BRIEF

Capitalizing on the sensitivity of our phenotypic profiling methodology in detecting marginal fitness defects, I endeavored to unravel novel cellular roles and functional links among the components and features of yeast ribosomes by providing quantitative chemogenetic data for a large set of deletion mutants concerning the cytoplasmic and to some extent, mitochondrial translational machineries.

The first task was to contribute in the unmasking of the elusive function of cytoplasmic ribosomal RNA modifications, believed to be the "fine tuners" of the protein synthesis machinery, and take snapshots of their potential roles in a wider physiological context. To achieve this, we ventured out in **paper I** with our phenotypic profiling of a limited number of box C/D snoRNA gene deletion mutants. Site-specific nucleotide modifications in the cytoplasmic rRNA are facilitated by snoRNA molecules. We extended our investigations in **paper II**, to include both box C/D and box H/ACA snoRNA in our near-complete set of snoRNA deletions mutants. The latter was also aimed at contributing to the Saccharomyces Deletion Project by constructing "bar-coded" snoRNA knockout strains in the same genetic background as the genome-wide protein-coding gene deletion collection.

In **paper III**, we profiled deletion knockouts of the non-essential genes encoding proteins annotated (in the Gene Ontology project) as structural components of the cytoplasmic and mitochondrial ribosomes. We uncovered distinct features in the chemogenetic landscape identifying previously known functional links and potentially novel stress-specific connections in the components of ribosomes.

Finally in **paper IV**, we investigated general translational features of deletion mutants lacking stress-specific classes of yeast cytoplasmic ribosomal proteins.

All in all, our quantitative phenotypic data provided evidence for an extensive phenotypic diversity within the eukaryotic ribosome, suggesting a high-degree of functional specialization in various stressful environments. This functional specialization revealed a new level of translational complexity indicating the existence of compositionally distinct ribosomes which are customized according to environmental cues and/or implies the direct participation of some ribosomal proteins and snoRNAs in cellular stress response.

BACKGROUND

If I have seen further it is by standing on ye shoulders of Giants.
Sir Isaac Newton

1. YEAST AS A MODEL ORGANISM

The first ever appearance of the budding yeast, *Saccharomyces cerevisiae* in a scientific setting happened when one curious Dutch fellow, by the name of Antony van Leeuwenhoek examined a drop of fermenting beer in a thin glass tube assembled in one of his many ingenuous single-lens contraptions (Leeuwenhoek 1680).

> *"I have made several observations with regard to the yeast formed by beer and have constantly seen that it consists of globules floating in a clear substance, which I took to be beer. I also saw quite distinctly that each globule of yeast in its turn consisted of six distinct globules and that it was of the same size and form as the globules of our blood…Some of them seemed to be quite round, while others were irregular, some of them were larger than the others and seemed to consist of 2, 3 or 4 of these particles joined together. Others, again, consisted of 6 globules forming a perfect globule of yeast. Although I was eager to see this coagulation of globules, I have not succeeded in spite of my efforts."* [2]

But mistakenly thought as mere constituents of the grain which was used to make the wort, it was indeed an unfortunate judgment to make in that very first microscopic description of the budding yeast; for it would take a century and a half more, when three independent pioneers, Cagniard-Latour, Kützing and Schwann, would ascribe alcoholic fermentation to living and dividing yeast cells (Barnett 1998).

Nonetheless, three hundred and twenty years hence, these "minute globular particles" have proven to be of great utility in relieving our insatiable thirst, not only for that fermented liquid which "make glad the heart of man" (Huxley 1893), but much more in finding answers to many of our most fundamental biological inquiries. Indeed the humble baker's yeast had made great strides, from being the workhorse of fermentation and food industries, to being one of the most valuable "tools" in biological discourse.

[2] Original letter in Dutch, dated 14 June 1680. Translated and annotated into English by a committee of Dutch scientists in *The Collected Letters of Antoni van Leeuwenhoek* (1948). Amsterdam, Swets and Zeitlinger, Ltd. Vol. III p. 245-251.

UNDERSTANDING HUMAN DISEASE GENES USING YEAST

The conservation of fundamental biochemical principles across the domains of life has made yeast one of the most important genetic model systems. In fact there are many human disease genes with functional homologues in yeast, specifically genes involved in heritable mitochondrial diseases (Steinmetz, Scharfe et al. 2002; Perocchi, Mancera et al. 2008). Not only are molecular mechanisms of these mitochondrial diseases being elucidated in yeast but drug screening has also been performed (Schwimmer, Rak et al. 2006). Yeast has also recently been used as a drug discovery platform in Huntington's and Parkinson's diseases (Outeiro and Giorgini 2006).

A striking number of considered rare (<1 person in 2000, US population) diseases have been found to be "translational" diseases, that is they involve defective components of the translational apparatus (Scheper, van der Knaap et al. 2007). For instance, mutations in at least three ribosomal proteins, *RPS19, RPS24* and *RPS17* have been found in patients with the blood disease Diamond-Blackfan anaemia (DBA), a congenital erythroid aplasia characterized by severe anaemia (Cmejla, Cmejlova et al. 2007; Morimoto, Lin et al. 2007; Choesmel, Fribourg et al. 2008). Interestingly, another disease arising from defective erythroid differentiation, the 5 q^- syndrome has been ascribed to the partial loss of function of another cytosolic ribosomal protein gene, *RPS14* (Ebert, Pretz et al. 2008).

Not only defects in "gross" ribosomal components have been found to be linked with human diseases, but also defects in the "finer" details of the ribosome such as rRNA modification. For instance, dyskerin which is the rRNA pseudouridylating enzyme when mutated has been linked to X-linked dyskeratosis congenita, a disease characterized by skin and bone marrow failure (Heiss, Knight et al. 1998; Ruggero, Grisendi et al. 2003; Yoon, Peng et al. 2006).

The fact that translation is a much conserved cellular process makes the elucidation of molecular mechanisms involved in these diseases possible in yeast. In **papers I, II** and **III**, we profiled deletion knockouts of the structural components of the cytoplasmic and mitochondrial ribosomes, and snoRNA knockouts, many of which have human homologs. We uncovered a previously unknown phenotypic diversity possibly linking these ribosomal features to new levels of translational control via changes in the ribosome or to processes outside translation.

YEAST IN FUTURE RESEARCH

To date, yeast is the best studied model organism, and has always been in the forefront of cutting edge technologies in the life sciences. Not only was yeast the first sequenced eukaryotic genome (Goffeau, Barrell et al. 1996), it was also the first genomic test bed of cDNA microarray technology, which allowed for the simultaneous analysis of the expression of thousands of genes (Lashkari, DeRisi et al. 1997). Indeed yeast has been leading the way in all the fields of "omics" of the post-genomics era, from transcriptomics and proteomics, to metabolomics and phenomics.

Currently with the advent of systems biology, the integration of massive amount of biological data has been of prime value. Therefore given the pioneering status of yeast in many facets of data-generating technological breakthroughs, it is not surprising that it is again paving the way in this exciting and very promising field (Mustacchi, Hohmann et al. 2006).

2. YEAST FUNCTIONAL GENOMICS

GENOME-WIDE STRAIN COLLECTIONS

One of the most important tools in the large scale analyses of the functions of genes and proteins in yeast is the collection of different strains harboring either "bar-coded" gene deletions (Winzeler, Shoemaker et al. 1999; Giaever, Chu et al. 2002) or gene constructs aimed at further elucidation of the biochemical functions of the gene products, such as those which can be used for complex purifications (Gavin, Bosche et al. 2002), determination of protein levels (Ghaemmaghami, Huh et al. 2003) and protein localizations (Huh, Falvo et al. 2003). Such collections are very important resources when performing parallel bioassays as they ensure uniform and well-defined genetic backgrounds.

Although the absence of genetic constructs involving non-coding RNA genes (ncRNA) in collections designed for protein-coding genes is understandable, their absence in genome-wide knockout collections is unfortunate. The yeast genome harbors close to 100 non-coding RNA genes (not including the tRNA and rRNA genes) comprised of the spliceosomal RNA genes, small nucleolar RNA genes, RNA components of the nuclear RNAse P and a small number of miscellaneous ncRNA genes with novel functions such as SRG1 RNA which regulates the transcription of its neighboring *SER3* gene (Martens, Laprade et al. 2004).

Moreover, there are at least two ncRNA genes with unknown functions such as the *RUF5* gene identified via computational methods (McCutcheon and Eddy 2003) and *RNA170* gene which is transcribed by RNA polymerase III (Olivas, Muhlrad et al. 1997). The inadvertent exclusion of these non-coding RNA genes in genome-wide phenotypic screens, such as in synthetic gene array (SGA) analysis (Tong, Evangelista et al. 2001; Tong, Lesage et al. 2004) or in a high-resolution phenotyping screen in salt stress (Warringer, Ericson et al. 2003) led to missed opportunities in unraveling their potentially novel functional networks.

In **paper II**, we successfully constructed 65 snoRNA gene deletion strains in the genetic background of the BY-strain series to complement the existing protein-coding gene deletion collection of the *Saccharomyces* Genome Deletion Project (Winzeler, Shoemaker et al. 1999). All mutants have also been labeled with sequence barcodes to enable large-scale microarray-based competition assays (Pierce, Davis et al. 2007; Yan, Costanzo et al. 2008). This will be an invaluable resource towards a more complete functional characterization of the yeast genome.

YEAST PHENOMICS

The genomic era saw the exponential accumulation of biological sequence data while functional characterization of the actual corresponding genes and their products lagged behind. The post-genomic era therefore saw the need to develop large scale bioassay methodologies, which in turn spawned the birth of various “omics”, aimed at elucidating the functional and regulatory networks of gene products *en masse.*

Phenomics compared with the other “omics” has an entirely different operational definition. Whereas transcriptomics deals with transcripts, proteomics deals with proteins and metabolomics deals with metabolic products, phenomics has no defined set of bio-molecules in which it is focused upon. Instead, it deals with identifying and integrating the diverse characteristics of the whole organism (phenotypes) observed under some administered genetic and/or environmental perturbations. The ultimate aim of phenomics is therefore the mapping of the complex relationship between genotype and phenotype of an organism in a defined environment.

A phenomics screen involves the scoring of phenotypic consequences of gene deletions or mutations. In yeast, a classical way to monitor phenotypes is to visually compare the growth

characteristics of the mutant versus the wild type in solid media. Such qualitative and rather subjective approach is straightforward and works very well, provided that the growth defects are clearly distinguishable. Indeed this is still the most widely used technique in yeast genetics when investigating a limited number of mutant strains. The so called drop test, where yeast populations are serially-diluted and plated on solid media is quite effective in demonstrating the effects of mutation with respect to the applied environmental perturbation. This approach has in fact been used as a final scoring method to identify 100 novel genes affecting yeast resistance to an immunosuppressive drug in a phenotypic screen involving 4787 knockout strains derived from the BY4742 genetic background (Desmoucelles, Pinson et al. 2002).

Robotics enabled phenotypic screening on solid media to be more expeditious by "pinning" cells on high-density ordered arrays of hundreds of colonies in a single plate. In one of the very first applications of the technique, close to 5000 viable gene deletion mutants were screened for sensitivity towards the DNA-damaging agent methyl methanesulfonate, identifying 103 genes required for growth in the said agent (Chang, Bellaoui et al. 2002). Despite the success of solid media-based phenotypic scoring in genome-wide phenotypic screens, the inherent subjectivity in judging colony sizes poses a problem for mutations conferring very subtle phenotypic effects. Indeed, many genes when deleted failed to produce easily identifiable phenotypes on plates (Oliver, van der Aart et al. 1992). Thus the main limitation of qualitative phenotypic assays on solid media plates is its lack of sensitivity to detect very small fitness benefit of apparently non-essential genes to cellular growth. Such genes are widespread in the yeast genome and the finding that many genes may never be essential for survival regardless of environmental condition but instead make small fitness contributions, led to the formulation of the "marginal benefit" hypothesis (Thatcher, Shaw et al. 1998).

To unravel marginal fitness phenotypes, quantitative approaches had to be devised. The "bar coding" technique, wherein knockouts were tagged with two unique 20-mer sequences serving as strain identifiers, allowed for a large number of deletion strains to be pooled together and their growth characteristics analyzed in parallel (Winzeler, Shoemaker et al. 1999). Essentially, the relative abundances of each strain in such competition experiment were measured by first amplifying the tags using fluorescently-labeled universal primers and then subsequently hybridizing them to high-density arrays containing the complementary

sequences. Relative growth rates for each strain could then be derived from the signals in the array data, providing quantitative fitness measure for each deletant. In the first screen of the near-complete set of yeast knockout mutants, the bar coding approach was able to uncover many previously unidentified phenotypes both in optimal growth conditions and in other six well-studied stress environments (Giaever, Chu et al. 2002).

In **papers I-IV**, the yeast phenotypic profiling approach employed, involved parallel micro-cultures of isogenic single gene knock-out mutants screened in a wide variety of environmental insults affecting a wide-range of cellular targets. This liquid-based culturing permitted high-resolution monitoring of the behavior of the growing cell population via optical measurements every twenty minutes. Relevant quantitative physiological parameters namely, lag time, rate and efficiency of growth (cell density reached) were extracted from the growth curves (Fig 1) (Warringer and Blomberg 2003; Warringer, Anevski et al. 2008). The preciseness and utility of this phenomics methodology has been established in previous studies (Warringer, Ericson et al. 2003; Warringer, Ericson et al. 2005; Ericson 2006; Osterberg, Kim et al. 2006; Warringer, Anevski et al. 2008), demonstrating its ability to uncover already known, and more importantly, marginal phenotypes with regards to specific cellular pathways and stress protection systems in the yeast cell.

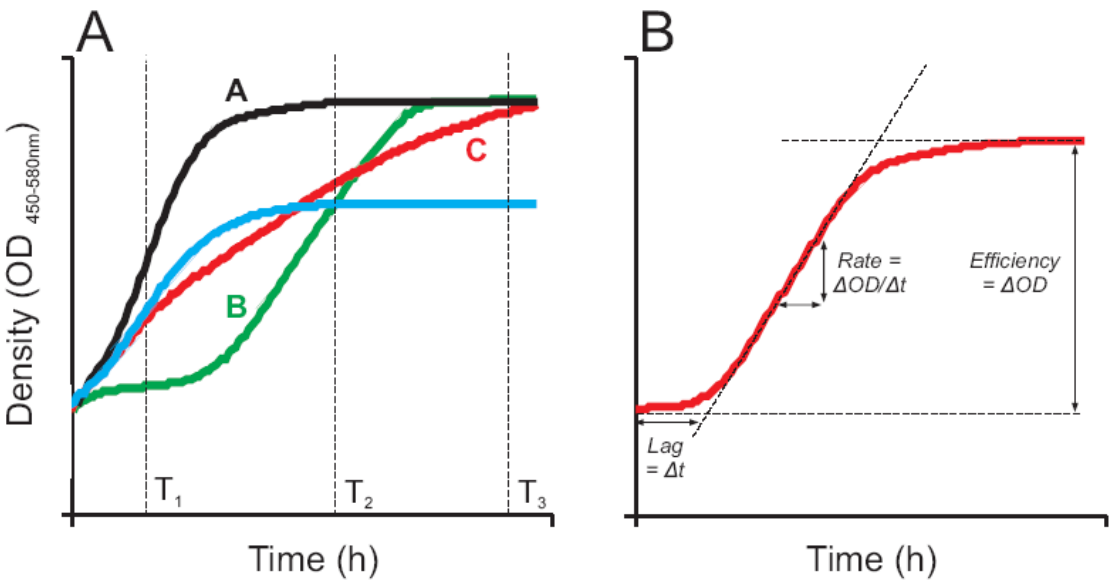

Figure 1. Phenotypic profiling via extraction of quantitative growth variables from high-resolution growth curves. A) Wild type yeast grown in different environments. Curve A represents no stress growth, while the other curves represent growth behavior affecting different aspects of growth dynamics. Classical phenotyping only considers composite growth at any time, T, which may not encapsulate specific features of cell physiology. B) The definition of the three quantitative variables derived from the high-resolution growth curve. Figure reprinted from (Warringer, Anevski et al. 2008) under the BMC Open Access License Agreement and with the authors' permission.

Yeast phenomics just like the other "omics", is not only about the methodology itself which yields vast amount of data. These "omics" also entail development of tools to analyze the data being generated from a given bioassay. Results of large-scale phenomics require some degree of algorithmic sophistication implemented in a computer. Indeed, all our phenomics data are stored, processed, and maintained in a specialized online database (http://cmb.gu.se/Prophecy/), aptly named PROPHECY for PROfiling of PHEnotypic Characteristics in Yeast (Fernandez-Ricaud, Warringer et al. 2007).

POST-GENOMICS BIOINFORMATICS

One of the main challenges of the genomic era was the identification of informative sequences, such as identification of genes and regulatory elements, from the sequenced genomes. The combination of existing knowledge about gene structures, statistical sequence analysis and probability models *e.g.* Hidden markovs was the main staple of bioinformatics approaches in analyzing astronomical amount of sequence data. Indeed, a successful implementation of such computational approach allowed for the complete identification of all snoRNAs in the yeast genome (Lowe and Eddy 1999; Schattner, Decatur et al. 2004). In this case, pre-existing biological information about the snoRNA classes, such as their characteristic secondary structures and sequence motifs have been of particular value to the search algorithm. However, for novel non-coding RNA gene classes or regulatory elements, their *de novo* identification required more powerful approaches.

Advances in DNA sequencing technologies contributed tremendously in genome-wide sequencing efforts not only of various species of organisms from distant niches of evolutionary tree to closely-related species and even within species. This gave birth to comparative genomics which unleashed new predictive power in the identification of new genes and regulatory elements in genomes.

In **paper V**, we employed comparative genomics-based computational approaches to predict novel non-coding RNA (ncRNA) gene families in yeast. As opposed to the methods to predict guide snoRNA, which were restricted and customized to such RNA classes, we used two *de novo* methods implemented in programs called QRNA (Rivas and Eddy 2001) and RNAz (Washietl, Hofacker et al. 2005). These programs rely on pairwise or multiple sequence alignments from related species. A total of 245 candidates were predicted by QRNA while RNAz predicted 47. We then designed short oligoprobes against 31 top scoring candidates

and checked their expression in 18 different physiological conditions by northern blot analysis. Unfortunately, despite repeated attempts and excellent positive control signals from two snoRNAs of different sizes (Fig 2), we have not been able to confirm any of the 31 top scoring predictions. The poor prediction power of the aforementioned computational methods was recently substantiated, especially pointing out their high false positive rates recall (Babak, Blencowe et al. 2007). Our expectation in the beginning of the project was to identify small ncRNAs such as the microRNAs that were abundant in higher eukaryotes. It turned out that yeast doesn't have the repertoire of enzymes to carry out RNA interference.

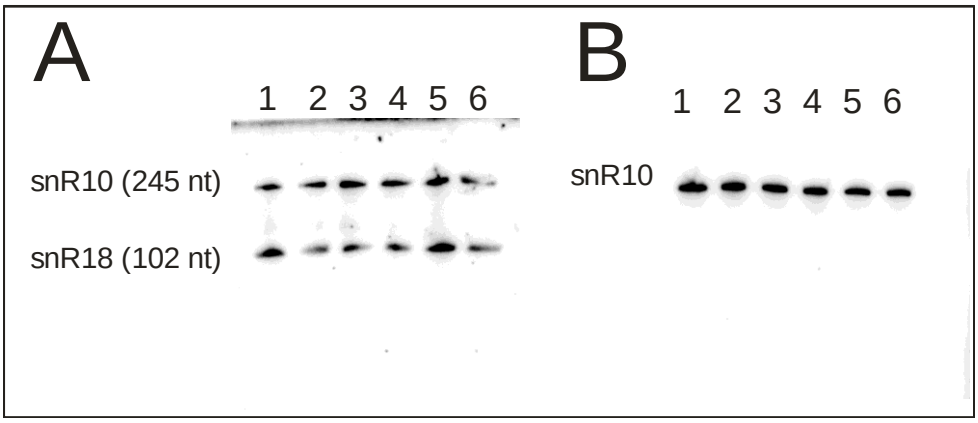

Figure 2. Northern analysis to confirm computationally-predicted ncRNA gene. Northern blots probed with **A)** oligoprobe against a top scoring QRNA candidate **B)** oligoprobe against a top scoring RNAz candidate. Both cases showed negative results. Lanes 1-6: Synthetic defined medium, salt, ethidium bromide, paraquat, caffeine and cadmium chloride. The blots were hybridized first with oligoprobes against the candidates then re-probed with the positive controls (snR10 and/or snR18). Excellent positive control signals were seen in all test blots.

3. YEAST RIBOSOMES

MITOCHONDRIAL RIBOSOME (MITORIBOSOME)

Mitochondria are known to be the power houses of most eukaryotic cells, responsible for energy production. They are also known to play a central role in ion homeostasis, and apoptosis (Westermann and Neupert 2003; Chan 2006). In yeast, mitochondria are considered to be redundant in the respiratory sense under anaerobic conditions due to the absence of oxygen as the final electron acceptor. Nonetheless, mitochondria could also assume other roles in anaerobic yeast cell physiology such as in ergosterol biosynthesis, synthesis and desaturation of fatty acids and membrane lipids, and in general physiological adaptation to stresses caused by ethanol, toxic oxygen radicals and high sugar concentrations (O'Connor-Cox, Lodolo et al. 1996).

Yeast mitochondria, like all eukaryotic mitochondria, contain their own translational machinery for the expression of its few genes. Nearly all the constituents of the mitoribosome are distinct from the cytoplasmic counterpart. The yeast mitoribosome contains two rRNA molecules, the 15S and 21S (Attardi and Schatz 1988) and estimated to have at least 90 mitochondrial ribosomal proteins (Graack and Wittmann-Liebold 1998). In **paper III**, 67 non-essential mitochondrial ribosomal proteins were included in the phenotypic screen.

The mitochondrial rRNA, unlike its cytoplasmic counterpart does not contain a large number of chemically-modified nucleotides or nucleosides. There are only three modified nucleotides, a pseudouridine and two *2´-O*-methylated riboses, which are carried out wholly by separate enzymes (Sirum-Connolly and Mason 1993; Ansmant, Massenet et al. 2000; Pintard, Bujnicki et al. 2002), unlike the modifying enzymes in cytoplasmic rRNA which depends on small nucleolar RNA (snoRNA) for proper targeting (see discussion below).

CYTOPLASMIC RIBOSOME

The cytoplasmic ribosome is made up of two asymmetric protein-RNA complexes of unequal sedimentation coefficients: the small and the large ribosomal sub-units. The 60S large sub-unit (LSU) contains the 25S (28S in higher eukaryotes), 5.8S and 5S rRNAs, while the 40S small sub-unit (SSU) harbors the 18S rRNA. At present, a total of 79 ribosomal proteins (r-proteins), 33 in the SSU and 46 in the LSU, are considered to be more or less regular residents of the yeast ribosome, per the criterion of non-dissociation *in vitro* of these proteins from purified ribosome preparations at 0.5 M KCl (Kruiswijk and Planta 1974).

To date, there is no high-resolution crystal structure reported yet for any eukaryotic ribosome. However, the high-conservation in the primary sequence level of ribosomal components, both for rRNA and many r-proteins from different organisms, gives ample confidence that the general structural outline of the ribosome may be inferred and extended across species. This allowed for the modeling of the yeast 80S ribosome, partly based on high-resolution crystal structures of subunits from the thermophilic bacterium *Thermus thermophilus* (Schluenzen, Tocilj et al. 2000; Wimberly, Brodersen et al. 2000) and the archaebacterium *Haloarcula marismortui* (Ban, Nissen et al. 2000).

Of the 79 r-proteins, only 44 could be represented in the 3D reconstruction based on individual homology models of r-protein components and analogous rRNAs assembled into an

11.7 Å cryo-EM map of the 80S yeast ribosome. The remaining unaccounted 35 r-proteins are presumed to dwell in the 19 unresolved clusters of protein densities in the cryo-EM map (Fig 3A) (Spahn, Beckmann et al. 2001; Spahn, Gomez-Lorenzo et al. 2004).

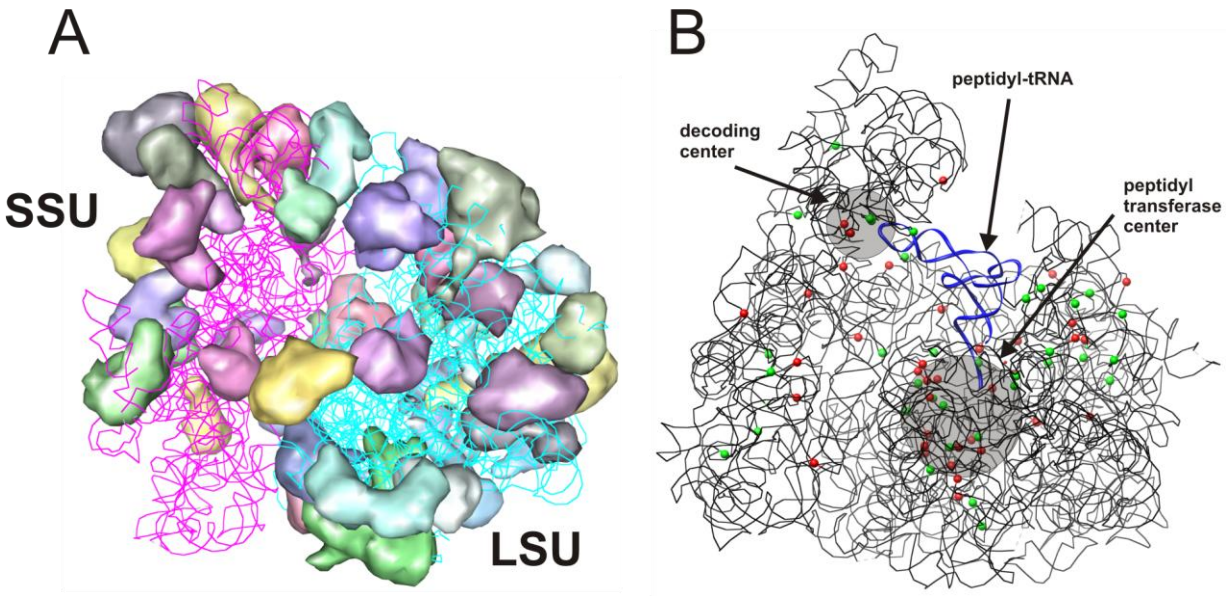

Figure 3. Three dimensional model of yeast 80S cytoplasmic ribosome.
A) Forty-four yeast r-protein homology models and analogous rRNAs from *H. marismortui* large subunit (LSU) and *T. thermophilus* small subunit (SSU) were fitted into an 11.7 Å cryo-EM map of the yeast 80S ribosome (Spahn, Beckmann et al. 2001; Spahn, Gomez-Lorenzo et al. 2004). (Low resolution surfaces, r-proteins; Chain traces: SSU rRNA, magenta; LSU rRNA, cyan). **B)** The yeast rRNA modification map indicating modifications considered in **paper II**. Green dots are pseudouridylations (34 sites) and red dots are *2'-O*-ribose methylations (41 sites), corresponding to modification sites targeted by 25 box H/ACA snoRNAs and 34 box C/D snoRNAs, respectively. Many snoRNAs have more than one target site of modification. Some modified residues in yeast cannot be mapped due to lack of correspondence between yeast rRNAs and the the *H. marismortui* or *T. thermophilus* rRNAs. Assignments of modified residues based on the work of (Piekna-Przybylska, Decatur et al. 2008). All visual manipulation and figures of the yeast ribosome and its components in this thesis were generated with PDB files (1s1h, 1s1i, 2j00, and 1ffk) downloaded from the Protein Data Bank (Berman, Westbrook et al. 2000) , using the molecular modeling system UCSF Chimera production version 1 build 2470 (Pettersen, Goddard et al. 2004).

CYTOPLASMIC RRNA AND THE SNORNAS

Modified nucleotides are ubiquitous in the cytoplasmic ribosomal RNA (Fig 3B). Their location are mostly evolutionarily conserved and are predominant in functionally-important regions of the ribosome (Decatur and Fournier 2002). The two major types of rRNA modifications are 2'-*O*-ribose methylation (Nm) and isomerization of uridine to pseudouridine (Ψ) (Bachellerie, Cavaille et al. 2002). The number of modifications in rRNA increases with increasing evolutionary complexity; *Escherichia coli* has ten Ψs and four Nms, *Saccharomyces cerevisiae* has 46 Ψs and 54 Nms, while human rRNA contains more than 90 Ψs and 100 Nms (Ofengand and Bakin 1997). While modifications in prokaryotic rRNAs are solely carried out by individual

protein enzymes, in eukaryotes the site-specificity of the modifications is facilitated by small nucleolar RNAs (snoRNA) guiding the modifying enzymes to their nucleotide targets via a base-pairing mechanism. Modification guide snoRNAs may belong in either two classes of snoRNAs as defined by sequence and structural motifs. Box C/D snoRNAs contain one or two pairs of small sequence elements called C and D and C' and D', while box H/ACA snoRNAs contain sequence elements called H and ACA. These motifs are required for processing of snoRNA precursors, and in the case of C/D boxes are also important in localization to the nucleolus (Decatur and Fournier 2003). Box C/D snoRNAs bind fibrillarin (Nop1p) and direct it to the sites of methylation in rRNA, while box H/ACA snoRNAs direct the binding of dyskerin (Cbf5p) to the site of pseudouridylation (Kiss 2002). The protein-snoRNA complex is called snoRNP (small nucleolar ribonucleoprotein).

It must be noted that there are cytoplasmic rRNA sites that are modified via snoRNP-independent manner. For example the lone pseudouridylation in 5S rRNA depends entirely on the activity of the multi-substrate synthase, Pus7p (Decatur and Schnare 2008), while in 25S rRNA the 2´-O-ribose methylation at G2922 is carried out by Spb1p (Lapeyre and Purushothaman 2004).

There are in total 76 box C/D and box H/ACA snoRNA genes annotated in the yeast snoRNA database (Piekna-Przybylska, Decatur et al. 2007; SGD 2008). Genetic analyses yielded only four lethal phenotypes, *i.e.* deletion mutants of MRP, U3a/b, snR30 and U14 are inviable, however, these are all required in pre-rRNA cleavage (Hughes, Konings et al. 1987; Bally, Hughes et al. 1988; Zagorski, Tollervey et al. 1988; Schmitt and Clayton 1993). One snoRNA mutant exhibited a clear fitness defect; the slow growing and cold-sensitive pseudouridylation guide snR10 deletion strain (Tollervey and Guthrie 1985) which later on was also shown to be involved in pre-rRNA cleavage (Tollervey 1987). For the remaining snoRNAs no strong link to cellular fitness has been reported despite several attempts (Parker, Simmons et al. 1988; Lowe and Eddy 1999; Qu, Henras et al. 1999). However, competition assays and the use of inhibitors directly targeting the ribosomal function have revealed subtle phenotypes for some knockouts for pseudouridylation yeast snoRNA. Five yeast mutants lacking a set of box H/ACA snoRNAs guiding pseudouridylations in the peptidyl transferase center were found to show marginal but significant fitness defects (Badis, Fromont-Racine et al. 2003; King, Liu et al. 2003). In addition, competition assays revealed subtle defects for strains lacking two highly conserved pseudouridine modifications guided by snR191 (Badis,

Fromont-Racine et al. 2003). As for the loss of 2'-*O*-ribose methylation with no reported impact on pre-RNA processing, no fitness defect has so far been reported. Remarkably however, deleting snR57, which is the box C/D snoRNA guiding the 2'-O-ribose methylation at position Gm1570 in the 18S rRNA, can actually suppress the lethal phenotype of the deletion of Nep1p, a protein that is involved in maturation of 18S rRNA (Buchhaupt, Meyer et al. 2006).

4. RIBOSOME AND PROTEIN SYNTHESIS

It has been demonstrated early on that each subunit of the ribosome performs specific aspects of protein synthesis. For instance, the actual decoding of the mRNA was found to occur in the small sub-unit (Okamoto and Takanami 1963), while incorporation of amino acid residues and peptide bond formation transpire in the large sub-unit (Gilbert 1963; Monro, Cerna et al. 1968). In this level of discourse, the ribosome in itself has almost become synonymous to translation. In truth however, protein synthesis, especially the eukaryotic one, is a very complex process which involves the concerted action of many protein factors interacting transiently with the ribosome.

Translation whether in pro- or eukaryotic system, occurs in four stages: initiation, elongation, termination and ribosome recycling. Each step requires a number of polypeptides, many of which associate together forming multi-subunit factors (Kapp and Lorsch 2004; Liljas 2004) (Fig 4).

The availability of high-resolution ribosomal structures and rich biochemical data allowed for the detailed description of the molecular events in each stage of translation. This also permitted the elucidation of roles of some ribosomal proteins in protein synthesis as will be discussed in the next section.

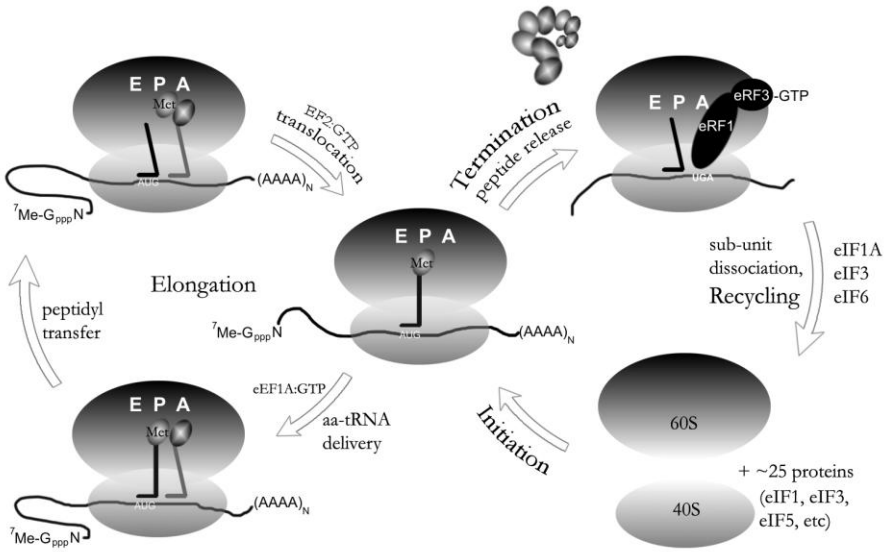

Figure 4. Schematic of the four stages of translation.
Each of the four stages: Initiation, Elongation, Termination and Recycling, is characterized by participation of protein complexes. E, P, and A are the exit, peptidyl tRNA, and amino-acyl tRNA sites respectively, in the large subunit (60S) of the ribosome. Modified from (Liljas 2004) to reflect the yeast system. Copyright permission from the author and the publisher (World Scientific).

5. Cytoplasmic ribosomal proteins (cRPs)

General properties

Kruiswijk and Planta (1974) started the isolation and characterisation of cRPs in yeast by two-dimensional electrophoresis, naming them according to their positions in the gel, as was originally done earlier with those in *E. coli* (Kaltschmidt and Wittmann 1970). However it was not until the publication of the complete sequence of *S. cerevisiae* genome that the exact number of genes encoding the cRPs was determined. Using three main criteria: 1) degree of homology with rp genes from other eukaryotes, 2) high codon adaptation index, and 3) promoter structure, Planta and Mager (1998) came up with the definitive compilation of cRP genes in yeast. Assuming functional equivalence between the duplicated cRP genes (59 pairs), they reported that the yeast genome contains 32 SSU and 46 LSU cRP gene families encoded by a total of 137 genes (Planta and Mager 1998).

The following year, a powerful approach that made direct use of mass spectrophotometry identified a novel component of the yeast and human 40S subunit (Link, Eng et al. 1999). This mammalian protein RACK1, Asc1p in yeast, was finally mapped in the SSU (Gerbasi, Weaver et

al. 2004; Sengupta, Nilsson et al. 2004) and is now considered a core ribosomal protein, bringing the total number of yeast cRPs to 79.

A typical characteristic of virtually all cRP genes, which they share with other highly expressed genes such as glycolytic enzymes, is the ability of their promoter regions to bind the multi-functional transcription factor Rap1p *in vivo* (Lieb, Liu et al. 2001). Indeed, it was shown bioinformatically that majority of cRP gene promoters have tandem Rap1p-binding sites or in places where this is absent, Abf1p or Reb1p binding sites are present (Lascaris, Mager et al. 1999). But as mentioned, these transcription factors are not exclusive to cRP genes. Rap1 for instance, is known to perform many functions such as regulation of many glycolytic enzymes, silencing of mating-type genes, maintenance of telomere ends (Shore 1994) and even controlling the osmotic stress-responsive gene, *GPD1* (Eriksson, Alipour et al. 2000). Only very recently was a cRP gene-specific transcription factor, Fhl1p, found (Lee, Rinaldi et al. 2002). This protein regulates cRP gene expression through TOR via the highly conserved PKA signaling pathway, a well-studied signal transduction pathway controlling cell growth in response to diverse environmental cues (Martin, Soulard et al. 2004). Regulatory elements, such as the Fhl1p binding-site on cRP gene promoters, are expected to modulate the coordinated transcription of cRP genes resulting in global attenuation of their mRNAs during stressed condition, such as rapamycin, heat shock treatment, nutrient depletion, and other environmental insults (Gasch, Spellman et al. 2000). The fact that not all cRP genes bind Fhl1p *in vivo* (two immunoprecipitation studies both identified only around 55% of all cRP genes that were strongly bound by Fhl1p (Schawalder, Kabani et al. 2004; Wade, Hall et al. 2004) point to other stress-specific regulatory mechanisms which might govern cRP genes.

Another feature of the cRP genes is the high frequency of introns among them. Of the 250 genes containing introns in the yeast genome, 99 are cRP genes (Planta and Mager 1998). Although the biological significance of selection for introns in many cRP genes is not understood, splicing of cRP mRNAs definitely adds another level of regulation in this class of genes (Warner 1989).

59 of the 79 of cRPs have paralogs which resulted from an ancient whole genome duplication event (Kellis, Birren et al. 2004). Nonetheless, only a handful of these paralog pairs retained 100% sequence identity in the amino acid level. Some diverged significantly even in the nucleotide level that the codon adaptation index (CAI) differs considerably between paralog

pairs, as in the case of RPL22A and RPL22B which have 0.86 and 0.29 CAI, respectively (Planta and Mager 1998). A high CAI indicates high gene expression (Akashi and Eyre-Walker 1998).

Of the 19 singletons, 15 are lethal when deleted, while only 5 of the 59 paralog pairs are essential. However, using conditional gene constructs, double deletion of both paralogs of 26 of 28 small subunit proteins resulted in lethality, which could be rescued by overexpression of any one of the two alleles (Ferreira-Cerca, Poll et al. 2005).

Ribosomal proteins are generally short, ranging from only 51 to 387 amino acid residues long. The crystal structures of many cRPs reveal a two-domain architecture consisting of globular and tailed regions (Fig 5). The tail parts, which might occur internally or in the termini regions of r-proteins, are highly-unordered and very flexible, mainly due to the presence of large amount of small residues such as glycine. This is one of the reasons why many cRPs containing tailed extensions or those completely lacking globular domains have been very difficult, if not impossible, to be structurally determined in isolation (Brodersen and Nissen 2005).

Chemically, cRPs are markedly enriched with Lys and Arg giving them a very alkaline character, with the exception of the four acidic r-proteins of the ribosomal stalk in the LSU. There is also a very low amount of negatively charged residues such as Glu and Asp (Planta and Mager 1998). This is comparable only to histones which bind and stabilise chromatin structures. The highly alkaline property of majority of r-proteins, particularly in their tail regions, is essential in counteracting the negative charges of the phosphate group of the rRNA backbone leading to its correct conformation during ribosome assembly (Wilson and Nierhaus 2005). Noteworthy as well is the high prevalence of small, hydrophobic amino acids such as Ala, Val and Gly (Lin, Kuang et al. 2002), relating to the observation that many other highly-expressed genes tend to contain such small amino acid residues (Jansen and Gerstein 2000).

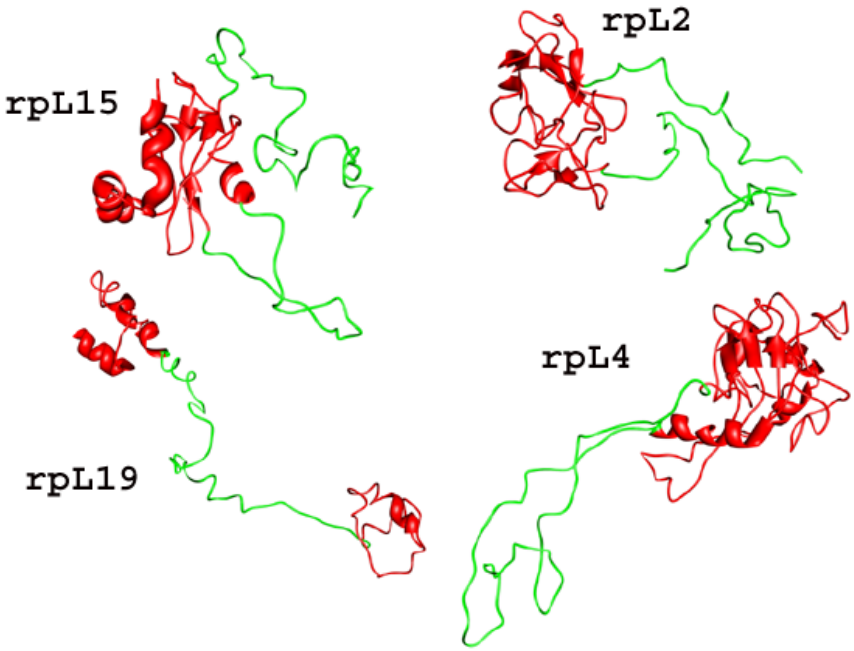

Figure 5. Globular and tail domains of r-proteins.
The globular regions are characterized by ordered secondary structures while the tail parts consist of flexible loops which extend into the ribosome making extensive contacts with the rRNA.

EVOLUTIONARY CONSERVATION

A systematic comparison of cRP genes from 66 complete genomes representing Bacteria (45 species), Archaea (14 species) and Eukarya (7 species) not only revealed a high degree of conservation for many of the cRP gene families but also the presence of kingdom-specific cRP genes (Lecompte, Ripp et al. 2002) (Fig 6). It is readily noticeable that the number of r-proteins also increases as one goes up the phylogenetic lineage, hinting that the protein components are recent additions to the ancestral ribosome of the postulated ancient RNA world (Wool 1996).

One interesting observation is the lack of r-proteins exclusively shared by Bacteria with either Archaea or Eukarya, in agreement with the theory that the last common ancestor of Archaea and Eukarya diverged from the Bacteria before their own separation occurred. Not only are many r-proteins kingdom-specific, but differences within each kingdom are also present. For example, within the eukaryotes, the mammalian L28 gene also present in the nematode (*Caenorhabditis elegans*), fruitfly (*Drosophila melanogaster*), and plant (*Arabidopsis thaliana*) has no homolog in the budding yeast. In higher plants, an additional P-protein called P3,

complexes to P1/P2 which are the usual acidic phosphoproteins forming the lateral stalk structure of the large subunits of animals, fungi and protozoans (Szick, Springer et al. 1998).

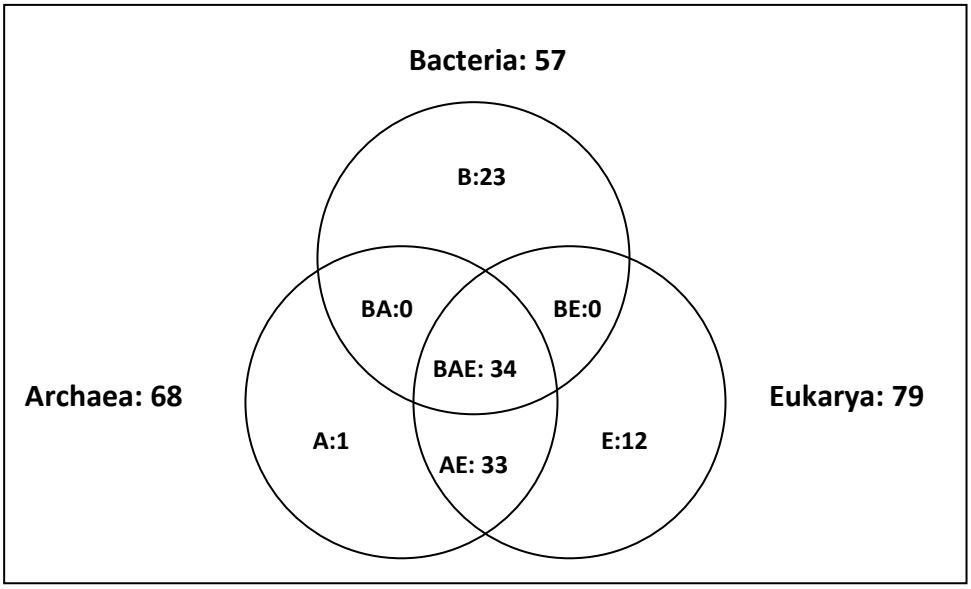

Figure 6. Intersection of r-proteins in the three kingdoms of life.
Only 34 r-proteins are universally conserved. The bacterial kingdom does not exclusively share any r-protein homologies with either the Archaea (BA:0) or the Eukarya (BE:0).The total number of r-proteins in eukarya increased by one with the recent addition of Asc1p in the small sub-unit. Modified from Lecompte et.al. (2002).

There are 34 universally-conserved cRPs, 15 in the SSU and 19 in the LSU (Table 1). Many of these have been shown to be involved in ribosome assembly in E. coli (Held, Ballou et al. 1974; Rohl and Nierhaus 1982) and virtually all were implicated in the maturation and transport of pre-18S rRNA in yeast (Ferreira-Cerca, Poll et al. 2005). A few act as inter-subunit bridges (E. coli numbering: S13, S15, S19, L2, L5, and L14) while three (L22, L29, L24) surround the exit tunnel of the large subunit (Yusupov, Yusupova et al. 2001). In E. coli, S9, S13, S17, L1, L11, L15, L24, L29 and L30, none of which has a duplicate, are among the conserved r-proteins which are non-essential (Dabbs 1986; Herr, Nelson et al. 2001). In yeast, 70% of the conserved r-proteins have duplicates, confounding the interpretation of the result of homozygous single-deletions of these r-proteins showing viability (Winzeler, Shoemaker et al. 1999). Nonetheless, complete deletion of paralog r-proteins in yeast generally result in lethality (Ferreira-Cerca, Poll et al. 2005).

Table 1. The universally conserved ribosomal proteins in the three kingdoms.
Modified from Wilson and Nierhaus (2005).
Primary data source: http://www.expasy.org/cgi-bin/lists?ribosomp.txt (22-Jul-2008 release)

Small subunit				Large subunit			
Bacteria	**Archaea**	**Low Eukarya**	**High Eukarya**	**Bacteria**	**Archaea**	**Low Eukarya**	**High Eukarya**
E. coli	***H. marismortui***	**Yeast**	**Rat**	***E. coli***	***H. marismortui***	**Yeast**	**Rat**
S2	S2	S0	Sa	L1	L1	L1	L10a
S3	S3	S3	S3	L2	L2	L2	L8
S4	S4	S9	S9	L3	L3	L3	L3
S5	S5	S4	S2	L4	L4	L4	L4
S7	S7	S5	S5	L5	L5	L11	L11
S8	S8	S22	S15a	L6	L6	L9	L9
S9	S9	S16	S16	L10	L10	P0(A0)	P0
S10	S10	S20	S20	L11	L11	L12	L12
S11	S11	S14	S14	L12	L12	P1/P2	P1/P2
S12	S12	S23	S23	L13	L13	L16	L13a
S13	S13	S18	S18	L14	L14	L23	L23
S14	S14	S29	S29	L15	L15	L28	L27a
S15	S15	S13	S13	*	L7Ae	L8	L7a
S17	S17	S11	S11	L18	L18	L5	L5
S19	S19	S15	S15	L22	L22	L17	L17
				L23	L23	L25	L23a
				L24	L24	L26	L26
				L29	L29	L35	L35
				L30	L30	L7	L7

*Ortholog absent in *E.coli* but present in *Bacillus subtilis* and many *Staphylococcal* species as L7Ae-like putative ribosomal protein genes.

ROLES IN TRANSLATION

With the availability of atomic resolution ribosome structures, investigating the role of individual ribosomal protein components has become more and more mechanistic in the context of actual events in protein synthesis. Coupled with chemical probing techniques to study rRNA conformations, targeted mutagenesis, biochemical and genetic approaches, various studies have successfully assigned specific roles during translation for many ribosomal proteins.

In prokaryotes, the roles of r-proteins in distinct steps of translation are well-documented (Brodersen and Nissen 2005; Wilson and Nierhaus 2005). Extensive studies on *E. coli* r-proteins revealed their diverse functions from recognition and decoding of translational substrates (mRNAs and tRNAs), to conferring resistance against antibiotics which inhibit translation. A number of r-proteins were also localized in functionally important sites such as

surrounding the PTC (L2 and L3) or lining the exit tunnel (L4, L22, L23 and L24). Many of these prokaryotic r-proteins are universally conserved and have been modeled in the corresponding sites of the yeast ribosome. It is therefore possible that their functions might be conserved as well. Table 2 lists some yeast ribosomal proteins implicated in specific steps during translation.

Table 2. Yeast ribosomal proteins involved in specific steps during translation.

Ribosomal protein	Function during protein synthesis	Reference
rpL3	Allosteric switch in the A-site coordinating binding of elongation factors	(Meskauskas and Dinman 2007)
rpS0	Involved in the recruitment of the translation initiation factor eIF3 to the small subunit	(Valasek, Mathew et al. 2003)
rpS9	Accurate decoding of mRNA	(Vincent and Liebman 1992; Alksne, Anthony et al. 1993)
rpS2	Control of translational accuracy	(Alksne, Anthony et al. 1993; Synetos, Frantziou et al. 1996)
rpL39	Translational accuracy	(Dresios, Derkatch et al. 2000)
rpL5	Anchoring of peptidyl-tRNA to P-site	(Meskauskas and Dinman 2001)
rpL41	Translocation of t-RNAs	(Dresios, Panopoulos et al. 2003).
rpL10	Subunit joining	(Eisinger, Dick et al. 1997)

The heart of the translational process, that is peptide formation in the PTC, occurs within the exclusive domain of the ribosomal RNA. However, four tailed-proteins have extensions protruding close to this region (Fig 7), which could influence the optimal positioning of the tRNA-bound amino acid substrates undergoing peptidyl transfer.

RPL10 and *RPL3* are both singletons and are lethal when deleted in yeast. *RPL2* and *RPL4* both have duplicates which are non-essential, and are therefore amenable to our phenotypic profiling approach. In **paper III**, we found that the deletion mutants of *RPL2B* and *RPL4A* showed fairly similar phenotypic profiles, specifically exhibiting sensitivity towards tert-butyl hydroperoxide, placing both of them in the oxidative sub-cluster. This is a rare instance where we found ribosomal proteins in close proximity to each other, both in the spatial position in the three dimensional structure and in the context of phenotypic clustering of their corresponding deletion mutants. In general, we did not observe any correlation between the spatial positioning of the cRPs in the ribosome, and the phenotypic clustering of the corresponding gene deletion mutants.

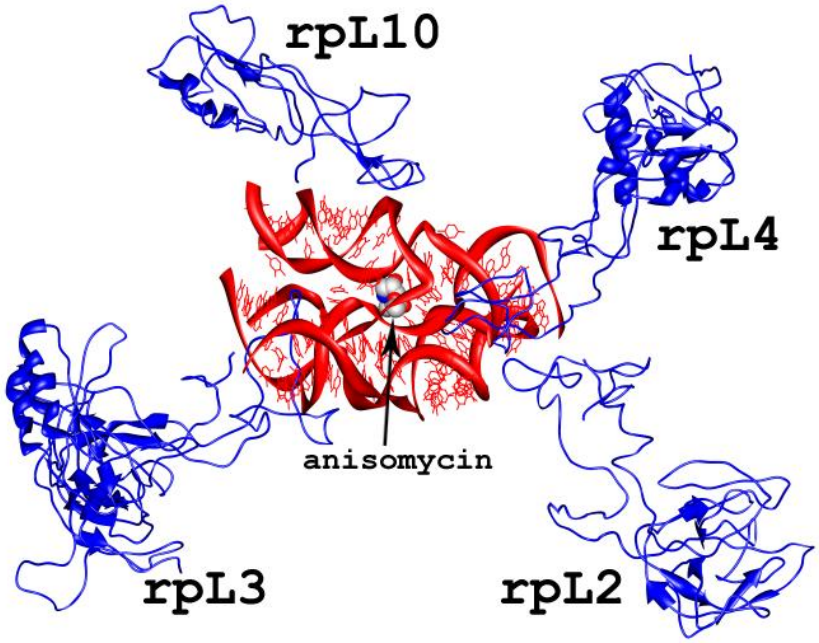

Figure 7. Some r-proteins reach into the peptidyl transferase centre via their tails. Although the PTC is completely surrounded by rRNA helices (red ribbon), four r-proteins can reach the vicinity via their tail extensions. The anisomycin antibiotic which binds in the PTC is shown for reference.

ROLES IN RIBOSOME BIOGENESIS

Making a functional ribosome is a huge task requiring the cooperative functioning of more than 170 pre-ribosomal factors in the post-transcriptional processing of rRNAs which involves splicing, modification, folding, transport and assembly (Fatica and Tollervey 2002). Despite the abundance of these trans-acting factors, the r-proteins themselves have also been shown to play specific roles in the maturation, assembly and transport of the rRNAs. An example is rpS14, whose tail extension was shown to be necessary for maturation of 43S pre-ribosomes (Jakovljevic, de Mayolo et al. 2004). Another is rpS15 required for exit of the 40S subunit precursors (Leger-Silvestre, Milkereit et al. 2004).

A systematic approach was used to address the specific roles of yeast small subunit proteins in pre-18S rRNA processing, localization and export (Ferreira-Cerca, Poll et al. 2005). Using strains whose r-proteins are under the strict control of the *GAL1* promoter, the authors were able to monitor the effect of specific r-protein depletion in the distinct steps of 18S rRNA processing pathway. The conclusion was that, except for rpS7, rpS30 and rpS31, virtually all of

the SSU r-proteins play a role in the maturation and transport of 18S rRNA precursors. In a recent study, an LSU r-protein, Rpl3p, was also shown to be involved in early assembly of 60S ribosomal subunit and in the subsequent steps of their maturation and export (Rosado, Kressler et al. 2007). In **paper IV**, polysomal analyses of knockouts of 20 ribosomal protein genes of the small subunit and 16 ribosomal protein genes of the large subunit in basal conditions confirmed the importance of individual ribosomal proteins in the final assembly of functional ribosomes.

ROLES IN OTHER CELLULAR PROCESSES

A decade ago, the extra-ribosomal functions of some r-proteins from various organisms were compiled (Wool 1996). The diversity of enumerated functions, ranging from control of viral replication in *E. coli* (Yancey and Matson 1991) to developmental regulation in higher eukaryotes (Fisher, Beer-Romero et al. 1990; Watson, Konrad et al. 1992) was seen as evidence "that ribosomal proteins might have been co-opted for other cellular functions" and that they were not originally designed specifically for the ribosome. It was an intriguing pronouncement in support of the widely-held view regarding the evolutionary history of ribosomal proteins, *i.e.* they came later into the ancient ribosome prototype exclusively made up of RNA. This view only strengthens by the day as new bi-functional r-proteins working outside the periphery of the translational apparatus are found:

i) The human L13A which is released from the 60S subunit into the cytoplasm upon phosphorylation in response to *in vivo* levels of interferon-γ. In the cytoplasm, L13A specifically binds a structural element at the 3'-end of the ceruplasmin mRNA, effectively silencing its translation (Mazumder, Sampath et al. 2003).
ii) The mammalian ribosomal protein RACK1, yeast Asc1p, serves as a scaffold among diverse kinases and receptors ultimately linking signals from various transduction pathways (McCahill, Warwicker et al. 2002). Interestingly, it has also been observed to exist as ribosome-bound and unbound forms in *S. cerevisiae* (Baum, Bittins et al. 2004), humans (Ceci, Gaviraghi et al. 2003) and fission yeast (Shor, Calaycay et al. 2003).
iii) Another novel function being recently attributed to ribosomal proteins, specifically those of the 40S small sub-unit, is their possible role in translational control via recruitment of specific mRNAs. Termed as the "ribosome filter" hypothesis, it posits

that components of the ribosomal subunit may act as regulatory elements, acting as filters, which may either enhance or inhibit the translation of various mRNAs via differential binding interactions (Mauro and Edelman 2002). Selective translation occurs depending on the affinity of a class of mRNAs to the rRNA or to ribosomal proteins.

iv) In *Schizosaccharomyces pombe*, RPL32-2 was shown to act as a potential transcriptional regulator in the nucleus (Jing, Sheng et al. 2006).

v) A number of ribosomal protein genes have been found to have increased gene expression in mammalian tumor cell lines. Human RPS3a specifically has been shown to fulfill an important role in cell transformation and apoptosis (Naora 1999). Interestingly, in zebra fish, at least 11 cRP genes were found to be haplo-insufficient tumor suppressors (Amsterdam, Sadler et al. 2004).

vi) Increased replicative life span (RLS) was found in yeast with deletion in 60S ribosomal protein genes (Steffen, MacKay et al. 2008). In another study, strains deleted for small subunit ribosomal protein genes were also shown to have increased RLS (Chiocchetti, Zhou et al. 2007).

Taking some of the above instances of r-proteins possibly working off the translational apparatus, it is not difficult to imagine the ribosome serving also as a repository of functionally important proteins (Mazumder, Sampath et al. 2003). In fact, the exchange of r-proteins on and off the ribosome has been known long ago for the acidic r-proteins in yeast that forms the ribosomal stalk in the large sub-unit (Zinker and Warner 1976). The amount of ribosome-bound forms is somehow correlated with the metabolic activity of the cells, being substantially reduced during the stationary phase of growth (Saenz-Robles, Remacha et al. 1990). Also, Qsr1p (RPL10) which was found to be essential in subunit joining is also exchangeable, cycling on and off large subunits in the cytoplasm (Dick, Eisinger et al. 1997).

THESIS SUMMARY

Discovery consists in seeing what everybody else has seen,
and thinking what nobody else has thought.
Albert Szerg-Györgyi

6. DIVERSE CELLULAR ROLES OF SNORNA GENES

The "notoriety" of snoRNA genes as "zero phenotype genes" in genetic depletion analysis in the wild type background is widely-known in the RNA field. As mentioned in an earlier section, only when the growth assays of some snoRNA gene knockouts were shifted from traditional phenotyping methodology on solid media to more sophisticated competition assays that a handful was shown to have fitness contributions to cellular growth.

Our high-resolution phenomics methodology confirmed in both **paper I and paper II** the generally marginal nature of phenotypic response of snoRNA gene deletion knockouts in basal conditions. Indeed, in our phenotypic screen in **paper II** we found only 4 out of 65 snoRNA gene deletion mutants showing strong growth rate defects in the basal medium. Nonetheless, we were able to capture a plethora of phenotypes in many growth conditions providing evidence on their potential roles in cellular stress response. Specifically we uncovered an apparent link of snoRNA genes to respiratory functions (see 9. FUNCTIONAL SPECIALIZATION IN YEAST RIBOSOMES).

The biochemical role of snoRNAs as guides in the site-specific modifications in the rRNA has long been known. However, the fact that snoRNAs had to make contacts with the rRNAs during the modification process forming transient snoRNA-rRNA duplexes leaves the question whether the snoRNA has some chaperone activity during the folding process of the rRNA. Indeed, it has been shown using computer simulation that archaeal box C/D snoRNAs could provide chaperone effects for the proper folding of rRNA by restricting folding domains (Schoemaker and Gultyaev 2006). This confounds the interpretation of genetic depletion experiments involving snoRNA genes. There's always the lingering question whether the observed phenotype displayed by a snoRNA gene knockout mutant is due to the failure of modification or due to the lack of snoRNA molecule itself which might function in some other biochemical context. This can probably be resolved by looking at the phenotypes of snoRNA gene knockouts, whose otherwise present snoRNA gene products target the same modification site. We were able to partly address this question in **paper II** where we observed

that *snr39Δ* and *snr59Δ* showed dissimilar phenotypic profiles. *SNR39* and *SNR59* are intron-encoded box C/D snoRNA genes contained in *RPL7A* and *RPL7B* respectively (Ghazal, Ge et al. 2005). *RPL7A* and *RPL7B* are nearly-identical paralogous cRP genes and the intronic snoRNAs they harbor both guide the 2'-O-ribose methylation at position A807 in the 25S rRNA. We saw from **paper II** that while *snr39Δ* displayed extreme sensitivity to the alternative carbon sources thus belonging to the "non-respiratory" sub cluster, *snr59Δ* did not show sensitivity to any particular growth inhibitor at all. The fact that residue A807 is modified (via snR59) even when *SNR39* has been deleted excludes the lack of *2'-O*-ribose methylation as the cause of phenotypic defect seen in *snr39Δ*. This result implies that snR39 could be involved in a yet unknown fitness-contributing cellular process.

7. Ribosomal protein genes as "turbo" genes

The "turbo" growth phenomenon was used to describe the importance of genes for maximal growth in optimal conditions while losing their importance during growth-perturbing conditions. In an earlier genome-wide phenotypic screen this phenomenon was noted in many of the core cellular components, such as general transcription factors and regulators of central metabolic processes (Warringer 2003).

Our analysis of phenotypic data in **paper III** indicated that turbo growth phenomenon was a widespread feature among cRPs and mRPs, with approximately 66% of the 110 cRPs and 43% of the 67 mRPs to have reduced importance in many stress conditions. These ribosomal proteins seemed to be required only when there is unperturbed proliferation during optimal conditions when the translational machinery is working at maximal capacity. However, when cell growth is slowed-down by various external stresses the translational rate might no longer be limiting for growth.

8. Paralogous cRP genes: "same, same but different"

A large proportion of cytoplasmic ribosomal protein genes have paralogs. Our phenotypic study in **paper III** contained 110 cRP deletion knockouts, and 94 of them have paralogous associations. Many of these duplicated cRPs are very well-conserved in fact, 11 pairs in this study were identical. Assuming that the biochemical function of ribosomal proteins is confined within the perimeters of translation, it was very surprising that in general, we found paralogous pairs to be phenotypically-divergent. Our findings were in fact corroborated by

earlier studies focused on specific paralogous pairs. For instance, in the *RPS9A* and *RPS9B* pair, the omnipotent suppression phenotype was shown only for *RPS9B* (Vincent and Liebman 1992). *RPS9B* was also found to have higher transcription levels than *RPS9A* (Vincent and Liebman 1992; Mager, Planta et al. 1997; Pnueli and Arava 2007). Another example is the deletion of *RPL7A* which moderately impairs growth and affects budding while deletion of *RPL7B* has no effect on growth (Mizuta, Hashimoto et al. 1995). In studies on yeast replicative life span (RLS), it was often the case that only one of the paralogs contribute to increased RLS (Chiocchetti, Zhou et al. 2007; Steffen, MacKay et al. 2008).

In **paper III,** analysis of evolutionary histories of the cRP paralogs based on the studies of Kellis *et al* (Kellis, Birren et al. 2004) allowed us to relate the "ancestral" and "derived" paralogs variants to their differential importance in growth conditions. Whereas we found the evolutionary most conserved cRP paralogs (ancestral) to be of higher importance during growth in optimal conditions, the derived cRP paralog variants appeared to be selected and optimized for functionality during various environmental challenges.

9. FUNCTIONAL SPECIALIZATION IN YEAST RIBOSOMES

We systematically established functional links among the ribosomal proteins and the cytoplasmic rRNA modifications in yeast ribosomes. Integrating the phenotypic data of snoRNA (from **paper II**), cytoplasmic and mitochondrial ribosomal protein (from **paper III**) deletion mutants, we observed distinct functional features delineating specific phenotypic sub clusters (Table 3 and Fig 8).

THE "NON-RESPIRATORY" SUB CLUSTERS

Remarkably, the chemogenetic landscape was heavily dominated by the extreme response of select groups of deletion mutants to non-fermentable or alternative carbon sources (Fig 8). It was striking that this characteristic phenotypic response could be resolved further into three levels according to the magnitudes of sensitivity toward galactose, raffinose, maltose and ethanol. These sugars were able to set clear demarcation lines between particular groups of cRP, mRP and snoRNA gene knockouts.

Table 3. Number of knockouts in each sub cluster according to type of gene deletion.

Some phenotypic sub clusters contain almost exclusively only a single type of gene deletion mutant, such as the anisomycin sub cluster while some contain mix of all three such as the oxidative stress sub cluster.

Phenotypic sub cluster	snoRNA	cRP	mRP
Low response	7	10	1
Mitochondrial non-respiratory	0	1	51
snoRNA non-respiratory	27	0	1
Cytoplasmic non-respiratory	6	25	5
Rapamycin cluster	8	23	0
Heat shock	1	26	3
Oxidative stress	6	25	6
Anisomycin	10	0	0
TOTAL	65	110	67

For instance, the "mitochondrial non-respiratory" sub-cluster did not grow in all the alternative carbon sources and was dominated almost exclusively by mitochondrial ribosomal proteins (mRPs) (except for a single cRP gene deletion mutant, *rpl1bΔ*) (Table 3). The next level of non-respiratory sensitivity was those not growing only in maltose and ethanol, and moderate growth defects in galactose and raffinose. Except for *mrpl27Δ*, this was comprised of snoRNA deletion mutants, hence "snoRNA non-respiratory". The last level of alternative carbon source sensitivity was the "cytoplasmic non-respiratory" which only exhibited slow growth in maltose and ethanol, while growing normally on galactose and raffinose. This sub-cluster is interesting as it is the only non-respiratory sub-cluster that contains a considerable mix of all three snoRNA, cRP and mRP deletion mutants.

The extreme sensitivity of majority of the mRP gene knockouts to alternative carbon sources was not a surprise since mRPs are required in the production of important respiratory chain components encoded in the mitochondrial genome. As for the sensitivity of a very tight group of snoRNA deletion mutants to alternative carbon sources, we confirmed in **paper II** via respirometry that it is due to defective respiratory capacities. Although it could be linked to negative influence on mitochondrial functions, a more complex physiological explanation is warranted since they were also the only deletion mutants to exhibit hyper resistance towards anisomycin, for which no other mutants belonging to the other two non-respiratory sub clusters showed resistance to.

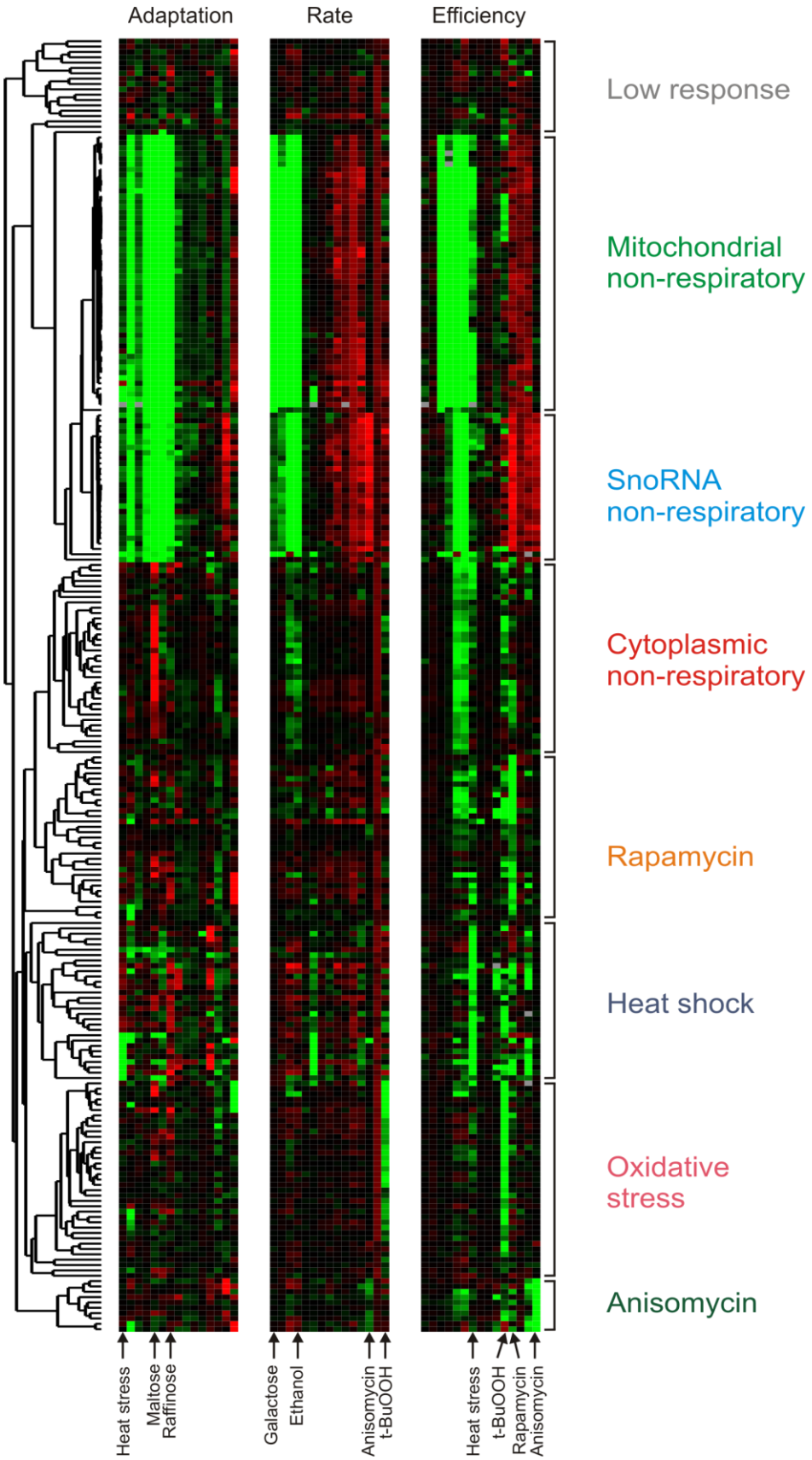

Figure 8. Hierarchical clustering of all profiled deletion mutants from papers II and III.
Hierarchical clustering of gene-by-environments interactions between 110 cRP, 67 mRP and 65 snoRNA gene deletion mutants in 19 stress environments. Colors indicate increased (red) or reduced (green) stress tolerance. Complete names of environments may be found in **paper II and III** separately for snoRNAs and r-protein gene deletion strains, respectively.

OTHER STRESS-SPECIFIC SUB CLUSTERS

It is noticeable that some sub clusters include only specific classes of gene deletion mutants. For example, the "anisomycin" sub cluster comprised only of snoRNA gene knockouts. Anisomycin is a macrolide that competitively inhibit peptide bond formation by blocking the hydrophobic crevice of the peptidyl transferase center from accepting incoming amino acid residues bound to amino-acyl tRNA (Hansen, Moore et al. 2003).

The "rapamycin" sub cluster was comprised only of cRP and snoRNA knockouts. This is consistent with the fact that there is a degree of coordination between the biosynthesis of snoRNA and components of the cytoplasmic translational machinery (Bachellerie, Cavaille et al. 2000). Eight snoRNA genes are intronic to cRP genes, and many monocistronic snoRNA genes are also found adjacent to cRP genes implying co-transcriptional regulation. Indeed canonical cRP gene promoter-binding sites such as Rap1, Abf1p and Fhl1p are also found upstream in a number of snoRNA gene loci (Qu, Henras et al. 1999; Harbison, Gordon et al. 2004). Since Fhl1p is controlled via the TOR pathway which is inhibited by rapamycin, the sensitivities observed in both cRP and snoRNA gene deletion mutants could be a direct consequence of the antibiotic inhibition.

GENERAL TRANSLATIONAL FEATURES OF MUTANTS HARBORING STRESS-SPECIFIC CLASSES OF CYTOPLASMIC RIBOSOMAL PROTEINS

For the identified stress-specific clusters discussed above, we investigated in **paper IV** some general translational features of a substantial number of deletion mutants by velocity sedimentation in sucrose gradient. Such studies have been done before in specific rp gene knockouts however, to our knowledge this is the first systematic polysome profiling analyses on a substantial number of rp gene knockouts of the same genetic background. We confirmed the observed importance of individual ribosomal proteins in the final assembly of a functional ribosome. While we observed the general translational inhibition in the growth of wild type as a consequence of ethanol or rapamycin-induced stress, we obtained surprising results regarding the behavior of the cRP gene mutants grown in the same stress conditions. Specifically, we identified cRP gene deletions strains which were resistant to such translation inhibitory effects of stress.

10. LINKING PHENOTYPES TO RIBOSOMAL STRUCTURE

We attempted, in **paper I**, to relate the phenotypic responses of 20 C/D snoRNA gene deletion mutants to the location of their cognate 2'-O-ribose methylation targets in the cytoplasmic rRNA. Specifically we spatially mapped the locations of the modifications into high resolution structural models of ribosomes to see whether the clustering among the phenotypes of C/D snoRNA gene deletion knockouts in ribosome-specific antibiotics, will correlate with their spatial positioning in the ribosome. However, no correlation has been found. New phenotypic data from **paper II** also did not show any specific phenotype-spatial location correlations, both in the level of ribosome-specific phenotypes, *i.e.* anisomycin-sensitives (Fig 9A) and environment-wide level phenotypic clustering, *i.e.* "respiratory" vs "non respiratory" clusters (Fig 9B).

The lack of correlation between the tight phenotypic responses of the box C/D snoRNA deletions and the spatial location of the corresponding modified residues probably reflects the highly complicated nature of allosteric physical interactions in the ribosome, *i.e.* local structural change in rRNA can trigger significant conformational changes in distant parts of the ribosomal structure (Bashan, Zarivach et al. 2003).

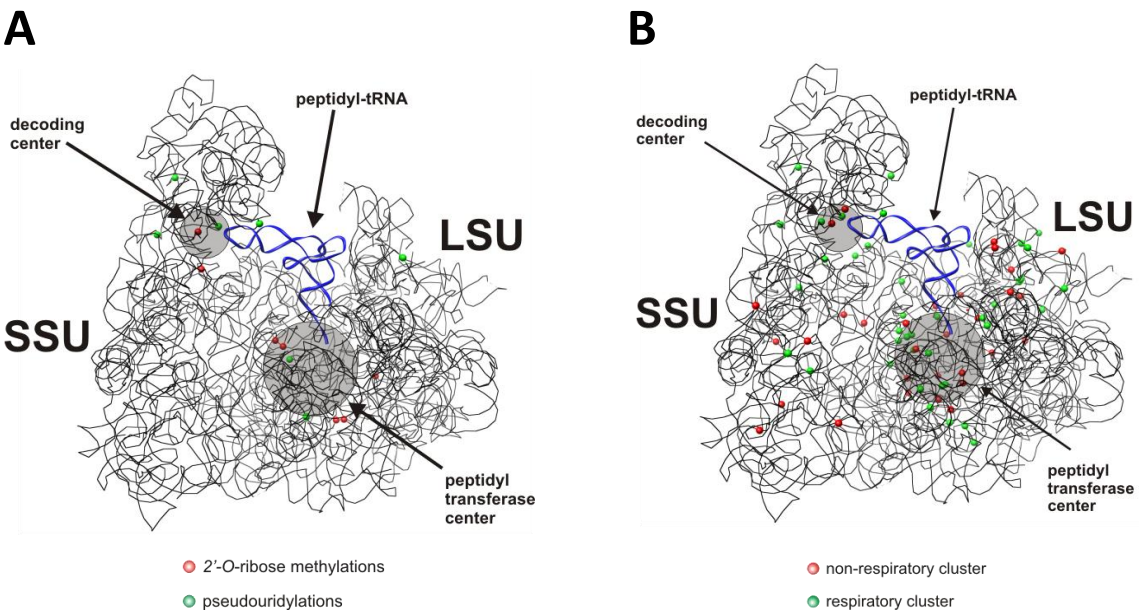

Figure 9. Spatial mapping of modified rRNA modifications on the ribosome.
A) The target modification sites of snoRNAs whose gene deletion knockouts exhibited sensitivity to anisomycin were mapped into the rRNA. The snoRNA knockouts belong to the tightly clustered "Anisomycin sub cluster" in Fig 8. Anisomycin inhibits translation by blocking the hydrophobic crevice in the peptidyl transferase center (Hansen, Moore et al. 2003). The scattered distribution of modification sites point to complex allosteric interactions affecting antibiotic binding. **B)** No spatial correlation was observed in the level of environment-wide phenotypic clustering for snoRNA gene knockouts (LSU: large subunit, SSU: small subunit).

PERSPECTIVES

If at first the idea is not absurd, then there is no hope for it.
Albert Einstein

11. SNORNAS IN CELLULAR STRESS RESPONSE

The diversity of phenotypic responses, ranging from extreme sensitivity to extreme resistance in various stresses, we uncovered for snoRNA gene deletion knockouts in **paper I and II** indicated a hitherto unknown functional importance of snoRNA molecules in cellular stress response. The link to cellular respiration is particularly intriguing and deserves further attention. Functional networks connecting snoRNAs to such cellular processes would be expected to be complex. Indeed the potentially multiple fitness contributions of snoRNA molecules to the cell, first as chaperones during rRNA maturation and secondly via the rRNA modifications they caused to exist, might confound the problem. Nevertheless results from **paper II** indicated functional separation between the snoRNA molecule itself and the rRNA modifications they cause, as illustrated by two snoRNAs having the same target modification site yet the two mutants lacking the snoRNAs showed highly divergent phenotypes. Whatever the case, there are a number of techniques currently available that should be able to help resolve the intricacies of snoRNA functions as will be described below.

SYNTHETIC GENE INTERACTION OF SNORNA GENES

The landmark publication of Tong *et al.* which made use of a large-scale systematic construction of double deletion mutants referred to as synthetic genetic array (SGA) analysis allowed for the identification of comprehensive functional genetic networks for a number of genes with roles in cytoskeletal organization, DNA synthesis or repair, and two genes with uncharacterized functions (Tong, Evangelista et al. 2001). In the current SGA set up, a query strain carrying a deletion or mutation of the gene of interest is crossed against an array of approximately 5000 viable protein-coding gene deletion mutants. The resulting meiotic progeny can then be scored for fitness defect. Newer SGA analysis variants patterned from the original synthetic lethal screens include genetic suppression, plasmid shuffling, and dosage lethality (Tong and Boone 2006). The power of this brute force approach lies in the systematic identification of functional relationships between genes leading to global mapping

of gene function. Unfortunately, non-coding RNA genes have so far been neglected in such SGA screens.

In **paper II,** we constructed 65 snoRNA gene knockouts in the same genetic background as the rest of the *Saccharomyces* Deletion Project collection. This gives us a great opportunity to perform synthetic lethal screens among these snoRNA gene deletion mutants and versus the whole protein-coding gene knockout collection. Future SGA analysis of other genes will also be more complete upon the inclusion of the snoRNA gene knockouts. The idea seems promising since one study already found a synthetic suppression effect of the deletion of *SNR57* on the lethal phenotype of *NEP1* gene which encodes a protein involved in 18S rRNA maturation (Buchhaupt, Meyer et al. 2006).

With the currently available variants of SGA analysis, a more global view of snoRNA gene function is within reach.

DIFFERENTIAL RIBOSOMAL RNA MODIFICATION

The identification of resistant phenotypes in a number of stress conditions upon deletion of specific snoRNAs led us to postulate that the modification of sites in the ribosomal RNA may be differentially-regulated, *i.e.* environment-dependent rRNA modification patterns. Not so much is known about the transcriptional regulation of snoRNA genes except that they are thought to be co-regulated with the components of the translational apparatus (Qu, Henras et al. 1999; Bachellerie, Cavaille et al. 2000). In a large-scale identification of *cis*-regulatory sequences within the yeast genome involving 203 known DNA-binding transcriptional regulators, a map of transcriptional regulatory code was produced (Harbison, Gordon et al. 2004). Genome-wide location analysis was used to pinpoint the genomic occupancy for each of the transcription factors, essentially mapping the target promoter regions for all the genes. We mined their data to determine which transcriptional regulators were associated with snoRNA genes. Remarkably, not only the known ribosomal protein transcription factors, such as Rap1p, Fhl1p, and Sfp1p were identified to be present in a number of snoRNA genes, we also found transcription factors involved in stress response to be bound to some snoRNA genes. Some specific examples are Adr1p, which is a carbon-source responsive transcription factor known to be required for the transcription of genes needed for transcription of the glucose-repressed gene *ADH2*, of peroxisomal protein genes, and of genes required for ethanol, glycerol, and fatty acid utilization. Gcn4p, a transcriptional activator of amino acid

biosynthetic genes in response to amino acid starvation, was also found to bind some snoRNA genes. Interestingly, Skn7p required for optimal induction of heat-shock genes in response to oxidative stress was also found to bind a snoRNA gene. All in all, a total of 21 transcription factors, a number of which are known to be activated during stress conditions, were shown to bind to promoter sequences of some of the snoRNA genes. This can be taken as an evidence of environment-specific expression of snoRNA genes, and hence differential rRNA modifications. Such a scenario is consistent with the prevailing consensus hypothesis regarding rRNA modifications as "fine-tuners" of the translational apparatus (Decatur and Fournier 2002). Indeed it is during stressful environments when such fine-tuning activity would be expected to be of great utility.

To be able to test the environment-dependent modification patterns of rRNAs, mapping the location of close to a hundred of modified residues would be a challenging task, especially when the most commonly used procedures in mapping pseudouridylation and *2´-O*-ribose methylation are reverse transcription-based approaches involving radioactive-labeling procedures and expensive reagents (Maden, Corbett et al. 1995; Maden 2001). Recently, the novel use of RNA-cleaving deoxyribozymes (DNAzymes) were used to detect *2´-O*-ribose methylations and pseudouridylations in the yeast rRNA (Buchhaupt, Peifer et al. 2007). The method was fast and cost-effective, requiring only DNA oligos designed to specifically cleave at desired target sites. We tried to utilize this method and tested on a number of our snoRNA deletion constructs (Fig 10). We designed two DNAzymes directed against a modification site in the 25S rRNA and another in the 18S rRNA. The procedure was indeed straightforward requiring minimum amount of reagents (only reaction buffers and low concentration polyacrylamide gels). However the fact that the cleavage efficiency never reaches 100% could be a cause of concern when trying to detect differential modification of rRNA. For instance, the hypothesized presence of heterogeneous rRNA populations in some environmental condition, wherein some are modified while some are not, would be difficult to resolve if the DNAzyme-catalyzed cleavage reaction has low cleavage efficiency. A possible solution would be to determine the exact cleavage efficiencies of DNAzymes by using standard amounts of RNA substrates. However, to ensure pure preparation of RNA *i.e.* without modification sites, one might need to do separate *in vitro* synthesis of RNA molecules.

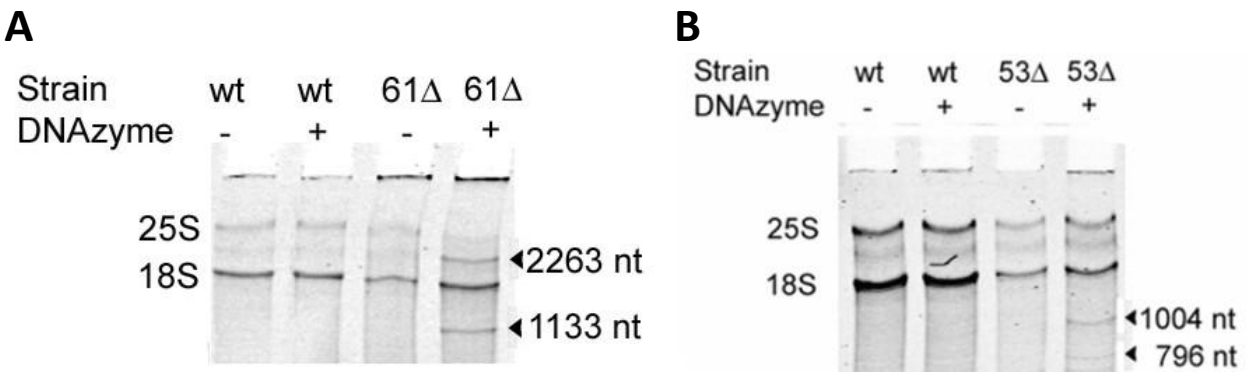

Figure 10. Use of DNAzymes to detect lack of *2'-O*-ribose methylations in the rRNA.
DNAzymes are RNA-cleaving DNA molecules identified by *in vitro* selection methods. The cleavage reaction requires the *2'*-hydroxyl group. Thus methylated rRNA nucleoside prevents cleavage. In the wild type, the target nucleoside is methylated while in snoRNA deletion mutants, the target nucleosides retain the *2'*-hydroxyl group allowing the DNAzyme-catalyzed cleavage of RNA to proceed. In A) snR61 guides the *2'-O*-methylation at A1133 in the 25S rRNA and in B) snR53 guides the *2'-O*-methylation at A796 in the 18S rRNA. The expected cleavage products are shown in both cases when the snoRNAs were deleted. Note that in both cases, 100% cleavage of 25S or 18S was not reached. Materials and methods were based on (Buchhaupt, Peifer et al. 2007).

12. THE "RIBOSOME CODE"?

With the observation that paralogous ribosomal proteins have different specialized functions, Komilii *et al.*, drew a parallel comparison between transcriptional regulation, known as the "histone code" and translational regulation, which they proposed to call the "ribosome code" (Komili, Farny et al. 2007). The "histone code", which is the canonical model for transcriptional regulation, posits that the transcriptional state of a given region of chromatin is determined by distinct combinations of histone proteins, their post-translational modifications, and DNA modifications (Jenuwein and Allis 2001). In the same manner, the heterogeneity of the ribosome, and thus its various translational states, could be realized by the different combinations of paralogous ribosomal proteins, their post-translational modifications, and possible differential rRNA modifications. The tantalizing prospect of finding demethylases that could reverse methylated nucleoside or nucleotide in the rRNA would be an exciting discovery just like when the first histone demethylase was found just a couple of years ago (Shi, Lan et al. 2004) after the dogmatic status of histone methylations as being static and enzymatically irreversible since the 1970s.

Our finding about the existence of stress-specific sub clusters of cytoplasmic ribosomal protein and snoRNA gene deletion mutants supports the idea of compositionally distinct ribosomes which are customized according to environmental cues. That or, the direct participation of many ribosomal protein components and snoRNAs in cellular stress response are two possible explanations of the rich phenotypic diversity we uncovered in this study.

13. TOWARDS A SYSTEM-WIDE ANALYSIS OF THE TRANSLATIONAL APPARATUS

We have come a long way in understanding the inner workings of the ribosome since its discovery almost 50 years ago. From its first description by George Palade as a small particulate component of the cytoplasm (Palade 1955), we can now literally see through the ribosome and explain what makes it tick. The simplicity of the mono-functional protein synthesis machinery whose parts are well-kept within the perimeters of translational burden is indeed a marvelous conception and perhaps best kept that way. However, evidence regarding the bi-functionality of many ribosomal proteins, and their apparent involvement in other cellular processes continues to pile up.

In this age of innovative high-throughput bioassays, it is imperative that we investigate the functional links of ribosomal components to other aspects of cellular physiology. The many variants of synthetic gene array (SGA) analysis such as the synthetic lethal screens, genetic suppression, and dosage lethality (Tong and Boone 2006) applied to the whole genome including the non-coding RNA genes, using all the non-essential ribosomal proteins and snoRNA genes as "query" genes would provide a more holistic understanding of their functions that is not limited within the confines of translation.

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To serve humanity alone is the sole purpose of life.
Leo Tolstoy

As this is ~~perhaps~~ the only page of the thesis comprehensible to any reader (or the only part people actually read), my intention was to write one kickass acknowledgment. One which will melt your heart, bring you to tears and leave the most profound impression on your soul. But as I am a man of repressed emotions (at least when I'm sober; fortunately I am at this very moment) I will skip the drama and go straight to the point without the obligatory aggrandizing descriptions of persons without whom this *obra* wouldn't have seen the light of day. Besides, you already know how great you all are! That might sound boring but I really want to avoid sounding like I'm leaving this world for good, and like, you know will not see you anymore. *sniff*

So without much further ado, my sincerest gratitude to THE man, Anders Blomberg.

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I take a humble bow and, thank you all from the bottom of my heart.

And as the Swedes say, "*Vi ses!*"

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There are no facts, only interpretations.

Friedrich Nietzsche

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